Astrocyte Activation in the Brainstem Evoked by Inflammatory Stimulation of the Masticatory Muscle

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Abstract

Objective: To identify a suitable treatment for masticatory muscle pain disorder, two stimuli were provided to the masseter muscle to cause inflammation, and the time course of changes in the number of astrocytes in the brainstem was determined.

Materials and Methods: A solution of 50 μl of lipopolysaccharide (LPS, 2 μg/kg) dissolved in saline was administered to the left masseter muscle (LMM) of Sprague–Dawley rats. After 24 h of recovery, the experimental group received 50 μl of a 6% sodium chloride solution 5 times every 90 min and the control group received another 50 μl of the LPS solution (2 μg / kg) dissolved in saline. The LMM and brainstem were dissected 1, 3, or 7 days after administering the reagents, and frozen sections were prepared. The expression of tumor necrosis factor α (TNFα) and bradykinin receptor B2 (BKRB2) in the masseter muscle and of an astrocytic marker and phosphorylated extracellular signal-regulated kinase (pERK) in the brainstem was examined by immunohistochemical staining.

Results: The expression of TNFα and BKRB2 in the LMM markedly increased 1 day after stimulation in the experimental and control groups, but the levels returned to nearly normal 7 days after stimulation. In the trigeminal subnucleus caudalis (Vc), there was a marked increase in the number of glial fibrillary acidic protein (GFAP) and pERK-immuno-reactive (IR) cells 7 days after stimulation in the experimental group. pERK-IR cells were observed in the dorsomedial, laterodorsal and central regions of the Vc.

Conclusions: When inflammation in the masticatory muscle was induced by the administration of LPS, which is an inflammatory factor, and a 6% sodium chloride solution, which is an infringing factor, activated astrocytes and pERK in the Vc persisted for around 1 week, even after local inflammation had subsided, which suggests a transition to chronic pain.

Keywords: Masseter, Glia, Inflammation

Introduction

The central nervous system is formed of neurons and glia, and there are approximately 10 times as many glia as neurons. Glia was initially thought to fill gaps between neurons, nourish neurons, and maintain nerve tissue by insulating axons [1]. However, it has recently become clear that glia express various neurotransmitter receptors.

Astrocytes release neurotransmitters, such as glutamic acid, ATP, and cytokines [2]. In addition, glia has been linked to inflammatory hyperalgasia, and it has been suggested that the activation of glia in the spinal cord is involved in mechanisms underlying inflammatory pain, neurogenic pain, hyperalgasia, and allodynia. For example, there is a marked increase in the number of phosphorylated-p38 mitogen-activated protein kinase (p-p38 MAPK)-immuno-reactive (IR) microglia in the spinal cord during peripheral inflammation or nerve injury [3-5]. Pre-treatment of microglia in the spinal cord with 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)-1H-imidazole (SB 203580), which is an inhibitor of p38 MAPK, suppresses allodynia [6-8]. Furthermore, glia in the trigeminal subnucleus caudalis (Vc) cause central sensitization [9] and astrocytes in the Vc cause plastic changes following dental pulp injury [10]. Transient receptor potential vanilloid 4, which is a member of the TRP family, is also involved in the activation of glia in the brain [11].

Many patients suffer from masticatory muscle pain disorder, which is a major pathological condition in temporomandibular disorders. Masticatory muscle pain is mostly due to myofascial pain syndrome (MPS), which is chronic myalgia confined
to 2-3 muscles, and it can be complicated by hyperalgiesia and related pain at the trigger point of the muscle and fascia [12,13]. At the active trigger point of MPS, inflammatory cytokines, such as substance P and TNFα, have been detected at high concentrations [14]. For this reason, the sensitization of polymodal receptors associated with local inflammation is thought to be involved in the development of pain.

Previous studies have elucidated numerous aspects of chronic pain caused by nerve injury in the oral maxillofacial area. However, the pathophysiology of masticatory muscle pain disorder has not yet been fully elucidated, and currently, there are no treatments. We hypothesized that glia are involved in masticatory muscle pain disorder and MPS. Therefore, we experimentally induced sustained pain, such as chronic pain, and examined the activity of glia, the role of glial activity, and the signal transduction pathway within glia.

