Effects of Danggui-Shaoyao-San on the Influence of Spatial Learning and Memory Induced by Experimental Tooth Movement

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Abstract

Background: The pain caused by orthodontic treatment has been considered as tough problems in orthodontic practice. There is substantial literature on pain which has exactly effected on learning and memory; orthodontic tooth movement affected the emotional status has been showed positive outcomes. Danggui-Shaoyao-San (DSS) is a Traditional Chinese Medicine prescription that has been used for pain treatment and analgesic effect for orthodontic pain via inhibiting the activations of neuron and glia. We raised the hypothesis that DSS could restore the impaired abilities of spatial learning and memory via regulating neuron or glia expression in the hippocampus.

Methods: A total of 36 rats were randomly divided into three groups: (1) Sham group ($n = 12$), rats underwent all the operation procedure except for the placement of orthodontic forces and received saline treatment; (2) experimental tooth movement (ETM) group ($n = 12$), rats received saline treatment and ETM; (3) DSS + ETM (DETM) group ($n = 12$), rats received DSS treatment and ETM. All DETM group animals were administered with DSS at a dose of 150 mg/kg. Morris water maze test was evaluated; immunofluorescent histochemistry was used to identify astrocytes activation, and immunofluorescent dendritic spine analysis was used to identify the dendritic spines morphological characteristics expression levels in hippocampus.

Results: Maze training sessions during the 5 successive days revealed that ETM significantly deficits in progressive learning in rats, DSS that was given from day 5 prior to ETM enhanced progressive learning. The ETM group rats took longer to cross target quadrant during the probe trial and got less times to cross-platform than DETM group. The spine density in hippocampus in ETM group was significantly decreased compared to the sham group. In addition, thin and mature spine density were decreased too. However, the DSS administration could reverse the dendritic shrinkage and increase the spine density compared to the ETM group. Astrocytes activation showed the opposite trend in hippocampus dentate gyrus (DG).

Conclusions: Treatment with DSS could restore the impaired abilities on ETM-induced decrease of learning and memory behavior. The decreased spines density in the hippocampus and astrocytes activation in DG of hippocampus in the ETM group rats may be related with the decline of the ability of learning and memory. The ability to change the synaptic plasticity in hippocampus after DSS administration may be correlated with the alleviation of impairment of learn and memory after ETM treatment.

Key words: Astrocytes; Danggui-Shaoyao-San; Dendritic Spines; Experimental Tooth Movement; Hippocampus; Spatial Learning and Memory

INTRODUCTION

The pain accompanied with orthodontic treatment has been considered as tough problems in orthodontic practice. Most orthodontic patients complained about pain and discomfort during the first few days after treatment.[1-5] The majority of patients experienced subjective pain during treatment and pain is a determining variable of adherence to orthodontic treatment.[6,7] These side effects can produce a considerable amount of distress in the daily life of patients, such as difficulties in chewing and biting.[8] In addition, 10% of patients choose to terminate their therapy, and potential orthodontic patients avoid treatment due to the fear of feeling pain.[9,10] Some studies including clinical orthodontic treatment and experimental tooth movement (ETM) to compare the emotional status before and after tooth movement show positive outcomes.[11,12] There is substantial

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literature on both chronic and acute pain has substantial effects on learning and memory.[13-15] However, few studies about the learning and memory that patients endure during orthodontic treatment, it is of interest to investigate the learning and memory and their relationship with pain evoked by ETM in rats by behavioral testing, immunofluorescent histochemistry and immunofluorescent dendritic spine analysis.

