Localization of glial cell marker proteins in the rat mesencephalic trigeminal nucleus

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Running title: Neuron-glia network involved in jaw movement

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Abstract

Oral dyskinesia, defined as repetitive involuntary movements appearing in the oral and maxillofacial region, is an oral function abnormality that decreases the quality of life of patients. However, the pathogenesis of oral dyskinesia remains unknown and the condition is often difficult to treat. Since 2000, glial cells have attracted attention as a target for therapeutic agents for Parkinson's disease, which is characterized by the same involuntary movements as oral dyskinesia. However, the distribution and role of glial cells in brainstem regions such as the pons and the medulla oblongata, where many neurons involved in oral and maxillofacial movements are located, have not been studied in detail. Thus, this study performed immunohistochemical analysis to investigate the localization of glial cell marker proteins in the mesencephalic trigeminal nucleus (Me5), which is involved in the control of jaw movement. To do so, the brainstem regions of rats were removed and coronal sections were prepared, followed by immunofluorescence staining for the glial cell markers glial fibrillary acidic protein (GFAP), S100 protein, and neuron-glial antigen 2 (NG2), which showed a large number of GFAP-, S100-, and NG2-positive cells around neurons in the Me5. These results suggested that glial cell marker-positive cells in the Me5 secrete ATP and other signaling

molecules and are involved in jaw reflexes such as the mandibular tensor and periodontal masseter reflexes, which regulate mandibular movements. Future studies will evaluate the expression of these marker molecules during postnatal development and investigate the effects of glial cells on neural circuit formation in the Me5 in detail, to elucidate the pathogenesis of oral dyskinesia and other oral dysfunctions.

Keywords: mesencephalic trigeminal nucleus, GFAP, S100, NG2

Introduction

Jaw movements such as chewing and sucking are established by the peripheral jaw movement system based on excitatory and inhibitory output from the central nervous system [1, 2]. The development of treatments for oral dysfunction related to these activities requires the elucidation of neural pathways, including postnatal development plasticity. Oral dyskinesia is a symptom that is particularly difficult to address in clinical dentistry. Adverse reactions to a number of the drugs used to treat neurological disease are regarded as the cause of oral dyskinesia' however, many cases are idiopathic (unknown cause), and almost all cases are difficult to treat [3]. Thus, this study used rats as model organisms to determine the role of glial cells in the neural network controlling jaw movement, which has not been studied to date, and to elucidate the cause of oral dysfunction, including oral dyskinesia.

Unlike humans, rodents, including rats, have an immature cell structure in the central nervous system at birth; thus, their peripheral nerves subsequently grow from the sucking to chewing developmental stages after the lactation period [4-6]. Therefore, rodents are considered useful models to analyze jaw movement from the perspective of

embryology or growth and development.

“Glial cells” is the generic term to describe non-excitatory cells in the cranial nervous system. These cells are classified as astrocytes, oligodendrocytes, Schwann cells, and microglia. The function of glial cells was traditionally thought to be related to nerve function, including providing physical protection and substance metabolism in the nervous system. However, the dynamics of glial cells are being elucidated with the development of methods to measure intracellular calcium levels. The findings suggest the involvement of glial cells in many central phenomena of nerve function, including neurotransmission and plasticity changes [7-11]. Previous studies on glial cells have mainly focused on physiological changes in the culture system; however, the elucidation of actual conditions in nerve tissue is required.

We previously investigated the morphology of neuron-glia communication in the trigeminal motor nucleus, which is the main component that forms the neural network for jaw movement. We reported that some glial marker-positive cells in the trigeminal motor nucleus may be involved in the neural network that controls masticatory movement, particularly the jaw opening movement [12].

Therefore, this study observed the distribution of glial cells in the mesencephalic trigeminal nucleus, which controls the mechanoreceptor of the periodontal ligament and the muscle spindles of jaw closing, which are the main components that form another neural network for jaw movement [13]. This study selected three types of glial cell markers; namely, glial fibrillary acidic protein (GFAP), a cytoskeleton protein that is an astrocyte-specific marker used in many studies to date [14-16]; S100 protein (S100), also a marker molecule for astrocytes and oligodendrocytes [17, 18]; and chondroitin sulfate proteoglycan (neuron-glial antigen 2, NG2), a marker molecule for oligodendrocytes [19]. We conducted immunohistochemical investigations of glial cell localization in the mesencephalic trigeminal nucleus of the rat.

Materials and methods

1. Experimental animals

The experimental animals were 10 male Wistar rats weighing 250 to 350 g (8-12 week old; Clea Japan, Inc., Tokyo). Two of these rats were used for Nissl staining; the remaining 8 rats were used for immunostaining. The animals were housed in a room with a 12-hour light and dark cycle, room temperature of 20℃, and free access to water and food. This study was approved by The Animal Care and Use Committee of Kanagawa Dental University and was conducted in compliance with established experimental animal guidelines (Approval No.13-012). The test animals were handled ethically, pursuant to the provisions of The Standards on the Care and Storage of Animal Experiments (Prime Minister's Office Advertisement No.6, March 27, 1980). The animals were put under deep anesthesia via the intraperitoneal injection of sodium barbiturate (50 mg/kg), underwent thoracotomy. After perfusion fixation using 0.1 M phosphate buffer (pH 7.4) with 4% paraformaldehyde, into the left ventricle, the brainstem was removed, including the mesencephalic trigeminal nucleus area, which was fixed overnight (4℃, 12 hours) in the same fixing solution.

