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FULL PAPER

A BOLD-fMRI study of cerebral activation induced by injection of algesic chemical substances into the anesthetized rat forepaw

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Abstract

This study was performed to examine whether the brain activities induced by noxious algesic chemical substances in anesthetized animals could be detected by blood oxygen-level-dependent functional magnetic resonance imaging (BOLD-fMRI). Multislice gradient echo images of the primary somatosensory cortex were obtained using a 7.05 T superconducting system and a one-turned surface coil centered over the primary somatosensory cortex of the 1.0%-isoflurane-anesthetized rat. The Z-score *t*-map of BOLD signals and its time-course analysis revealed that subcutaneous injection of formalin into the left forepaw immediately induced an early response in the contralateral primary sensory cortex lasting for a few minutes, followed by a late response until 20 min after stimulation. In contrast, injection of capsaicin into the left forepaw evoked only the early response. Furthermore, pretreatment with morphine completely abolished these responses induced by the chemical algesic substances. Thus BOLD-fMRI is a useful method to analyze the brain activities of painful stimulation in anesthetized animals.

Key Words: anesthetization, formalin, functional MRI, pain, somatosensory cortex

Introduction

The assessment of pain in veterinary science is an important problem. Veterinarians usually recognize the signs of the pain by very careful observation of changes in the appearance and behavior of animals, and these signs are quantified by a score based on the observation[3]. Recently, from the view point of quality of life (QOL) of animals and their owners, analgetic treatments are recommended for postoperative animals, and pain-control is performed according to evaluation of ani-

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mal behavior by the veterinarian[6, 23]. Generally, two phenotypes of pain, acute and chronic, are well-known[3]. The acute pain sensation is elicited by noxious stimulation of nociceptive receptors within several seconds after stimulation. In contrast, chronic pain, a persistent state of pain, is known to continue for a long period after surgery, accidental injuries or inflammation. This chronic pain sometimes induces a reduction of threshold of secondary stimulation, termed hyperalgesia, and is frequently accompanied neuropathic pain such as allodynia[3].

The formalin test, which is based on the change of behavior in awake animals, is widely used to evaluate both acute and chronic pain. By using this conventional test, it was demonstrated that the early phase response (acute pain) was caused predominantly by C-fiber activation due to the peripheral stimulus, while the late phase (chronic pain) appeared to be dependent on the combination with an inflammatory reaction in the peripheral tissue[12, 20, 22]. However, from the standpoint of animal welfare, a technique to evaluate the pain in anesthetized animals is essential. Recently, blood-oxygen-level-dependent functional magnetic resonance imaging (BOLD-fMRI) is known to be useful as a noninvasive method to evaluate the brain's functional responses, including quantification of the brain responses to various types of pain[14, 16, 21]. BOLD-fMRI is an imaging technique employing the difference in the oxygenation level of hemoglobin, which is an endogenetic contrast agent in anesthetized animals. BOLD responses reflects alterations in local cerebral blood flow and volume and is considered to closely relate to neuronal activation[14]. Several investigators have reported the early responses to electrical or capsaicin stimulation in α-chloralose anesthetized animals using this BOLD-fMRI technique[1, 2, 5, 9-11, 15, 22]. Tuor *et al.*[22] observed an early phase response to formalin stimulation in α-chloralose anesthetized rats, but they could not detect a late phase response by BOLD-fMRI. In our preliminary experiments using α-chloralose anesthetized rats, we also observed the early phase response by BOLD-fMRI imaging but failed to detect the late phase response in formalin-stimulated rats. These results may indicate that α-chloralose abrogates the formalin-induced late phase response but preserves the early phase response, because early and late phase responses induced by formalin were reported by observation of nociceptive behavior in the awake rats[20]. Therefore, the condition of anesthetization, i.e., anesthetic drug, anesthetic depth and respiratory condition, is considered to be important to detect the late phase of pain.

This study was performed to examine whether the brain activities induced by noxious algesic chemicals in anesthetized animals could be detected by using the BOLD-fMRI technique. Since, with inhaled analgesics, it is relatively easy to control the depth of anesthesia and BOLD-fMRI imaging of brain activation induced by electrical stimulation of the forepaw has been reported in rats anesthetized with isoflurane[11], we employed isoflurane anesthetization with artificial ventilation in this experiment. Herein, we report BOLD-fMRI visualization of brain activities evoked by two noxious algesic chemical substances, formalin and capsaicin, in 1.0%-isoflurane-anesthetized rats.