In this basic research study focused on developing a treatment for masticatory muscle pain disorder, we experimentally induced inflammation in the masseter muscles using two stimuli. We investigated the role of astrocytes in the Vc by examining histological changes in the masseter muscle and Vc over time.

Materials and Methods

This experiment was conducted in accordance with the guidelines of the International Pain Society [15] and with approval from the Animal Research Committee of Osaka Dental University (approval number: 17-02002). After anesthetizing Sprague–Dawley rats (males, n = 30, 250 g; Japan SLC Inc., Japan) with sodium pentobarbital (70 mg/kg, i.p.; Nembutal, Dainippon Sumitomo Pharma, Japan), stimulation was conducted under the following conditions [16-19]:

① The L-L group (control group, n = 30) received 2 μg/kg (50 μl) of lipopolysaccharide (LPS, Sigma-Aldrich, MO, USA) that was injected into the left masseter muscle LMM. After 24 h, LPS (50 μl) was injected at the same site.

② The L-S6 group (experimental group, n = 30) received 2 μg/kg (50 μl) of LPS that was injected into the LMM. After 24 h, a 6% sodium chloride solution (S6; 50 μl, 5 times every 90 min) was injected at the same site [16-19].

LPS was administered as an immune and inflammatory factor, and S6 was administered as an inhibiting factor [16-19]. On days 1 (n = 10), 3 (n = 10), and 7 (n = 10) after administering the reagents, the LMM and brainstem were dissected after perfusion fixation with 4% paraformaldehyde (PFA, 100 ml). After post-fixation with 4% PFA (LMM: 24 h, brainstem: 48 h), tissues were immersed in 30% sucrose for 48 h. Immunostaining was performed after the preparation of frozen sections (LMM: 15 μm, brainstem: between -7.22 mm and -15.84 mm; distance from the bregma): 30 μm, -20°C, Leica CM 3050 S cryostat (Leica Microsystems, Wetzlar, Germany) [19].

The following primary antibodies were used for the LMM:

- Rabbit anti-TNFα (tumor necrosis factor α) (1:200, 1h, room temperature (RT); Chemicon, USA)
- Rabbit anti-bradykinin receptor B2 (BKRB2, 1:250, 1h, RT; ENZO Life science, USA)

TNFα is an inflammatory cytokine that is synthesized and secreted by keratinocytes and fibroblasts during inflammation and after injury [20,21]. After injury in the peripheral nervous system, TNFα is synthesized by Schwann cells [22]. BKRB2, which is expressed in macrophages and fibroblasts, is involved in acute pain [23].

The following primary antibodies were used for immunostaining the brainstem:

- Rabbit anti-GFAP (glial fibrillary acidic protein) (1:1000, 1h, RT, Millipore, CA, USA)
- Rabbit anti-phospho ERK1/ERK2(T202/Y204) (pERK) (1:200, 1h, RT; R&D SYSTEMS, MN, USA)

GFAP is a marker of activated astrocytes and stains thick astrocytic processes [24]. ERK is a signaling molecule belonging to the mammalian representative MAPK family.

For enzyme-labeled antibody staining of the LMM and brainstem, the following secondary antibodies were used [19]:

- Biotinylated goat anti-rabbit IgG (1:400, 30min, RT; Vector Laboratories, USA)
- Avidin-biotin-peroxidase complex (30 min, RT, Elite ABC kit; Vector Laboratories)

Distilled Water (DW) containing 0.05% 3,3’-diaminobenzidine (DAB) (30 min, RT; Vector Laboratories), 0.2% nickel sulfate and 0.01% hydrogen peroxide.

Sections of the LMM and brainstem were examined using an Olympus BX41 light microscope (Olympus, Tokyo, Japan), and photographs were taken with an Olympus FX380 3CCD digital camera system (OS: Windows 7; Microsoft, CA, USA) using Flvel software (Flvel Image Filling System, Tokyo, Japan) [19]. In the LMM, 10 sites (area = 200 × 200 μm² square) were selected and the number of TNFα and BKRB2-IR cells was counted [19]. Statistical analysis of the time course of changes in the number of IR cells was conducted by one-way ANOVA (Excel 2010; Microsoft, USA, statistically significant: *p< 0.05) [19]. In the brainstem, 3 sites (area = 400 × 400 μm² square) were selected from the Vc (between -13.68 mm and -15.84 mm from the bregma) and the number of GFAP- and pERK-IR cells was counted [19]. Statistical analysis of the time course of changes in the number of IR cells was conducted by one-way ANOVA (Excel 2010; Microsoft, USA, statistically significant: *p< 0.05) [19].