2. Nissl staining and fluorescent Nissl staining with Neuro Trace green

The post-fixed brainstem was immersed in 0.1 M phosphate-buffered saline (PBS) (pH 7.4) with 25% sucrose, then embedded with Tissue-Tek O.C.T. Compound (Sakura Finetek Japan Co., Ltd., Tokyo). Frozen coronal sections of the brainstem were prepared to 40 μm thickness using a cryostat, classified into two groups, stained as follows, and observed microscopically.

1) The group 1 sections were prepared with Nissl stain using a 1% cresyl violet solution in a 95% ethanol aqueous solution. The sections were dehydrated using ethanol and xylene, treated using a tissue clearing technique, and sealed. The cell structure of the pontine tegmentum area from the midbrain tegmentum, including the trigeminal mesencephalic nucleus, was then observed under an optical microscope.

2) The group 2 sections were immersed in Neuro Trace green (Molecular Probes, Oregon, USA ; 1: 500, 0.1 M PBS, pH 7.4, 4℃) for 30 minutes and washed in 0.1 M PBS (room temperature) for 30 minutes. Each section was then mounted on a glass slide and sealed and the same field that was observed by optical microscopy was observed with a fluorescence microscope. A BZ-8000 all-in-one fluorescence microscope (Keyence Corporation, Tokyo) was used for the microscopic

observations, and both optical and fluorescence microscope images were photographed.

3. Immunohistochemical analysis using GFAP, S100, and NG2 antibodies

After fixing the tissue in the same way as described in 2., 40-μm-thick post-fixed brainstem frozen coronal serial sections were prepared using a cryostat. The sections were divided into three groups, and the sections from each group were immersed in rabbit anti-GFAP antibodies (Shima Laboratories, Co., Ltd., Tokyo; 1: 500, 0.1 M PBS, pH 7.4, 4℃), mouse anti-S100 antibodies (Chemicon, Pittsburgh, PA, USA ; 1: 100, 0.1M PBS, pH 7.4, 4℃), or rabbit anti-NG2 antibodies (Abcam, Tokyo ; 1: 100, 0.1M PBS, pH 7.4, 4℃) for 24 hours, then immersed in biotinylated secondary antibodies (Dako, Glostrup, Denmark ; 1: 500, 0.1 M PBS, pH 7.4, 4℃) for one hour, and then immersed in Cy3-streptavidin (Sigma, Missouri, USA ; 1: 500, 0.1 M PBS, pH 7.4, 4℃) for one hour. After immersion in the primary antibodies, secondary antibodies, and fluorescently labeled streptavidin, each section was washed with 0.1 M PBS (room temperature) for 1 hour. The immunohistochemically prepared sections were then immersed in

Neuro Trace green (1: 500, 0.1 M PBS, pH 7.4, 4℃) for 30 minutes and washed with 0.1 M PBS (room temperature) for 30 minutes. Each section was then mounted on a glass slide and sealed using a mounting medium. Finally, the cell structure of the mesencephalic trigeminal nucleus area was observed with a fluorescence microscope.

Results

1. Nissl staining and fluorescent Nissl staining images using Neuro Trace green

Observation of the cell structure of Nissl-stained coronal sections of the brainstem area of rats, including the mesencephalic trigeminal nucleus, revealed relatively large neuronal aggregates rich in Nissl substances in the ventromedial part of the brachium conjunctivum (superior cerebellar peduncle) of the pontine tegmentum area from the midbrain tegmentum of the brainstem in the experimental animals. Furthermore, the locus ceruleus, a group of noradrenalinergic neurons, was observed in a group of medium to small neurons on the medial side of the mesencephalic trigeminal nucleus (Fig. 1a). However, labeling the same site with Neuro Trace green showed groups of neurons such as those in the mesencephalic trigeminal nucleus and locus ceruleus under fluorescence microscopy (Fig. 1b). Insert Fig.1

2. Distribution of GFAP-positive cells

Immunofluorescence staining with GFAP antibodies of coronal sections of the pontine tegmentum area from the midbrain tegmentum, including the mesencephalic trigeminal nucleus revealed a large number

of GFAP-positive cells with protrusions in the mesencephalic trigeminal nucleus and locus ceruleus. The GFAP immunopositive findings, in particular, showed a strongly positive image around the nerve cell bodies within the mesencephalic nucleus and the vascular walls peripheral to the mesencephalic nucleus. GFAP-positive cells were also observed among the ependymal cells lining the fourth ventricle (Fig. 2).

3. Distribution of S100 positive cells

Immunofluorescence staining with S100 antibodies of coronal sections including the mesencephalic trigeminal nucleus in the same manner as GFAP revealed a large number of S100-positive cells in the mesencephalic trigeminal nucleus and locus ceruleus, nearly the same as observed for GFAP. Positive cells were also observed around the nerve cell bodies within the mesencephalic nucleus and around the vascular walls peripheral to the mesencephalic nucleus, similar to the GFAP-immunopositive findings. Moreover, although the signal was weaker than that of GFAP, S100 positive cells were also observed in the ependymal cells lining the ventricles (Fig. 3).

4. Distribution of NG2-positive cells

Immunofluorescence staining using NG2 antibodies revealed a large number of NG2-positive cells in the mesencephalic trigeminal nucleus and locus ceruleus, similar to both GFAP and S100, and strongly positive findings around the nerve cell bodies in the mesencephalic nucleus. However, unlike the GFAP and S100 protein findings, there were no positive findings around the vascular walls peripheral to the mesencephalic nucleus; however, as seen with GFAP and S100, NG2-positive cells were observed among the ependymal cells lining the fourth ventricle (Fig. 4).