Materials and Methods

Animal preparation : Specific-pathogen-free Sprague-Dawley (SD) rats (Slc: 300±25 g body weight, 10-11 weeks old, n=30) were obtained from Japan SLC, Inc. (Hamamatsu, Japan). The rats were housed in accordance with the Guide for the Care and Use of Laboratory Animals, Graduate School of Veterinary Medicine, Hokkaido University. They were acclimated to an air-conditioned special animal room in the Experimental Animal Facility, Graduate School of Veterinary Medicine, Hokkaido University. All rats had *ad libitum* access to food and filtered ion-exchanged water in sipper bottles. Each animal was initially anesthetized with isoflurane (5% for induction, 2% for surgery) in 30% oxygen and air (1.5 l/min). Catheters

(PE 50) containing heparinized saline were placed in a femoral artery and vein cannulated to monitor mean arterial blood pressure and heart rate and to use for administering saline or morphine (3 mg/kg, Tanabeseiyaku, Osaka, Japan), respectively. The right external jugular vein was cannulated to achieve muscle relaxation by i.v. injection of gallamine (Sigma-Aldrich, St. Louis, MO). The rats were tracheotomized and artificially ventilated. Then gallamine was administered (initial 50 μg/kg; supplemental 50 μg/kg/h) after the artificial ventilation (volume: 3.0-4.0 ml, rate: 75/min). Lidocaine (1%, AstraZeneca, Osaka, Japan) was administered to all surgical wound sites prior to closure. For chemical stimulation, plastic cannula tubing (22gage, TERUMO, Tokyo, Japan) was placed subcutaneously into the left dorsal forepaw and secured in position with surgical tape. The tubing was filled with 50 μl of either saline, buffered formalin (5%), or capsaicin (10 μg). After surgery, rats were placed on a plastic MR holder and wrapped with a hot-water-circulating blanket that kept them at 37.5±1.0℃ throughout the experiment. The rat's head was held in position using ear pins and an incisor bar.

fMRI measurements: All functional imaging experiments were carried on a 7.05-Tesla Varian Unity INOVA system (Varian Inc. Palo Alto, CA). A DSI-1083 one-turned surface coil (16 mm in diameter, Doty, Columbia, SC) was centered over the primary somatosensory cortex. After setting up the MRI experiments, anesthesia was reduced to 0.6, 0.8, 1.0, 1.2, or 2.0 % to investigate the most appropriate concentration of isoflurane. As for the appropriate concentration of isoflurane with artificial ventilation and muscle relaxation, the following two conditions were requested; 1) to maintain stable anesthetization, BOLD signal was under 2% before stimulation, and 2) to observe the early phase and the late phase responses following formalin stimulation, two phases responses were clearly observed and each BOLD signal was 2% or more. Coronal slice shimming at the center of the coil was performed automatically to obtain half height linewidths of the water signals of less than 20 Hz for a 5 mm-thick coronal slice. Five sequential coronal slices 0.6 mm thick centered 0.2 mm posterior to the bregma[17] for covering the primary somatosensory cortex of the forepaw (Fig. 1) were acquired for fMRI using a T2* weighted gradient echo sequence (GRE, TR = 375 ms, TE = 25 ms, flip angle = 50° and a resolution of 64 × 64 in 2.56 × 2.56 cm^2 resulting in a voxel size of 0.4 × 0.4 × 0.6 mm^3). In each experiment, 8 dummy scans were used to establish steady-state magnetization before data acquisition. In the fMRI experiments, a total of 80 sets of images per rat acquired over 28 min, *i.e.* 20 sets of images were acquired for 4 min before stimulation, and then 60 sets of images for 24 min after stimulation. After the fMRI experiment, high-resolution GRE images were acquired for the same slice positions in the primary somatosensory cortex used of fMRI experiments. The following parameters were employed: field of view, 2.56 × 2.56 cm^2; slice thickness, 0.6 mm; acquisition number, 4; image matrix size, 256 × 256; TR, 375 ms; TE, 25 ms. Upon completion of all MRI experiments, rats were euthanized with pentobarbital (200 mg/kg, i.v.).