Danggui-Shaoyao-San (DSS, Pharmaceutical ingredients including Dang Gui, Bai Shao, Chuan Xiong, Ze Xie, Fu Ling and Bai Zhu, Table 1) is a traditional herbal medicine which is widely used in China, Japan, and Korea to treat visceral pain. It was first recorded in "*JinKuiYaoLue*" and consists of six medicinal herbs. DSS possesses antioxidative,[16] cognitive enhancing[17] and antidepressant effects.[18] Our previous study also added evidence that DSS had neuroprotective effects in the aged animals,[19] and the beneficial effect of DSS was related with the reversion of brain noradrenaline and dopamine concentrations under depressive conditions.[20] Our recent study proved that DSS also possesses analgesic effects for orthodontic pain, and the possible analgesic mechanism for DSS inhibit the activation of the neuron.[21]

In this study, by employing a rat ETM model, we aimed to explore the potential spatial learning and memory effects of DSS and its underlying mechanism on activation of neuron and glia in orthodontic therapy with morphological and biochemistry techniques.

METHODS

Preparation of Danggui-Shaoyao-San

Six herbs of DSS were purchased from Nanjing Traditional Medicine Clinic, Jiangsu Province, China, and identified by Dr. Yu in the Department of Complex Prescription of Traditional Chinese Medicine of our University. Aqueous extract of DSS was prepared similarly as described before.[22] In brief, six medicinal materials were mixed in proportion and were macerated for 1 h with 8 times (v/w) distilled water, and then decocted for 1 h, after which the filtrate was collected and the residue was decocted again for 1 h with 6 times (v/w) distilled water. The filtrates were mixed and condensed and then lyophilized to DSS powder with the yield of 29%. The HPLC fingerprint of DSS was detected according to our previous report,[23] in that gallic acid, paeoniflorin, ferulic acid, senkyunolide I, tetra-galloyl-glucose Penta-O-galloyl-D-glucose (PGG) and alisol B in DSS were well identified.

Animal and groups

Thirty-six male Sprague-Dawley rats (180–200 g) were housed in plastic cages, and maintained on a 12:12 h light/dark cycle under conditions of 22–25°C ambient temperature with food and water available. All experimental procedures were performed in accordance with the Animal Use and Care Committee for Research and Education of Institute of Stomatology, Chinese PLA General Hospital (Beijing,

China) and the ethical guidelines. The Animal Care and Use Committee of the Chinese PLA General Hospital approved this study (Permit number: 20110089).

Rats were randomly divided into three groups: (1) Sham group (*n* = 12), rats underwent all the operation procedure except for the placement of orthodontic forces and received saline treatment; (2) ETM group (*n* = 12), rats received saline treatment and ETM; (3) DSS + ETM (DETM) group (*n* = 12), rats received DSS treatment and ETM. All DETM group animals were administered with DSS at a dose of 150 mg/kg twice daily (bid) via gastric tube from preoperative day 5 to postoperative day (POD) 5, and vehicle (1 ml/kg saline) was administered via gastric tube to ETM rats [Figure 1]. The selection of DSS and the rationale for the dosing regime was within the range of being neuroprotective in rodents.

Appliance for experimental tooth movement

A fixed, Ni-Ti alloy closed-coil spring appliance was constructed for mesial movement of the right maxillary first molar based on the method previously described by Ong *et al*.[24] The Ni-Ti alloy closed-coil spring appliance was placed on both the right maxillary first molar and upper incisors of rats after ketamine anesthesia (0.02 ml/kg, intraperitoneal injection). A 0.2 mm shallow groove between adjacent gingival margins of two maxillary incisors was cut, and stainless steel wires (diameter 0.2 mm) were placed in the groove, which encircled the two incisors as a whole. Then another stainless steel ligature wire with the same diameter was placed crossing the gap of right maxillary

Table 1: Recipe of DSS formulation

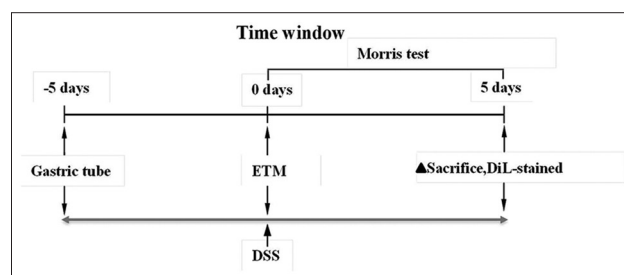| Components	Ratio
Dang Gui (*Angelica sinensis* (Oliv.) Diels., root)	3
Bai Shao (*Paeonia lactiflora* Pall., root)	16
Chuan Xiong (*Ligusticum chuanxiong* Hort., rhizome)	8
Bai Zhu (*Atractylodes macrocephala* Koidz., root and rhizome)	4
Fu Ling (*Poria cocos* (Schw.) Wolf., fungus nucleus)	4
Ze Xie (*Alisma orientale* (Sam.) Juzep., rhizome)	8

DSS: Danggui-Shaoyao-San.