Insert Fig.2
Insert Fig.3
Insert Fig.4

Table 1

Discussion

1. Nissl stain and fluorescent Nissl stain images using Neuro Trace green

Nissl staining is a standard histology method used to visualize neurons in the brain and spinal cord. The Nissl substance comprises ribosomal RNA derived from the rough endoplasmic reticulum in the perikaryon and dendrites, which is redistributed in the nerve cell body if the nerve is damaged or regenerating. Therefore, it also serves as a marker for the physiological condition of the neuron. Neuro Trace green, which was also used in this study, binds selectively to the Nissl substance, characteristic of neurons based on the findings. Thus, Neuro Trace green can also be used similarly to other histological Nissl stain reagents such as toluidine blue and cresyl violet and is a useful counterstaining reagent in immunofluorescent antibody analyses such as those used in the present study.

2. Distributions of GFAP-, S100-, and NG2-positive cells

This study investigated the localizations of the glial cell (particularly astrocyte) markers GFAP and S100, as well as the oligodendrocyte marker molecule NG2 [14-19].

GFAP is a cytoskeleton protein (intermediate filament) with a molecular weight of approximately 50 k that is found specifically in astrocytes. GFAP expression is increased in neurological disorders such as brain injury, dementia, prion disease, and multiple sclerosis [20, 21]. Experiments measuring ATP in an astrocyte-specific culture system showed that GFAP-positive cells (astrocytes) can release ATP [22]. ATP is a neurotransmitter; moreover, there are seven types of ion channel P2X receptors and eight types of G-protein coupled P2Y receptors distributed as ATP receptors on neurons [22].

S100 is a calcium-binding protein with a molecular weight of approximately 10 k that is found in astrocytes in the central nervous system, similar to GFAP. It is also found in Schwann and satellite (ganglion cells) cells in the peripheral nervous system [23, 24]. Clinically, S100 is a marker for glioma [25-28]. Sakatani et al. [29] reported that S100 was secreted by hippocampal neurons with increased neuronal activity due to the administration of kainic acid and that the secreted S100 acted as a signal transducer from astrocytes to neurons and regulated neuronal activity.

NG2-positive cells, generally called oligodendrocyte progenitor cells. are considered to be glial progenitor cells that differentiate into

oligodendrocytes and astrocytes [30]. However, the possibility that NG2-positive cells can also differentiate into inhibitory neurons, as well as oligodendrocytes and astrocytes, has recently been proposed' moreover, the possibility that these cells are also involved in neuron support and synaptic transmission as glial cells has also been reported [31]. NG2 is a type of neuroglycan involved in neural circuit formation and neurotransmission. NG2 also acts to control cell substratum interactions when melanoma cells differentiate and migrate in the basement membrane of the endothelium. However, studies have also suggested that NG2 inhibits neurite outgrowth and collapse of the nerve growth cone in axon regeneration [32-38]. Thus, NG2-positive cells are abundant in the adult brain and differentiate into various cells and serve various functions. They are now categorized as a fourth type of cell separate from nerve cells, astrocytes, and oligodendrocytes [39].

The results of this study revealed a large number of cells expressing the glial cell marker molecules GFAP, S100, and NG2 around nerve cell bodies in the mesencephalic trigeminal nucleus. Thus, as previously reported, these glial cell marker molecule-positive cells located in the mesencephalic trigeminal nucleus may be involved in the neural network controlling jaw movement, by secreting signal transducers

such as ATP and by differentiating into inhibitory neurons [22, 39]. In 1999, Araque et al. [40] proposed the concept of the tripartite synapse, whereby glia and neurons each express neurotransmitter receptors while also releasing transmitters, indicating that brain function was no longer considered to be simply a circuit created by neurons but rather that the brain may be produced from a more extensive circuit created by glial cells and neurons. In other words, this tripartite synapse adds the presence of synapses between the astrocytes peripheral to the synapses created by presynaptic neurons and postsynaptic neurons. If we consider the processing of information in the brain while also considering the presence of these kinds of synapses, the brain function originally considered to operate on a circuit created only by neurons would be a much more complex and profound system. Furthermore, since 2001, glial cells have attracted attention as a target for therapeutic agents for Parkinson's disease, which is characterized by the same involuntary movements as oral dyskinesia [41-44].

These results suggest that there is the tripartite synapse between the neurons in the mesencephalic trigeminal nucleus and the surrounding GFAP- or S100-immunopositive astrocytes, and that this synapse may control jaw movement. In addition, NG2-immunopositive glia in the

periphery of neurons in the mesencephalic trigeminal nucleus cannot be identified by the results of this study. However, if they differentiate into astrocytes, they may form tripartite synapse. Alternatively, if they differentiate into inhibitory neurons, they may release neurotransmitters such as glycine to suppress the activity of the mesencephalic trigeminal nucleus. In addition, the GFAP- and S100-immunopositive cells observed surrounding the blood vessels in the periphery of the mesencephalic trigeminal nucleus were thought to be astrocytes that probably form the blood-brain barrier. On the other hand, NG2-positive cells were not observed around blood vessels, suggesting that they are not involved in the formation of the blood-brain barrier. Moreover, it has been reported that there is also a neural network between the locus ceruleus and the mesencephalic trigeminal nucleus[45]. Therefore, it has been suggested that glial marker-positive cells in the locus ceruleus are also involved in the regulation of this neural network.

Further studies are needed to assess the expression of glial cell markers and tripartite synapse-related marker molecules in postnatal development, as examined in this study, as well detailed examinations of the effect of glial cells on the formation of neural circuits in the

mesencephalic trigeminal nucleus and link the findings to the identification of the cause of oral dysfunction, including oral dyskinesia.