Data Analysis: Image data processing and analyses of the significance of signal changes were processed off-line using MEDx (Sensor Systems, Sterling, VA) running on a LINUX workstation. For the series of multislice images, the parametric unpaired *t*-test was used and BOLD signal change (unpaired *t*-test Z-score) maps were semiautomatically created on a pixel-by-pixel basis from the images in the prestimulation (20 images for 4 min before stimulation) and poststimulation (60 images for 24 min after stimulation) periods. To locate the activation region in the primary somatosensory cortex (Fig. 1B), each Z-score *t*-map was linearly superimposed on a 256 × 256 image matrix anatomic image[10]. Activated regions were defined as pixels that revealed a statistically significant response at a level of $P<0.01$ (uncorrected Z-value> 2.34). Data are presented as the mean±SEM. A two-tailed *t*-test was used to show significant dif-

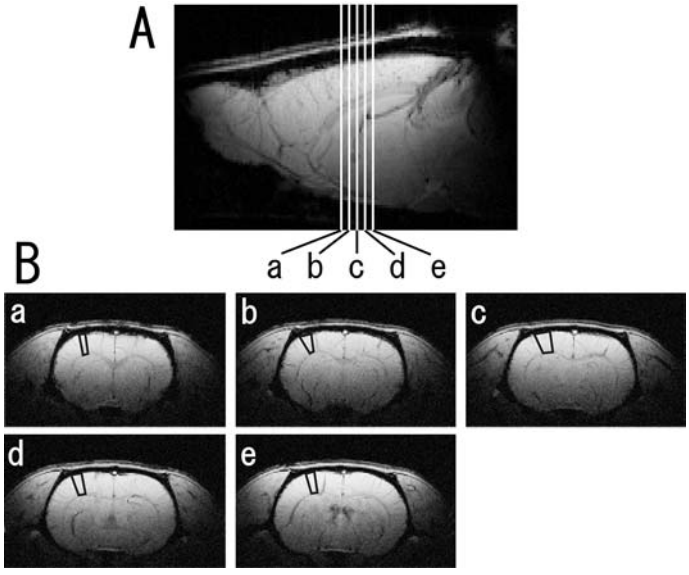

Fig. 1. Slice selection for primary somatosensory cortex. *A.* Sagittal slice of gradient echo image for selection of the coronal slices. a, b, c, d, and e are the slice positions 1.2, 0.7, 0.2, -0.3, -0.8 mm, with plus and minus being anterior and posterior to the bregma[17], respectively. *B.* The trapezium is the primary somatosensory cortex of the left forepaw in each slice position of coronal slices. a, b, c, d, and e are the slice positions 1.2, 0.7, 0.2, -0.3, -0.8 mm to the bregma[17].

ferences between the no-morphine and morphine pretreatments at each time period ($P<0.05$).

Systemic blood pressure: Systemic blood pressure was monitored through the right femoral artery to a pressure transducer using PowerLab (ADinstruments, Castle Hill, Australia) during fMRI experiments.

Results

Isoflurane concentration

The most appropriate concentration of isoflurane with artificial ventilation and muscle relaxation was investigated. Figure 2 shows representative time course of BOLD signal intensity were plotted for % changes from 5% formalin stimulation onset as a function of poststimulation time under various isoflurane concentrations (0.6, 0.8, 1.0, 1.2, and 2.0%, respectively). When muscle relaxation was administrated in animal, it was impossible to monitor stable anesthetization by the change of animal behavior against pain stimulation. Under 2% isoflurane anesthesia, which concentration is usually used for surgery, BOLD signal was under 2% after saline stimulation into the left forepaw (data not shown). Therefore, 2% or more BOLD signal were defined as the responses of pain. In addition, when the change in BOLD signal before stimulation was under 2%, the isoflurane concentration was regarded as stable anesthetization. Moreover, it was important that the early phase and the late phase responses following formalin stimulation were clearly observed and each response was 2% or more. As shown in Fig. 2, 1.0% and 1.2% isoflurane anesthesia satisfied these conditions, however, under 1.2% anesthesia, the early phase responses were lower than that under 1.0%. Thus, we determined that 1.0% isoflurane anesthesia was suitable to observe the early phase and the late phase responses following formalin stimulation.