Results

TNFα-IR cells in the LMM

There were numerous TNFα-IR cells in the control (①) and experimental (②) groups 1 day after stimulation, but they were not consistently observed 7 days after stimulation (Figure 1a). There was no significant difference between the control (①) and the experimental (②) groups (Figure 1b). Muscle fibers were ruptured in both groups 1 day after stimulation. However, 7 days after stimulation, images confirmed that except in a few muscle fibers, repair was occurring.

BKRB2-IR cells in the LMM

There were numerous BKRB2-IR cells 1 day after stimulation
in the control (①) and experimental (②) groups. However, few BKRB2-IR cells were observed 7 days after stimulation (Figure 2a). As seen with TNFα, there was no significant difference in the number of BKRB2-IR cells between the (①) and the experimental (②) groups (Figure 2b).

Intergroup comparison of changes in the number of GFAP-IR cells over time in the ipsilateral Vc

GFAP-IR cells were detected in both groups at days 3 and 7 after stimulation, but the number of positive cells was considerably higher in the experimental group (②) than in the control group (①) (Figure 3a). The highest number of GFAP-IR cells was detected in the experimental group (②) 7 days after stimulation (p<0.01, Figure 3b). In both groups, expression was more significant in the caudal side of the Vc than in the rostral side at days 3 and 7 after stimulation (Figure 3b). GFAP-IR cells were observed in the dorsomedial, laterodorsal, and central regions of the Vc.

Comparison of GFAP-IR cells in the ipsilateral and contralateral sides of the Vc in the experimental group (②) 7 days after stimulation

As there was a marked increase in the number of GFAP-IR cells in the experimental group (②), we compared the number of GFAP-IR cells in the ipsilateral and contralateral sides 7 days after stimulation. The number of GFAP-IR cells was significantly higher in the ipsilateral side than in the contralateral side (p<0.01, Figure 4a, b). In the ipsilateral and contralateral sides, expression was higher in the caudal side than in the rostral side of the Vc (Figure 4b).
bregma (Figure 5b). Similar to GFAP-IR cells, pERK-IR cells were observed in the dorsomedial, laterodorsal, and central regions of the Vc. In addition, pERK-IR cells were particularly numerous in the subnucleus zonalis and subnucleus gelatinosus of the Vc.

Comparison of the number of pERK-IR cells in the ipsilateral and contralateral sides of the Vc in the experimental group (②) 7 days after stimulation

As the number of pERK-IR cells was markedly increased in the experimental group (②), we compared the number of pERK-IR cells in the ipsilateral and contralateral sides 7 days after stimulation (Figure 6a, b). There was significant difference

Intergroup comparison of pERK-IR cells in the ipsilateral Vc

On days 3 and 7 after stimulation, the number of pERK-IR cells was considerably higher in the experimental group (②) than in the control group (①) (Figure 5a). The most significant number of pERK-IR cells was detected in the experimental group (②) 7 days after stimulation (p<0.01, Figure 5b). pERK-IR cells were particularly numerous 15 mm or more caudally from the
between the ipsilateral and contralateral sides (p<0.01, Figure 6b). Similar to GFAP-IR cells, pERK-IR cells were more numerous in the ipsilateral side at all points analyzed, from the rostral side to the caudal side.

Discussions

This study, our previous study [19], and studies by Hashimoto, et al. [16-18] counted the number of GFAP- and pERK-IR cells in the Vc and examined the role of astrocytes and pERK in the persistence of pain in masticatory muscle pain disorder. Following injury to the masseter muscle, pERK-IR cells in the ipsilateral side of the Vc were persistently observed until 1 week after stimulation in the experimental group (2). In the LMM, the expression level of TNFα, which is an inflammatory cytokine, was significantly elevated in the control (1) and experimental (2) groups 1 day after stimulation. Muscle fibers were ruptured by the needle during injection, and the original structure was not maintained. However, 1 week after stimulation, TNFα- and BKRB2-IR cells were occasionally observed and muscle fiber repair was observed. BKRB2, which is expressed in macrophages and fibroblasts, is involved in acute pain [25]. The number of BKRB2-IR cells also remarkably increased 1 day after stimulation; however, 1 week after stimulation there were extremely few cells. The number of cells expressing TNFα and BKRB2 was consistent with the that from the results of our previous study [19]. Based on these results, it can be inferred that the development of local inflammation was accompanied by transient pain and that local inflammation subsided within 1 week of stimulation.