Conclusion

The results of this study observed localization using glial cell markers to investigate the relationship between neurons and glial cells in the mesencephalic trigeminal nucleus, which is involved in the formation of the neural network controlling jaw movement. Moreover, a large number of glial cell marker molecule-positive cells were observed around the nerve cell bodies in the mesencephalic trigeminal nucleus. Previous studies have reported that glial cells not only provide physical protection and substance metabolism in the nervous system but are also involved in information transmission in neural networks. Therefore, the mesencephalic trigeminal nucleus glial marker-positive cells found in this study may also be similarly involved in the neural network controlling jaw movement. Further research is needed to establish treatments for oral dysfunctions such as oral dyskinesia, which often have unknown etiology.

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Conflicts of interest statement

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

1. Squire LR, Bloom FE, Spitzer NC. Fundamental Neuroscience; 2nd edition, Academic Press, London, 753–766, 2002.
2. Kandel ER, Schwartz JH, Jessell TM. Principles of Neural Science; 4th edition, McGraw-Hill, New York, 349–380, 982–996, 1227–1246, 2000.
3. Blanchet PJ, Rompré PH, Lavigne GJ, Lamarche C: Oral dyskinesia: a clinical overview. *Int J Prosthodont.* **18**(1): 10-19, 2005.
4. Nozaki S, Iriki A, Nakamura Y: Localization of central rhythm generator involved in cortically induced rhythmical masticatory jaw-opening movement in the guinea pig. *J Neurophysiol.* **55**(4): 806-825, 1986.
5. Nozaki S, Iriki A, Nakamura Y: Role of corticobulbar projection neurons in cortically induced rhythmical masticatory jaw-opening movement in the guinea pig. *J Neurophysiol.* **55**(4): 826-845, 1986.
6. Iriki A, Nozaki S, Nakamura Y: Feeding behavior in mammals; corticobulbar projection is reorganized during conversion from sucking to chewing. *Brain Res.* **44**: 189-196, 1988.

7. Nicholls JG, Kuffler SW: Na and K content of glial cells and neurons determined by flame photometry in the central nervous system of the leech. *J Neurophysiol.* **28**: 519-525, 1965.

8. Kuffler SW, Nicholls JG: The physiology of neuroglial cells. *Ergeb Physiol.* **57**: 1-90, 1966.

9. Kuffler SW, Nicholls JG, Orkand RK: Physiological properties of glial cells in the central nervous system of amphibia. *J Neurophysiol.* **29**(4): 768-787, 1966.

10. Haydon RG: Listening and talking to the synapse. *Nat Rev Neurosci.* **2**(3): 185-193, 2001.

11. Fields RD, Stevens-Grahan B: New insights into neuron-glia communication. *Science.* **298**(5593): 556-562, 2002.

12. Akagi T, Kawata A, Takahashi O: Study of neurons and glial cells in the trigeminal motor nucleus and inhibitory neurons projecting to the trigeminal motor nucleus. *J Kanagawa Odontol. Soc.* **48**(2): 71-79, 2013.

13. Fujise T, Yoshimatsu H, Kurokawa M *et al.*: Satiation and masticatory function modulated by brain histamine in rats. *Proc Soc Exp Biol Med.* **217**: 228-234, 1998.

14. Bignami A, Eng LF, Dahl D, Uyeda CT: Localization of the glial

fibrillary acidic protein in astrocytes by immunofluorescence. *Brain Res.* **43**(2): 429-435, 1972.

15. Bignami A, Dahl D: Astrocyte-specific protein and neuroglial differentiation. An immunofluorescence study with antibodies to the glial fibrillary acidic protein. *J Comp Neurol.* **153**(1): 27-38, 1974.
16. Benitz WE, Dahl D, Williams KW, Bignami A: The protein composition of glial and nerve fibers. *FEBS. Lett.* **66**(2): 285-289, 1976.
17. Ludwin SK, Kosek JC and Eng LF: The topographical distribution of S-100 and GFA proteins in the adult rat brain: an immunohistochemical study using horseradish peroxidase-labelled antibodies. *J Comp Neurol.* **165**(2): 197-207, 1976.
18. Tabuchi K, Kirsch WM: Immunocytochemical localization of S-100 protein in neurons and glia of hamster cerebellum. *Brain Res.* **92**(1): 175-180, 1975.
19. Dugas JC, Ibrahim A, Barres BA: A crucial role for p57 (Kip2) in the intracellular timer that controls oligodendrocyte differentiation. *J Neurosci.* **27**: 6185-6196, 2007.
20. Salinero O, Moreno-Flores MT, Ceballos ML, Wandosell F: β-Amyloid peptide induced cytoskeletal reorganization in cultured

astrocytes. *J Neurosci Res.* **47**(2): 216-223, 1997.

21. Lazarini F, Deslys JP, Dormont D: Regulation of the glial fibrillary acidic protein, beta actin and prion protein mRNAs during brain development in mouse. *Brain Res Mol Brain Res.* **10**(4): 343-346, 1991.

22. Price AB, Moneer Z, Brown GC: Nitric oxide induces rapid, calcium-dependent release of vesicular glutamate and ATP from cultured rat astrocytes. *Glia.* **40**(3): 312-323, 2002.

23. Stefansson K, Wollmann RL, Moore BW: Distribution of S-100 protein outside the central nervous system. *Brain Res.* **234**(2): 309-317, 1982.

24. Brockes JP, Fields KL, Raff MC: Studies on cultured rat Schwann cells. I. Establishment of purified populations from cultures of peripheral nerve. *Brain Res.* **165**(1): 105-118, 1979.