Formalin stimulation

Figures 3A and 3B show representative multislice BOLD fMRI activation maps obtained after 5% formalin (50 μl) stimulation of the left forepaw after no-morphine (saline) and morphine (3 mg/kg) administrations, respectively. The red pixels in Fig. 3A show the regions indicating significant formalin-induced increased responses in the Z-score *t*-map ($P<0.01$) and were superimposed on anatomical MR images. Obviously, the formalin-induced increased responses in BOLD signals were found in the left forepaw primary so-

matosensory area of the right hemisphere (Fig. 3A), whereas the morphine administration remarkably reduced the number of red pixels (Fig. 3B). Time courses of average of signal intensity were plotted for % changes from stimulation onset as a function of poststimulation time (Fig. 3C). In the saline-administered (no-morphine) group, increases of about 8% in BOLD signals (early phase) were immediately observed lasting for 4 min after stimulation. Furthermore, increases of about 2-3% in BOLD signals (late phase) were sustained from 4 to 20 min after stimulation (black circles in Fig. 3 C). In the morphine-administered group, no increase in BOLD signals was detected for 24 min (white circles in Fig. 3C), indicating that the morphine administration completely inhibited the early phase and the late phase induced by formalin stimulation. Time courses of the changes in the systemic mean blood pressure are shown in Fig. 3 D. As significant increase ($\Delta 47.8 \pm 8.9$ mmHg) was observed at 30 sec after stimulation, whereas no increase in the response of the blood pressure was induced from 4 to 24 min after stimulation (black circles in Fig. 3D). In the morphine-administered group, an increase ($\Delta 24.2 \pm 3.2$ mmHg) was observed at 30 sec after stimulation but the response was smaller than that in the saline group (white circles in Fig. 3D).

Capsaicin stimulation

Figures 4A and 4B show representative multislice BOLD fMRI activation maps obtained after 10 μg capsaicin (50 μl) stimulation of the left forepaw after no-morphine (saline) and morphine (3 mg/kg) administrations, respectively. The functional MRI maps in Figs. 4A and 4B show that significant increases in BOLD signals induced by capsaicin were detected in the left forepaw primary somatosensory area of the right hemisphere and that morphine preadministration remarkably decreased the number of red pixels. In the time course data for the left forepaw somatosensory cortex, as shown in Fig. 4C, the BOLD signals were immediately increased about 4% after stimulation and disappeared within 3 min (early phase). How-