When rat gastrocnemius muscle was damaged in previous study, reactivity at the non-injured side increased for 2 weeks after stimulation [16-18]. Based on these results, the method for creating an animal model of chronic pain was effective. Therefore, it was used in this study. This study examined the time course of changes due to stimulation for up to 1 week after stimulation. Our results indicated that the number of GFAP- and pERK-IR cells was markedly increased in the experimental group (2) 7 days after stimulation compared with that 3 days after stimulation. This was consistent with the results of the previous study. In a study using a rat model of trigeminal nerve injury, GFAP and pERK co-staining was observed in the Vc on days 1, 3, and 7 after injury [26]. This also supports the results of our study, which suggests that pERK is involved in the activation of astrocytes. In contrast, GFAP- and pERK-IR cells were not as common in the control group (1) as in the experimental group (2). These results suggest that sustained pain was caused by LPS-mediated inflammation and S6-mediated pain and that animal in this study may be useful models of chronic pain, similar to the results of the previous studies [16-19]. The Vc mainly relays information from regions dominantly innervated by the trigeminal nerve, and there have been many reports on oral maxillofacial pain [9,10,19,26-29]. In the present study, we examined astrocytes, which are glial cells involved in pain regulation [4,5,8-11,20-34]. Astrocytes are the most common glia in the brain, and they regulate nerve activity by releasing various neurotransmitters such as glutamic acid [10]. Once activated, astrocytes are involved in nociceptive stimulation and pain control [10]. The Vc responds to nociceptive stimuli from the trigeminal region induced by formalin, and astrocytes play an important role in the regulation and transmission of pain [31]. In our previous study, we conducted similar experiments on microglia and MAPK in the Vc [19]. The results showed that microglia and p-p38 MAPK were activated in response to noxious stimuli to the masseter muscles and that the response was enhanced over time [19].

In the present study, we observed high numbers of astrocytes in the ipsilateral side of the Vc in the experimental group (2), even after the expression of TNFα- and BKRB2-IR cells had subsided. In addition, pERK-IR cells were persistently observed in the ipsilateral side. Taken together, these results suggest that even if the local inflammatory reaction subsides, astrocytes and pERK were activated in the Vc of the central nervous system. This is consistent with the results of our previous study [19] and studies on injured rat gastrocnemius muscle [24-26]. The results of our study are also consistent with those of previous reports showing that the persistence of pain is due to changes in the plasticity of the central nervous system rather than that of the peripheral nervous system [16-19,35]. The number of GFAP-IR cells in the Vc was remarkably higher in the ipsilateral side at all points analyzed. This is consistent with the results of previous studies in which there was a clear difference in the number of astrocytes in the ipsilateral and contralateral sides 4 days after dental pulp injury in rats [10].

In pathological conditions that activate microglia, the delayed activation of astrocytes suppresses the continued activation of microglia [29]. Although activated astrocytes are involved in inflammatory reactions [33], they are also involved in localized inflammation, tissue repair, and nerve repair [34]. In the present study, the activation of microglia and astrocytes was not simultaneously monitored. However, in our previous study [19], the activation of microglia was monitored for up to 14 days after stimulation. To understand the persistent activation of microglia and astrocytes and their interactions, further studies are necessary. Furthermore, experiments using a mouse model of brain injury induced by cortical stab injury showed a significant increase in the expression of GFAP in the injured side compared with the expression in the healthy side, and astrocytes in the injured area were enlarged, peaking at 3–5 days post injury [32]. In the future, we would like to examine changes in the morphology of astrocytes during activation.

Conclusion

Our results suggest that when inflammation occurs in the
masticatory muscle due to the administration of LPS and S6, the activation of astrocytes and pERK in the Vc is maintained for approximately 1 week, even if local inflammation subsides and may lead to the development of chronic pain.

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References