25. Zimmer DB, Van Eldik LJ: Analysis of the calcium-modulated proteins, S100 and calmodulin, and their target proteins during C6 glioma cell differentiation. *J Cell Biology*. **108**: 141-151, 1989.

26. Zimmer DB, Cornwall EH, Landar A, Song W: The S100 protein family: history, function, and expression. *Brain Res Bull.* **37**(4): 417-429, 1995.

27. Salama I, Malone PS, Mihaimeed F, Jones JL: A review of the S100 proteins in cancer. *Eur J Surg Oncol.* **34**(4): 357-364, 2008.

28. Van Eldik LJ, Ehrenfried B, Jensen RA: Production and characterization of monoclonal antibodies with specificity for the S100 beta polypeptide of brain S100 fractions. *Proc Natl Acad Sci USA.* **81**(19): 6034-6038, 1984.

29. Sakatani S, Seto-Ohshima A, Shinohara Y *et al.*: Neural activity-dependent release of S100B from astrocytes enhances kainate-induced gamma oscillations *in vivo*. *J Neurosci.* **28**(43): 10928–10936, 2008.

30. Zhu X, Bergles DE, Nishiyama A: NG2 cells generate both oligodendrocytes and gray matter astrocytes. *Development.* **135**(1): 145-157, 2008.

31. Zonouzi M, Scafidi J, Li P *et al.*: GABAergic regulation of cerebellar NG2 cell development is altered in perinatal white matter injury. *Nat Neurosci.* **18**(5): 674-682, 2015.

32. Ozerdem U, Grako KA, Dahlin-Huppe K *et al.*: NG2 proteoglycan is expressed exclusively by mural cells during vascular morphogenesis. *Dev Dyn.* **222**(2): 218-227, 2001.

33. Al-Mayhani MT, Grenfell R, Narita M *et al.*: NG2 expression in

glioblastoma identifies an actively proliferating population with an aggressive molecular signature. *Neuro Oncol*. **13**(8): 830-845, 2001.

34. Girolamo F, Dallatomasina A, Rizzi M *et al.*: Diversified expression of NG2/CSPG4 isoforms in glioblastoma and human foetal brain identifies pericytes subsets. *PLoS One*. **8**(12): e84883, 2013.
35. Poli A, Wang J, Domingues O *et al.*: Targeting glioblastoma with NK cells and mAb against MG2/CSPG4 prolongs animal survival. *Oncotarget*. **4**(9): 1527-1546, 2013.
36. Levine JM, Reynolds R, Fawcett JW: The oligodendrocyte precursor cell in health and disease. *Trends Neurosci*. **24**(1): 39-47, 2001.
37. Stallcup WB: The NG2 proteoglycan: past insights and future prospects. *J Neurocytol*. **31**(6-7): 423-435, 2002.
38. Levine JM, Nishiyama A: The NG2 chondroitin sulfate proteoglycan: a multifunctional proteoglycan associated with immature cells. *Perspect Dev Neurobiol*. **3**(4):245-259, 1996.
39. Chang A, Nishiyama A, Peterson J, Prineas J, Trapp BD: NG2-positive oligodendrocyte progenitor cells in adult human brain and multiple sclerosis lesions. *J Neurosci*. **20**(17): 6404-6412, 2000.
40. Araque A, Parpura V, Sanzgiri RP, Haydon PG: Tripartite synapses:

glia, the unacknowledged partner. *Trends Neurosci*. **22**(5): 208-215. 1999.

41. Akerud P, Canals JM, Snyder EY *et al.*: Neuroprotection through delivery of glial cell line-derived neurotrophic factor by neural stem cells in a mouse model of Parkinson's disease. *J Neurosci*. **21**: 8108-8118, 2001.

42. Connor, B: Adenoviral vector-mediated delivery of glial cell line-derived neurotrophic factor provides neuroprotection in the aged parkinsonian rat. *Clin Exp Pharmacol Physiol*. **28**(11): 896-900, 2001.

43. Gill SS, Patel NK, Hotton GR *et al.*: Direct brain infusion of glial cell line-derived neurotrophic factor in Parkinson disease. *Nat Med*. **9**(5): 589-595, 2003.

44. Patel NK, Bunnage M, Plaha P *et al.*: Intraputamenal infusion of glial cell line-derived neurotrophic factor in PD: a two-year outcome study. *Ann Neurol*. **57**(2): 298-302, 2005.

45. Takahashi T, Shirasu M, Shirasu M *et al.*: The locus coeruleus projects to the mesencephalic trigeminal nucleus in rats. *Neurosci Res*. **68**(2): 103-06, 2010.

Figure legends

Figure 1　Nissl staining image

The brainstem area of rats, including the Me5, revealed relatively large neuronal aggregates rich in Nissl substances in the ventromedial part of the BC of the pontine tegmentum area from the midbrain tegmentum of the brainstem (a, b).

a: Nissl staining image of the pontine tegmentum area including the Me5.

b: Fluorescent Nissl staining image with Neuro Trace green of the pontine tegmentum area including the Me5.