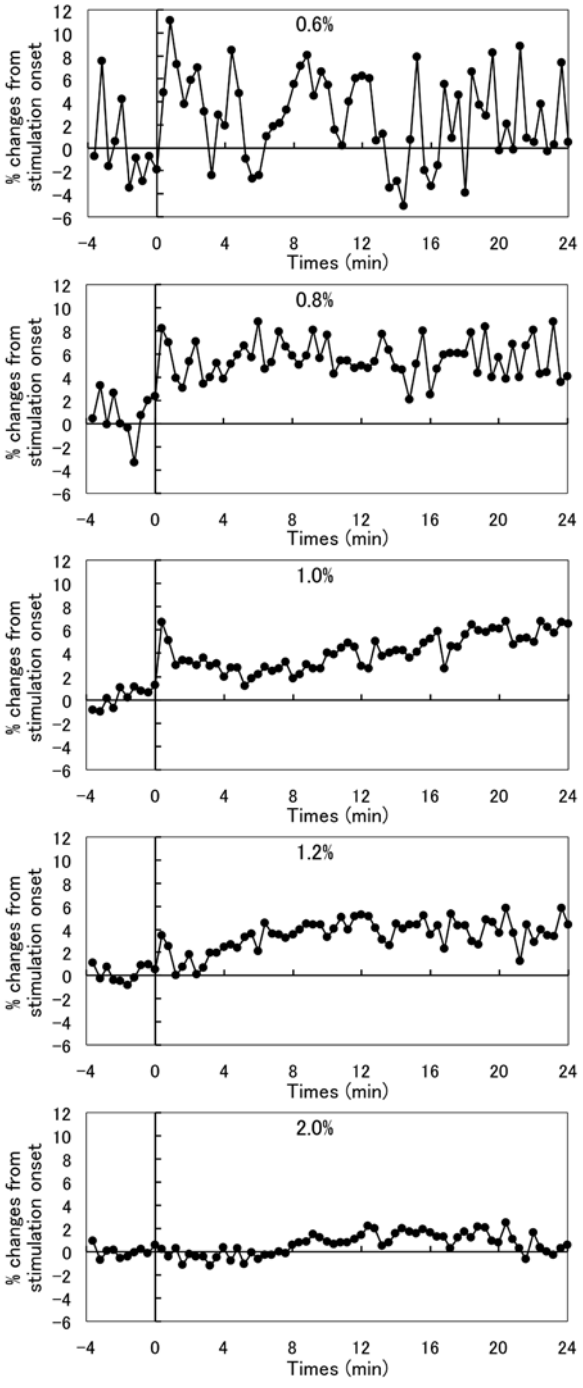

Fig. 2. BOLD-fMRI experiments under various isoflurane concentrations with 5% formalin stimulation of the left forepaw. Representative time course of signal intensity for each 24 sec was plotted for % changes from stimulation onset as a function of poststimulation time under each isoflurane concentrations (0.6, 0.8, 1.0, 1.2, and 2.0%, respectively).

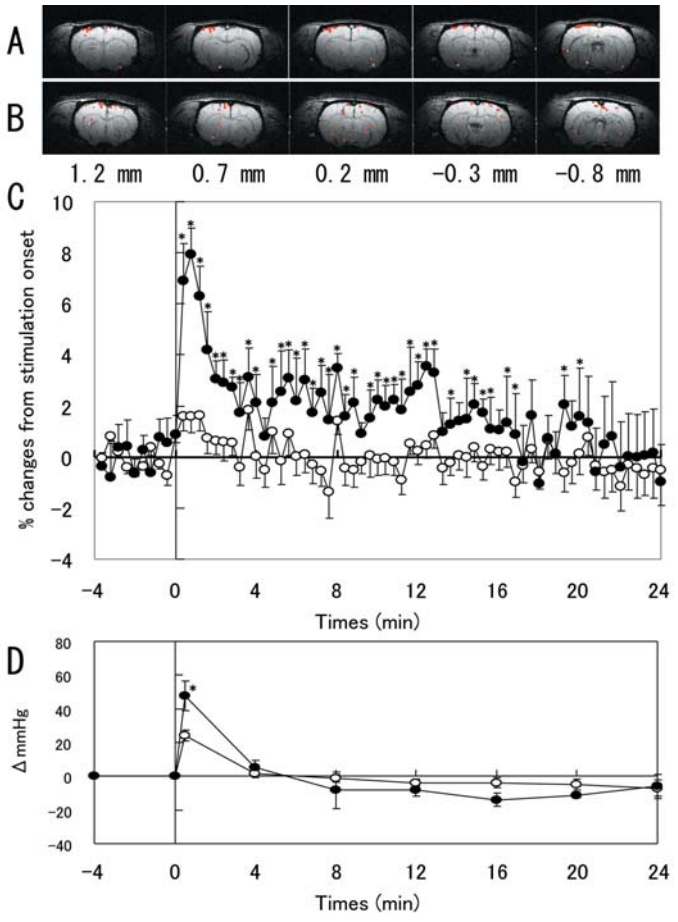

Fig. 3. BOLD-fMRI experiment with 5% formalin stimulation of the left forepaw. *A* and *B*. The representative multislice BOLD fMRI activation maps after no-morphine (saline) and morphine (3 mg/kg) administrations, respectively. Red pixels indicate the regions showing significant formalin-induced increased responses for 24 min after stimulation compared to the prestimulation period in the Z-score *t*-map (P<0.01, uncorrected Z-value>2.34). *C.* Time courses of average signal intensity for each 24 sec were plotted for % changes from stimulation onset as a function of poststimulation time. Black and white circles represent the saline-and morphine-administered groups, respectively. *P<0.05, saline- vs. morphine-administered groups in the same period. *D.* Time courses of changes in increased systemic blood pressure compared to prestimulation. Black and white circles represent the saline- and morphine-administered groups, respectively. *P<0.05, saline- vs. morphine-administered groups in the same period.

ever, in contrast to the data obtained from the rats exposed to formalin, late phase responses were not observed from 3 to 24 min after capsaicin administration (black circles in Fig. 4C). The morphine administration was shown to completely abolish the early phase response induced by capsaicin (white circles in Fig. 4C). Stimulation with capsaicin also induced a temporary increase ($\Delta 49.3 \pm 7.0$ mmHg) of systemic mean blood pressure immediately (black circles in Fig. 4D) and morphine treatment significantly reduced this capsaicin-induced response ($\Delta 18.0 \pm 6.4$ mmHg, white circles in Fig. 4 D).