Figure 1: Experimental procedures. Black bar represents the period during which behavioral test was performed. Gray bar represents the period when Danggui-Shaoyao-San was intragastric administered bid. Triangle represents the time points when rats were sacrificed for immunohistochemistry. Drugs were intragastric administered 5 days prior to experimental tooth movement and throughout the 5 days of observing window.

first and second molars. In addition, Ni-Ti helical tension spring (diameter 0.18 mm) was laid between the right maxillary first molar and incisors. The molar would lean to the middle after the wires connecting and dragging each other [Figure 2b]. The ETM and DETM groups were loaded with 60 g orthodontic force while the sham groups were implemented as the same protocol without orthodontic force.

Morris water maze test

Spatial memory of the rats was tested using the Morris water maze test.[25] The water maze apparatus consisted of a water tank of 1.20 m in diameter and 75 cm in depth, which was filled with water 50 cm deep (22 ± 1°C). Four points on the rim of the pool were designated as north (N), south (S), east (E), and west (W), thus dividing the pool into four quadrants. There was a Plexiglas platform (10 cm in diameter), 23 cm high and centered at one of the quadrants, which is placed 2 cm below the water surface in one of the quadrants, the target quadrant. Milk black powder was added to the water just before the experiment to make the water opaque. To provide extra-maze cues for allowing the rats to develop a spatial map strategy, four different shape pictures were hung on the walls. Positions of the cues were kept unchanged throughout the experimental period.

Acquisition phase

All the rats were allowed 3 days rest after arrival at the laboratory before behavioral observation began, and then the rats were given spatial memory training.

The rats were trained in the water maze in nine sessions on 5 consecutive days, two sessions on each day, except on the last day in which was given only one session. During each training session, the rats in the three groups (sham group, ETM group, DETM group; $n = 12$ per group) that received spatial memory training were given massed trials with duration of 60 s per trial, the rats were placed in water so that they faced the wall of the pool. Each session consisted of four trials. The trials were always initiated from different positions in the tank. For each rat, the quadrant in which the platform was located remained constant, but the point of immersion into the pool varied between N, E, S, and W so that the rat was not able to predict the platform location from the location at which it was placed into the pool. The rat was then given 1 min to search for the platform. Once the rat located the platform, it was permitted to remain on it for 5 s. In each training session, the latency (time) to escape onto the hidden platform was recorded. If the rat was unable to find the platform within 1 min, it was guided to the platform and allowed to remain on it still for 5 s. After each trial, the rats were dried and removed back to their home cages.

Probe trial

One-day following completion of the last training session, each rat was subjected to a probe trial (60 s) in which there was no hidden platform. The rat was placed into water. The time taken to reach the target quadrant and the time spent in the target quadrant were measured. All the events were video recorded using a video (SONY video with F29-100 lens) and illumination lights, etc., The data were analyzed using Imaris software (7.5, Olympus, Japan). Greater latency to reach the target quadrant and less time spent in the target quadrant suggested memory impairment. After the last trail in training day 5, the rats in three groups were sacrificed by decapitation immediately (within 1 min). The brains were rapidly removed, chilled on ice and the hippocampus of the brains were microdissected by hand. Tissues were stored in microcentrifuge tubes at − 80°C for immunofluorescent histochemistry and DiI-stained.[26]