Mo5: trigeminal motor nucleus, Me5: mesencephalic trigeminal nucleus, BC: brachium conjunctivum

scale bars = 300 μm

Figure 2　Distribution of GFAP-positive cells in the mesencephalic trigeminal nucleus and surrounding area

A large number of GFAP-positive cells are visible in the Me5 and LC. Strongly positive GFAP images are especially visible around the nerve cell bodies in the mesencephalic nucleus and around the vascular

walls peripheral to the mesencephalic nucleus. GFAP positivity is also visible in the ependymal cells lining the fourth ventricle (arrowhead).

a: Neuro Trace green (green), b: GFAP (red), c: merge

GFAP: glial fibrillary acidic protein, Me5: trigeminal mesencephalic nucleus, LC: locus ceruleus, *: blood vessel

scale bars = 100 μm

Figure 3 Distribution of S100 protein-positive cells in the mesencephalic trigeminal nucleus and surrounding area

S100 positivity is visible in the Me5 and around the nerve cell bodies and vascular walls in the LC, as seen with GFAP. Although the signal is weaker than that of GFAP, S100 positivity is also visible in the ependymal cells lining the ventricle (arrowhead).

a: Neuro Trace green (green), b: S100 (red), c: merge

Me5: mesencephalic trigeminal nucleus, LC: locus ceruleus, GFAP: glial fibrillary acidic protein, *: blood vessel

scale bars = 100 μm

Figure 4 Distribution of NG2-positive cells in the trigeminal mesencephalic nucleus and surrounding area

A large number of NG2 positive cells are visible in the Me5 and LC, with particularly strongly positive findings around the nerve cell body in the mesencephalic nucleus. However, unlike GFAP and S100, almost no positive findings are visible in the vascular walls. The ependymal cells lining the fourth ventricle are positive for NG2, as seen with GFAP and S100 (arrowhead)

a: Neuro Trace green (green), b: NG2 (red), c: merge

NG2: neuron-glial antigen 2, Me5: mesencephalic trigeminal nucleus, LC: locus ceruleus, *: blood vessel