Discussion

It is well-known that BOLD signals are affected by the various conditions of magnetic field strength of the MRI equipment, position of RF coil and physiological states such as the body temperature of the patient or animal[18]. Furthermore, unlike in human fMRI experiments, it is necessary to determine the optimum concentration of anesthesia that does not abolish the BOLD response induced by painful stimulus in animal models. Many investigators have reported the availability for assessment of acute pain induced by electrical or capsaicin stimulation in α-chloralose anesthetized rodents[1, 2, 5, 9-11, 15, 22]. In our preliminary study of BOLD-fMRI, we tried to assess the difference between early and late phase responses to formalin-induced pain using in α-chloralose anesthetized rats. However, a response for the acute phase of formalin was observed but no late phase response was observed until 1 hr after stimulation (data not shown), although early and late phase responses induced by formalin were reported by observation of nociceptive behavior in the awake rats[20]. It seems that estimation of the stable anesthesia

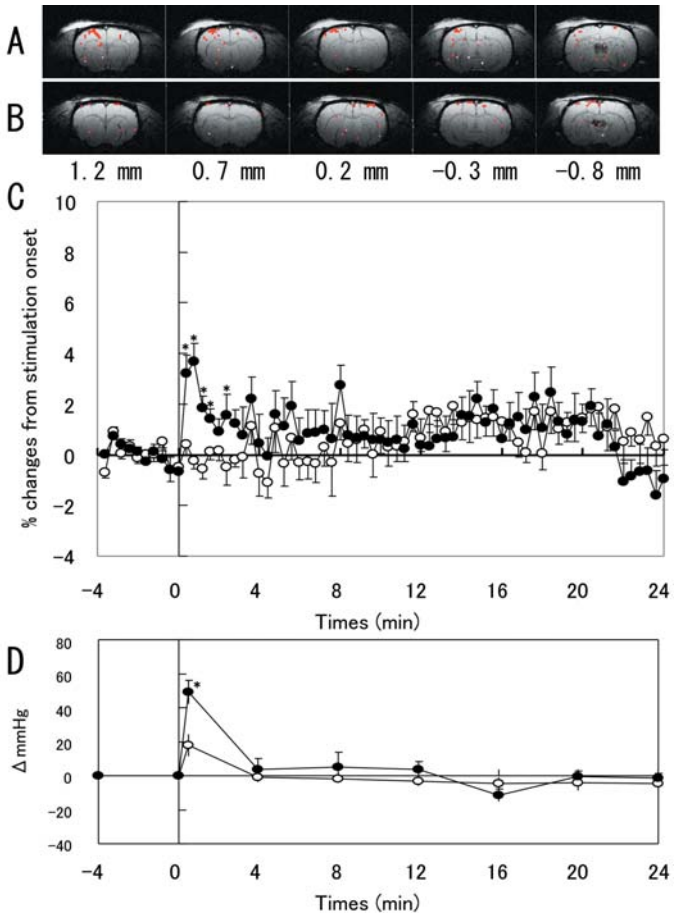

Fig. 4. BOLD-fMRI experiment by 10 μg capsaicin stimulation of the left forepaw. *A* and *B*. Representative multislice BOLD fMRI activation maps after no-morphine (saline) and morphine (3 mg/kg) administrations, respectively. Red pixels indicate the regions showing significant capsaicin-induced responses for 24 min after stimulation compared to the prestimulation period in the Z-score *t*-map (P<0.01, uncorrected Z-value>2.34). *C*. Time courses of average signal intensity for each 24 sec were plotted for % changes from stimulation onset as a function of poststimulation time. Black and white circles represent the saline- and morphine-administered groups, respectively. *P<0.05, saline- vs morphine-administered groups in the same period. *D*. Time courses of change in increased systemic blood pressure compared to prestimulation. Black and white circles indicate the saline-and morphine-administered groups, respectively. *P <0.05, saline- vs. morphine-administered groups in the same period.

level with α-chloralose is very difficult, because the depth of α-chloralose-anesthetization depends on the extent of surgical stress and differences between rat strains. Liu *et al.*[13] demonstrated that isoflurane provided a stable anesthesia level by BOLD-fMRI, although they used only electrical stimulation to induce acute pain. Therefore, we first investigated the most appropriate concentration of isoflurane (0.6, 0.8, 1.0, 1.2, and 2.0%) through artificial ventilation and we found that 1.0% isoflurane anesthesia with artificial ventilation was suitable to observe the early phase and the late phase responses following formalin stimulation (Fig. 2).