Immunofluorescent histochemistry

Rats were anesthetized with an overdose sodium pentobarbital (Shanghai Xinran Biotechnology Co. Ltd., China) and perfused through the ascending aorta with 100 ml of 0.9% saline followed by 500 ml of 0.1 mol/L phosphate buffer (PB, pH 7.4) that contained 4% paraformaldehyde and 2% picric acid. After perfusion, the brain was removed and postfixed in the same fixative for 2 h and then cryoprotected for 24 h at 4°C in 0.1 mol/L PB that contained 30% sucrose. Brainstems (30 μm thickness) were cut with a cryostat (Leica CM1800, Heidelberg, Germany) and collected serially in three dishes. Each dish contained a complete set of serial sections that were processed for immunofluorescent staining. The sections in the dish were rinsed in 0.01 mol/L phosphate-buffered saline (PBS, pH 7.4) three times (10 min each), blocked with 2% normal goat serum in 0.01 mol/L PBS that contained 0.3% Triton X-100 for 1 h at 22–25°C, and then incubated overnight at 4°C with primary antibodies: Mouse anti-Iba-1 (1:5000; Chemicon, Temecula, CA, USA), rabbit anti-Fos (1:200; Chemicon). The sections were washed three times in 0.01 mol/L PBS (10 min each) and then incubated for 4 h at 22–25°C with the secondary antibodies: Alexa Fluor 488-conjugated donkey anti-mouse IgG (1:500; Vector, Burlingame, CA, USA) and Alexa Fluor 594-conjugated donkey anti-rabbit IgG (1:500; Vector). The specificity of the staining was tested on the sections in another dish by omitting one of the specific primary antibodies. No fluorescent signal was observed on these sections (data not shown). Confocal images were obtained using a confocal laser microscope (FV1000; Olympus, Tokyo, Japan) and digital images were captured with Fluoview 1000 (Olympus, Tokyo, Japan).

The protocol of image analysis was similar to our previous study.[27] Five nonadjacent sections, containing the dentate gyrus (DG) of hippocampus that is mostly associated with spatial memory, were selected randomly. Images were evaluated by a computer-assisted image analysis program (MetaMorph 6.1, Molecular Devices, USA). Image data were collected using the same region and the same size of field within the DG of hippocampus. The same configuration was used to measure cell areas in all experimental groups.

Immunofluorescent dendritic spine analysis

To identify dendritic spines, we used specific morphological characteristics as reported previously.[28,29] We considered a protrusion without a visible neck structure (from the main

dendrite shaft), a spine only if there was a visible indentation on either side of the junction of the protrusion from the dendrite branch. A spine neck was defined as the structure between the base of the spine, the interface between the parent dendrite branch, and the base of the spine head where the appearance of the spine began to swell distally. Spine head structure varied greatly, but we defined these as the visible bulb-like structures located at the ends of protrusions. Thin- and mushroom-shaped spines were classified as follows: Thin spines have head diameters that are less than or equal to the length of the spine neck. Mushroom spines have head diameters that are greater than the length of the neck. There are three rationales for using these geometric categories for spines: First, two spine shapes allowed us to use simple but strict rules in classifying spine morphology; second, this approach, by precluding discrimination of subtle variations in spine shape, allows collection of a large sample size; third, there is a large body of literature describing the different physiological characteristics associated with the morphologies of thin- and mushroom-shaped spines.[30] Neurolucida software was used to reconstruct dendritic spines of hippocampal culture. We analyzed the complete three-dimensional reconstructions of hippocampal neurons for spine density and distribution. Three-dimensional Imaris 7.5 traces were made for each neuron. (1) An outline trace of the spinal cord section upon which the location of identified hippocampal neurons was marked. (2) A three-dimensional reconstruction of each neuron of hippocampus, which was created by tracing through the x-, y-, and z-axes. Dendritic spine type and location. We marked on these three-dimensional reconstructions. Dendritic spine density was expressed as spine number per 10 mm dendritic length. Imaris 7.5 software was used to analyze the spatial distribution of spines (relative to the cell body). Seven 50-mm-wide spherical bins were formed around the cell body, and spine density within each bin was averaged for each experimental group. Mean data were compared against equivalent bins across groups.[31]

Statistical analysis

The SPSS 13.0 package (SPSS Inc., USA) and GraphPad Prism 5 (GraphPad Software, USA) were used and one-way analysis of variance (ANOVA) or Student's t-test was employed where appropriate for statistical analysis. All the data were presented as mean ± standard error of mean; A $P < 0.05$ was considered as statistically significant in all cases.