scale bars = 100 μm

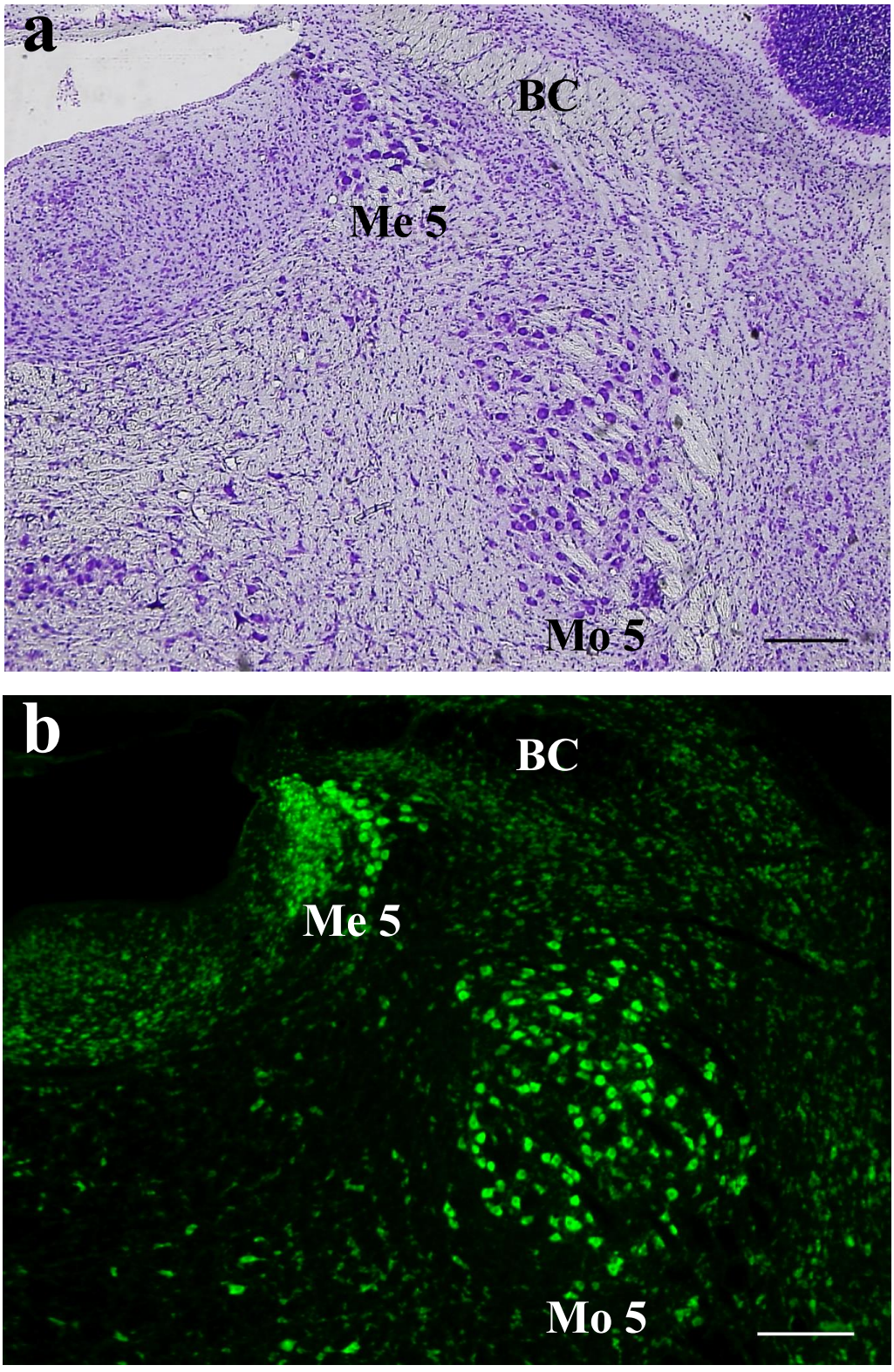

Figure 1 (Color)
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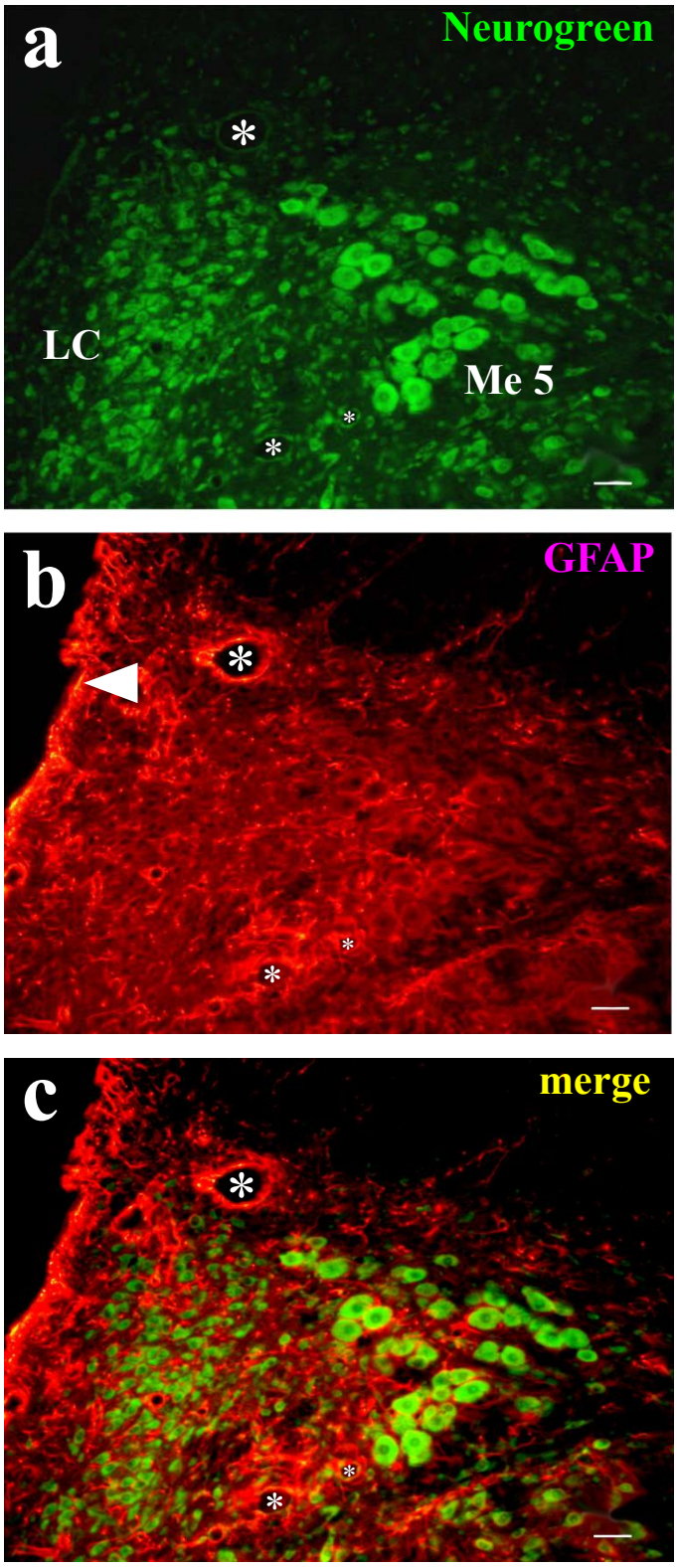

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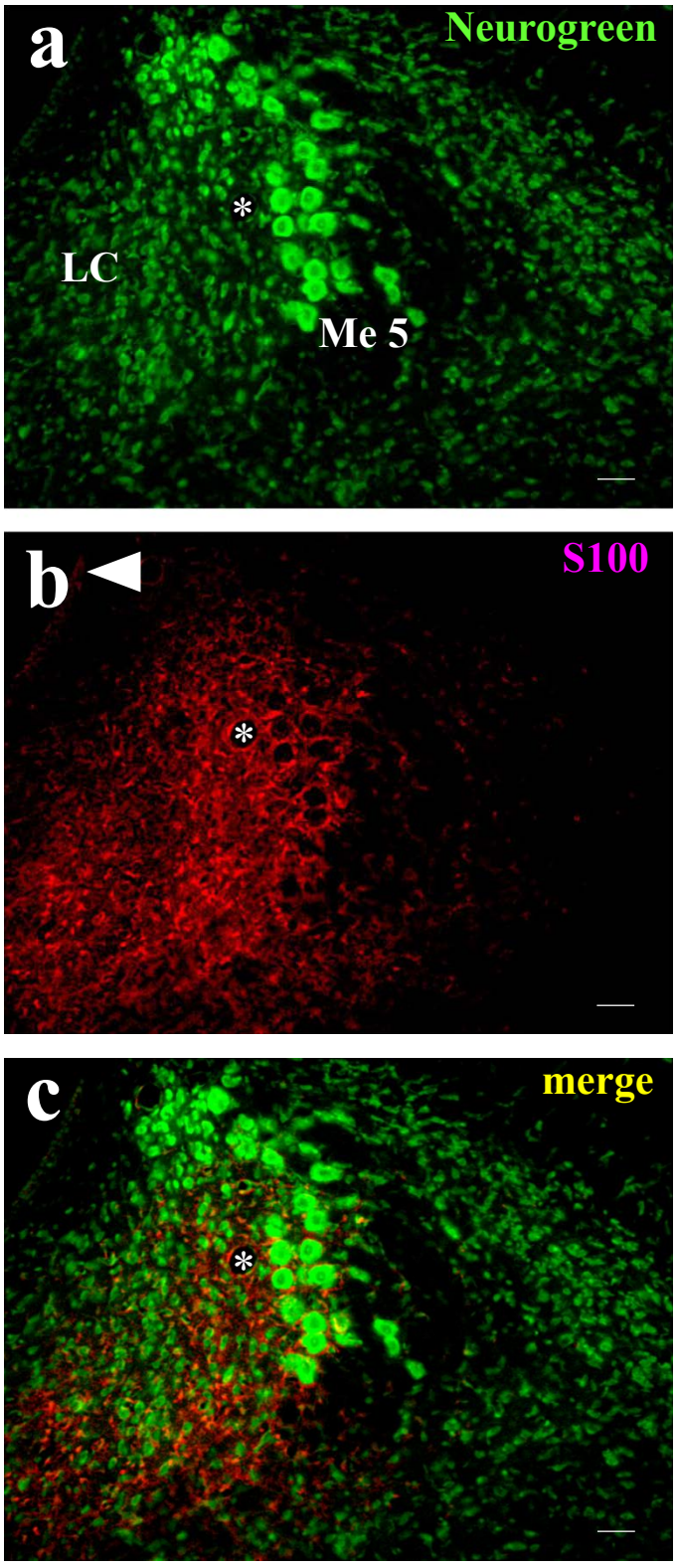