In this condition, as shown in Fig. 3, the Z-score *t*-map and time course of BOLD signals showed the two responses in the primary somatosensory cortex induced by formalin stimulation and inhibitory effect of morphine on these formalin-induced responses. Furthermore, capsaicin-induced temporary increases in BOLD signals were also observed as shown in Fig. 4, and this response seemed to be an early phase response (acute pain), because these BOLD signals were observed within 2-4 min after stimulation. Tjolsen *et al.*[20] reported two-phase responses by observation of nociceptive behavior in the rat, an early phase response immediately after the formalin injection, lasting for about 5 min, and a late phase response starting after an early phase. Caterina *et al.*[4] reported that paw licking behavior indicating a painful response was terminated within 55 sec after capsaicin injection, suggesting that capsaicin induced the early phase response but not the long-time persistent response. Our observations that formalin induced the early and late phase responses and capsaicin induced only the early phase response, are similar to those of previous studies. The BOLD-fMRI method was very useful to assess the difference of the phenotypes of

the brain responses to pain in anesthetized animals.

When early phase responses were induced by both stimulants, systemic mean blood pressure was temporally increased as shown in Figs. 3D and 4D. However, during late phase responses induced by formalin, systemic mean blood pressure was at the control level. Since the BOLD response was reported to be strongly influenced by circulatory dynamics that induced changes of the ratio of oxy-hemoglobin and deoxy-hemoglobin[16], the present observations led us to speculate that the early phase BOLD response was partly associated with a passive increase of cerebral blood flow followed by an increase of blood pressure, whereas the late phase BOLD-response depended on changes of heme-oxy-dynamics induced by unknown factors other than blood pressure. However, the accurate physiological mechanisms for the changes of BOLD signals evoked by noxious stimuli have not yet been clarified.

For animal research on pain and analgesics, various techniques for visualization of neuronal activity, i.e., immunohistochemistry for c-fos[12], autoradiography with [^{14}C]-2-deoxy-glucose[19], single photon emission tomography (SPECT) and positron emission tomography (PET)[7,8], have been widely employed. These techniques are based on the accumulation of stress marker protein and metabolic products, or alterations of blood flow and blood volume. However, for analysis of brain activation induced by pain stimuli, these methods have disadvantages such as the need to kill many animals, difficulty in measuring time course of one animal and poor spatial resolution. For the detection of the early phase response and the late phase response induced by noxious stimulation, a technique to monitor the time course of local brain activity has been desired. As demonstrated here, BOLD-fMRI experiments revealed not only topological information but also the time course of brain activity evoked by chemical algesic substances. In the future, this BOLD-fMRI technique may be useful for the assessment of animal pain and development of new analgesics in clinical veterinary medicine.

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References

1) Bock, C., Krep, H., Brinker, G. and Hoehn-Berlage, M. 1998. Brainmapping of alpha-chloralose anesthetized rats with T2*-weighted imaging: distinction between the representation of the forepaw and hindpaw in the somatosensory cortex. *NMR Biomed.,* **11**: 115-119.

2) Brinker, G., Bock, C., Busch, E., Krep, H., Hossmann, K. A. and Hoehn-Berlage, M. 1999. Simultaneous recording of evoked potentials and T2*-weighted MR images during somatosensory stimulation of rat. *Magn. Reson. Med.,* **41**: 469-473.

3) Carstens, E. and Moberg, G. P. 2000. Recognizing pain and distress in laboratory animals. *ILAR J.,* **41**: 62-71.

4) Caterina, M. J., Leffler, A., Malmberg, A. B., Martin, W. J., Trafton, J., Petersen-Zeitz, K. R., Koltzenburg, M., Basbaum, A. I. and Julius, D. 2000. Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science,* **288**: 306-313.

5) Chang, C. and Shyu, B. C. 2001. A fMRI study of brain activations during non-noxious and noxious electrical stimulation of the sciatic nerve of rats. *Brain Res.,* **897**: 71-81.