RESULTS

Experimental tooth movement with 60 g orthodontic force demonstrated well orthodontic results between the maxillary first molar and the maxillary second molar at POD 7 [Figure 2]. ETM was successfully drawn to the mesial side after POD 3 [Figure 2].

Morris water maze test

Water maze performance during the training sessions

The rats in the three groups had the same capacity for special learning and memory before ETM afflicted, and one-way
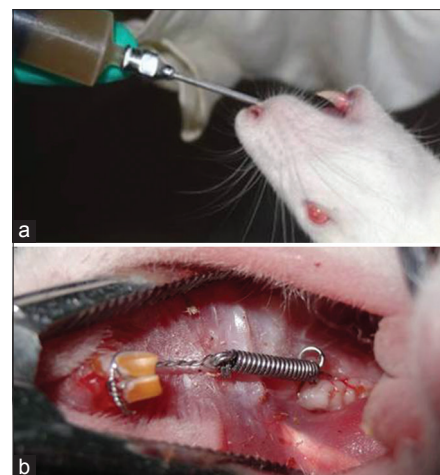

Figure 2: Representative graph (a) Danggui-Shaoyao-San (DSS) was intragastric administered bid to the rats; (b) The rat's molars with the experimental appliance of orthodontic tooth movement at POE 5. The appliance was added between the upper incisors and the right first molar of the rat. The upper first molar was successfully drawn to the mesial side at POE 5.

ANOVA statistical analysis demonstrated that there was no difference in the mean escape latencies between groups during training day 1 ($P > 0.05$). Maze training sessions during the 5 successive days revealed deficits in progressive learning in rats that subjected to ETM and those pretreated with DSS prior to ETM [Figure 3]. However, all rats of other groups began to identify the platform from the 1st day and continued to retain their learning ability throughout the training period. At the end of the 5th day, results showed enhanced progressive learning in sham and DSS groups, the ETM treated rats took longer to identify and to reach the hidden platform, indicating memory impairment ($P < 0.05$, Figure 3). The progressive learning over days in these groups was much lower as compared to the other groups. These results suggest a possible facilitation of progressive learning in DSS-treated animals during the first 5 days of ETM period ($P < 0.05$, Figure 3).

Time spent on current goal during probe trial

Analysis of swimming performance during the probe trial evidenced that the ETM rats cross target quadrant less times than sham group ($P < 0.01$, Figure 4a), whereas rats pretreated with DSS before ETM-treated animals do it more times to reach the target quadrant ($P < 0.05$, Figure 4a) and ETM group animals submitted to significantly less time in the quadrant of the former platform position ($P < 0.01$, Figure 4b). Rats in the ETM group pretreated with DSS spent more time in the target quadrant ($P < 0.05$, Figure 4b).

Danggui-Shaoyao-San and astrocytes activation

Daily DSS pretreatment starting at 5 days prior to ETM operation significantly inhibited the ETM induced astrocytes activation at POD 5. At the section level, relative expression of GFAP was significantly decreased (Figure 5, $P < 0.05$, vs. ETM). The decreased expression of GFAP was accompanied with the shrinkage of the astrocytes cell bodies and withdrawal of cell elongations [Figure 5c].