Figure 3 (Color)
Kota Watanabe

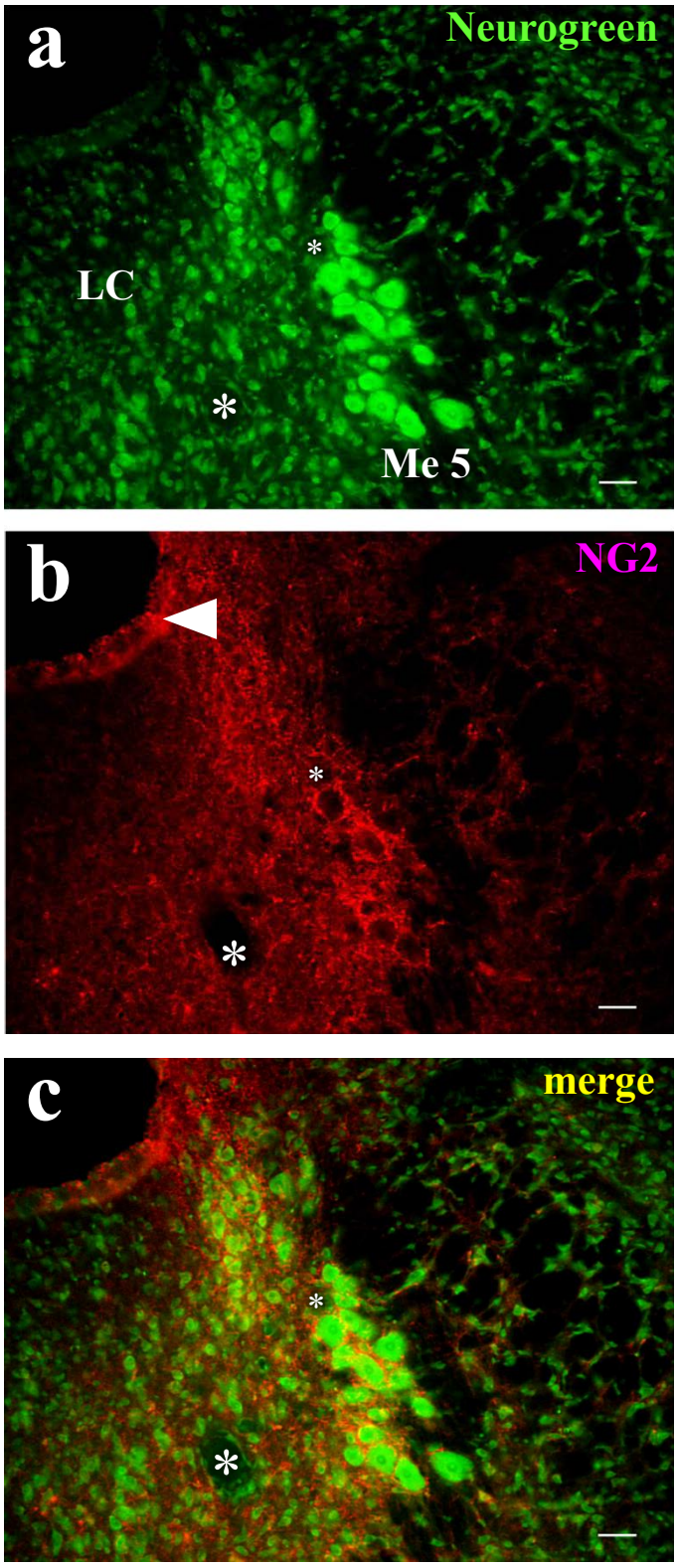

Figure 4 (Color)
Kota Watanabe

Table 1 Summary of immunostaining results

Immunofluorescence staining using for GFAP, S100, and NG2 antibodies revealed positive signals for all marker proteins in the mesencephalic trigeminal nucleus and locus ceruleus. Ependymal cells on the same sections also showed positive signals for all markers. In addition, immunopositive images for GFAP and S100 were observed in the vessel wall around the mesencephalic trigeminal nucleus, but almost negative image for NG2 was observed.

＋: positive, －/＋: almost negative

	neuronal periphery (Me5)	locus ceruleus	vascular periphery	ependymal cell
GFAP	＋	＋	＋	＋
S100	＋	＋	＋	＋
NG2	＋	＋	－/＋	＋

Table 1
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