6) Deneuche, A. J., Dufayet, C., Goby, L., Fayolle, P. and Desbois, C. 2004. Analgesic comparison of meloxicam or ketoprofen for orthopedic surgery in dogs. *Vet. Surg.,* **33**: 650-660.
7) Graff-Guerrero, A., Pellicer, F., Mendoza-Espinosa, Y., Martinez-Medina, P., Romero-Romo, J. and de la Fuente-Sandoval, C. 2008. Cerebral blood flow changes associated with experimental pain stimulation in patients with major depression. *J. Affect. Disord.,* **107**: 161-168.
8) Heiss, W. D. and Herholz, K. 2006. Brain receptor imaging. *J. Nucl. Med.,* **47**: 302-312.
9) Hofstetter, C. P., Schweinhardt, P., Klason, T., Olson, L. and Spenger, C. 2003. Numb rats walk -a behavioural and fMRI comparison of mild and moderate spinal cord injury. *Eur. J. Neurosci.,* **18**: 3061-3068.
10) Hyder, F., Behar, K. L., Martin, M. A., Blamire, A. M. and Shulman, R. G. 1994. Dynamic magnetic resonance imaging of the rat brain during forepaw stimulation. *J. Cereb. Blood Flow Metab.,* **14**: 649-655.
11) Kida, I., Hyder, F. and Behar, K. L. 2001. Inhibition of voltage-dependent sodium channels suppresses the functional magnetic resonance imaging response to forepaw somatosensory activation in the rodent. *J. Cereb. Blood Flow Metab.,* **21**: 585-591.
12) Labuz, D., Chocyk, A., Wedzony, K., Toth, G. and Przewlocka, B. 2003. Endomorphin-2, deltorphin II and their analogs suppress formalin-induced nociception and c-Fos expression in the rat spinal cord. *Life Sci.,* **73**: 403-412.
13) Liu, Z. M., Schmidt, K. F., Sicard, K. M. and Duong, T. Q. 2004. Imaging oxygen consumption in forepaw somatosensory stimulation in rats under isoflurane anesthesia. *Magn. Reson. Med.,* **52**: 277-285.
14) Logothetis, N. K., Pauls, J., Augath, M., Trinath, T. and Oeltermann, A. 2001. Neurophysiological investigation of the basis of the fMRI signal. *Nature,* **412**: 150-157.
15) Malisza, K. L. and Docherty, J. C. 2001. Capsaicin as a source for painful stimulation in functional MRI. *J. Magn. Reson. Imaging,* **14**: 341-347.
16) Ogawa, S., Lee, T. M., Kay, A. R. and Tank, D. W. 1990. Brain magnetic resonance imaging with contrast dependent on blood oxygenation. *Proc. Natl. Acad. Sci. U.S.A.,* **87**: 9868-9872.
17) Paxinos, G. and Watson, C. 1998, In: *The rat brain in stereotaxic coordinates.* Academic Press, San Diego.
18) Reijnders, K., English, S. J., Krishna, M. C., Cook, J. A., Sowers, A. L., Mitchell, J. B. and Zhang, Y. 2004. Influence of body temperature on the BOLD effect in murine SCC tumors. *Magn. Reson. Med.,* **51**: 389-393.
19) Sokoloff, L., Reivich, M., Kennedy, C., Des Rosiers, M. H., Patlak, C. S., Pettigrew, K. D., Sakurada, O. and Shinohara, M. 1977. The [^{14}C]deoxyglucose method for the measurement of local cerebral glucose utilization: theory, procedure, and normal values in the conscious and anesthetized albino rat. *J. Neurochem.,* **28**: 897-916.
20) Tjolsen, A., Berge, O. G., Hunskaar, S., Rosland, J. H. and Hole, K. 1992. The formalin test: an evaluation of the method. *Pain,* **51**: 5-17.
21) Tracey, I., Becerra, L., Chang, I., Breiter, H., Jenkins, L., Borsook, D. and Gonzalez, R. G. 2000. Noxious hot and cold stimulation produce common patterns of brain activation in humans: a functional magnetic resonance imaging study. *Neurosci. Lett.,* **288**: 159-162.
22) Tuor, U. I., Malisza, K., Foniok, T., Papadimitropoulos, R., Jarmasz, M., Somorjai, R. and Kozlowski, P. 2000. Functional magnetic resonance imaging in rats subjected to intense electrical and noxious chemical stimulation of the forepaw. *Pain,* **87**: 315-324.
23) Wagner, A. E., Worland, G. A., Glawe, J. C. and Hellyer, P. W. 2008. Multicenter, randomized controlled trial of pain-related behaviors following routine neutering in dogs. *J. Am. Vet. Med. Assoc.,* **233**: 109-115.