Dendritic spines remodel on hippocampal neurons in experimental tooth movement

To determine whether ETM pain associated with changes in dendritic spine morphology, we first analyzed dendritic spines from hippocampal tissue collected immediately from rats of ETM after Morris water maze test [Figure 6a and 6b]. Neurons were identified in DiL-stained coronal sections of hippocampal tissue. To control for possible variation in cell morphologies, we compared the dimensions of sample cells for the following: Total spine density and density of thin and mature spine. The spine density in ETM group were significantly decreased compared to the sham group (Figure 6c; $P < 0.05$), and thin and mature spine density were decreased, too (Figure 6d; *$P < 0.05$, †$P < 0.05$), but the DSS administration could reverse the dendritic shrinkage and increase the spine density compared to the ETM group (Figure 6c and 6d; ‡$P < 0.05$).

DISCUSSION

In our experiments, rats were trained to learn (in the navigation task) and then remember the location of the hidden platform (probe trail 8 h later) in the Morris water maze. Under ETM conditions, the rats exhibited a decreased performance in both navigation task and probe trail compared with the rats in the sham groups. The results demonstrated that the learning and memory abilities of the rats were decreased by ETM. We offered evidence that DSS may help improved

Figure 3: Progressive learning of rats in different groups during 5 successive days of water maze training. Data represent mean ± standard error of mean ($n = 12$). Sham/DSS + experimental tooth movement (ETM) groups versus ETM group, *$P < 0.05$; Sham/DSS + ETM groups vs. ETM group; †$P < 0.05$.

the ETM decreased performance in both navigation task and probe trail of rats, which was accompanied with the neuronal inhibition within hippocampal culture. These findings consolidated the experimental basis for the application of DSS in the orthodontic pain;[21] however, lots of efforts need to be contributed to future more detailed experiments before it can be applied in clinic settings.

Spatial learning and memory is one of the advanced neurophysiologic activities of the brain. It involves in many brain regions, neurotransmitters and intracellular signaling molecules. Spatial learning and memory are a very complex process of neurophysiologic activities. Hippocampus is an important structure of the brain, and the integrity of the hippocampal formation is essential for spatial learning. Using Morris water maze test, which was developed by Morris,[32] it is particular sensitivity to the effect of hippocampus lesions in rats. It has been shown that the hippocampal formation is particular important in spatial learning and memory in Morris water maze.[33-35] Morris water maze as behavioral observation parameters, we detected beneficial effect on the ETM induced impairments of spatial learning and memory, and we also identified that the spine density in ETM group was significantly decreased compared to the sham group by DiL-stained coronal sections of hippocampal tissue. Though we do not really know the underlying mechanisms of the Morris water maze in ETM model, ETM really decreases performance in both navigation task and probe trail of rats. Further efforts should be put on revealing the mechanism of this behavioral parameter for ETM model.

Daily DSS pretreatment for 5 days ameliorated ETM induced behavioral disturbances of the Morris water maze, but there remains one very important issue to address, which effective essential component(s) account for its beneficial effects. Previous studies have shown that multiple components such as gallic acid, paeoniflorin and from DSS exert significant active effects in visceral hyperalgesia or migraine.[36-39] Recently, we also identified PGG as a novel analgesic compound from DSS in acetic acid and formalin-induced mouse models and obtained Chinese patent. In addition, treatment with DSS had significant analgesic effects on ETM-induced pain, which was accompanied with inhibition of both neuronal and microglial activation.[21,40] Whether or not these constituents would contribute to the effect of DSS in spatial learning and memory after ETM and exert similar
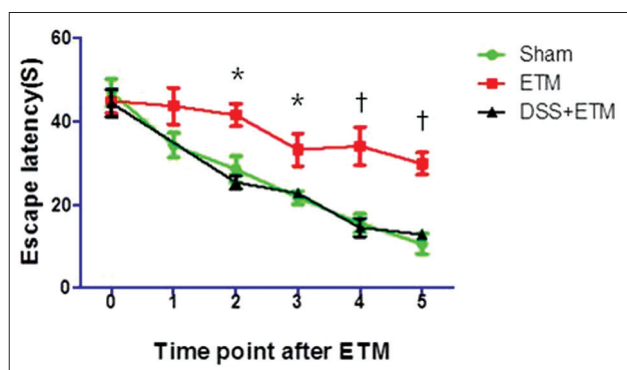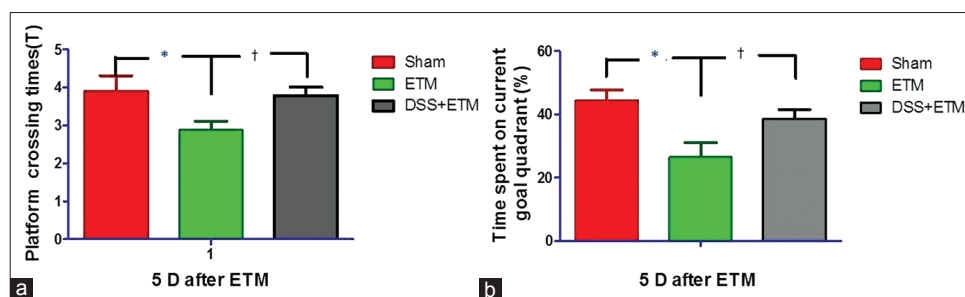
Figure 4: Platform crossing times during the probe trial (a) Data represent mean ± standard error of mean ($n = 12$). Sham group versus experimental tooth movement (ETM) group, *$P < 0.01$, DSS + ETM group vs. ETM group; †$P < 0.05$; Time spent on current goal quadrant (%) during the probe trial (b) data represent mean ± SEM ($n = 12$). Sham group vs. ETM group, *$P < 0.01$; DSS + ETM group versus ETM group; †$P < 0.05$.

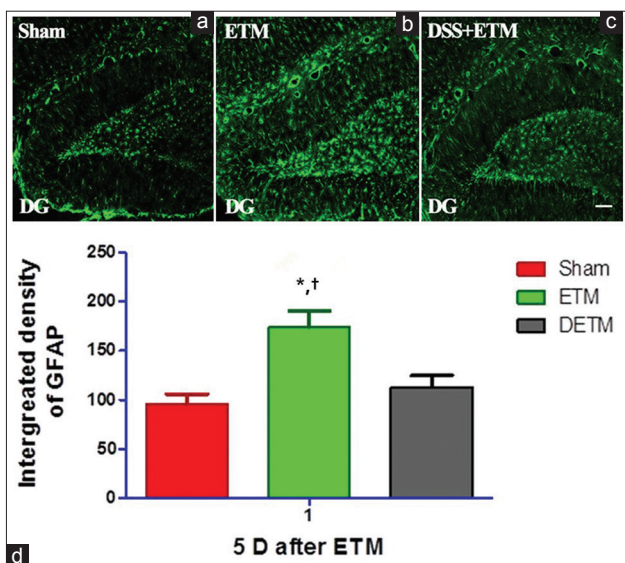regulatory role on activation of neuron is of great interest to explore in the future.

Figure 5: Astrocytes activation and GFAP expression levels in the ipsilateral dentate gyrus (DG) of hippocampus. (a) Expression of GFAP in the sham group; (b) Expression of GFAP at 5 days after experimental tooth movement (ETM) operation, ETM induced a remarkable astrocytes activation indicated by GFAP up-regulation in the ipsilateral DG of hippocampus at 5 day; (c) DSS pretreatment starting at 5 days prior to ETM operation significantly inhibited the ETM induced astrocytes activation at postoperative day 5. Bars = 200 μm; *$P < 0.01$ versus sham group; $^\dagger P < 0.05$ versus DETM group.

It has been shown that the hippocampal formation is particular important in spatial learning and memory. The plasticity of hippocampal synaptic may be the neuronal basis for hippocampus-dependent learning. Using immunofluorescent DiL-stained techniques, we offered evidence that the positive effect of DSS on ETM-induced the spatial learning and memory was accompanied with the inhibited neuronal activations in hippocampal culture. It is reported that inflammatory pain can cause the decline of the rats' learning and memory function and anti-inflammatory drugs could improve the learning and memory function of the inflammatory pain rats,[41-43] and we also found that astrocytes obvious activation and increase of GFAP expression levels in the ipsilateral DG of hippocampus at 5 days after ETM operation. Daily DSS pretreatment starting at 5 days prior to ETM operation significantly inhibited the ETM-induced astrocytes activation at POD 5. The positive effect of DSS on ETM-induced the spatial learning and memory was accompanied with the inhibited astrocytes activations in hippocampus. In addition, studies have shown that the significant up-regulation of astrocytic GFAP expression in the present results in trigeminal spinal nucleus caudalis (SpVc), which is the brain primary central pain perception, is observed on days 7 and 14 of ETM, indicating a nociceptive facilitation by astrocytes.[44] Alternatively, our previous results have proved that early microglial activation, as seen by the significant increase in iba-1 in SpVc on days 1, 3 after POD, is consistent with the development of behavioral hypersensitivity.[21] Although it is unclear whether that there
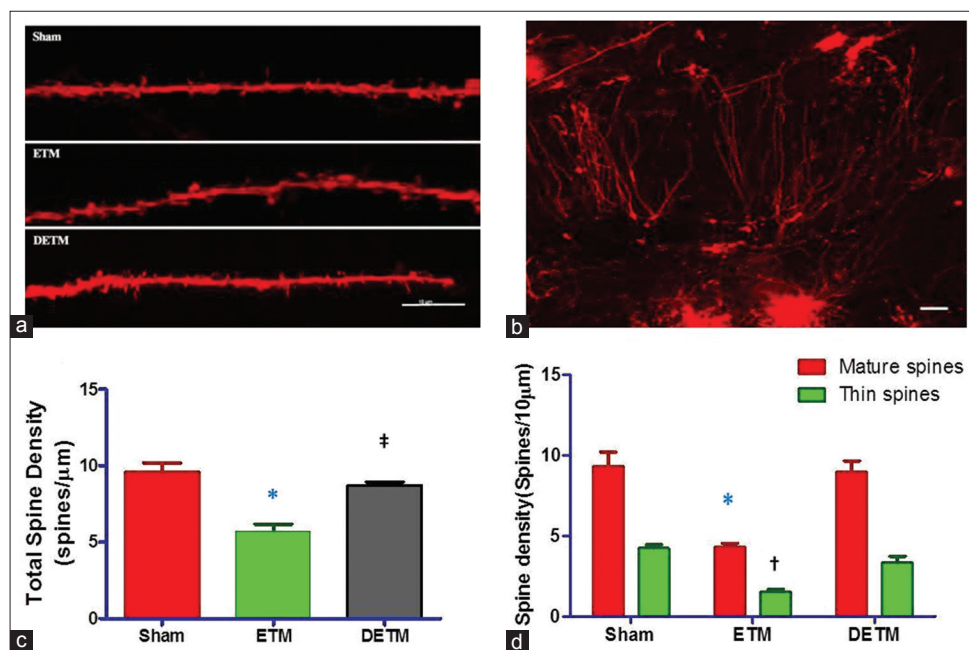

Figure 6: DiL-stained coronal sections of hippocampal tissue reveal dendritic spine remodeling on effects of experimental tooth movement (ETM) in neurons of hippocampal culture. (a) A representative image of the neuron located in hippocampal culture); (b) A high-power field of the neuron shown in a (inset); (c, d, a) Blinded observer analyzed dendritic spines from 10 to 13 randomly sampled neurons from each treatment arm for spine total spine density; (c) and spine density of mature and thin; (d) Data represent mean ± standard error of mean ($n = 6$). Sham group versus ETM group, *$P < 0.01$; $^\dagger P < 0.05$; DSS + ETM group versus ETM group, $^\ddagger P < 0.05$; Bars = 10 μm.

are distinct plastic changes in glia-neuron interactions in hippocampus after ETM which could cause a rule change of the spatial learning and memory in rats, our results suggest that DSS seems to improve spatial memory ability through the inhibition of hippocampal astrocytes in rats after ETM.

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