Hirosaki Med. J. 61 (Suppl.) : S192—S196, 2010

NEUROVASCULAR COUPLING STUDIES IN AWAKE-BEHAVING MICE

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Abstract The mechanism regulating cerebral blood flow (CBF) during brain function (neurovascular coupling) has been widely investigated in animals under anesthetized conditions, though anesthesia is known to greatly affect neurovascular physiology. The present study aims to develop a novel model for neurovascular coupling studies in awake-behaving mice. Male C57BL/6J mice were initially anesthetized with isoflurane in preparation for attaching the study apparatus to the head. The animal was tethered to the study apparatus but allowed to move spontaneously on a floated ball. The animal behavior and regional CBF in the somatosensory barrel cortex were simultaneously measured with optical motion sensor and laser-Doppler flowmetry (LDF), respectively. Anesthesia was discontinued during recovery, while whisker stimulation (frequency 10 Hz and duration 10 or 20 sec) was induced at either the contralateral or ipsilateral side of the LDF recording site. During the experiments, the animals showed no signs of struggling against the head restraint. The intensity of baseline CBF was higher while the animal was under 2% isoflurane aesthesia than it was after anesthesia was stopped. CBF response to stimulation was not observed under anesthesia. After the animal was recovered from anesthesia, an increase in CBF (34 ± 18%) was observed during contralateral stimulation but not during ipsilateral stimulation. The fluctuation levels of CBF baseline during resting and walking conditions were ±2.7% and ±3.5%, respectively. We observed that these fluctuations were not due to vibration noises caused by such as air-puff and animal motion in our experimental conditions.

Hirosaki Med. J. 61, Supplement : S192—S196, 2010

Key words: laser-Doppler flowmetry; Functional brain imaging; whisker stimulation; behavioral analysis

Introduction

There have been a number of studies examining neurovascular coupling, which is the mechanism that regulates cerebral blood flow (CBF) induced by neural activity[1-4]. In these studies, experiments were performed under when the animals were anesthetized. Since anesthesia has strong effects on systemic physiology (e.g., arterial blood pressure and heart rate, etc.), the implications from the anesthetized animal studies may not be directly applicable to human studies which are usually performed on subjects who are awake. Therefore, it was needed to develop the methods for measuring neurovascular functions in awake-behaving animals.

There are few reports on the *in vivo* imaging of neural activity in awake-behaving mice. Some of the studies that have been done have utilized two-photon microscopy[5,6]. In addition, neural activity-induced hemodynamic response in un-anesthetized animals has been measured with functional magnetic resonance imaging (fMRI)[7-9] and intrinsic optical imaging[10]. The awake-behaving animal is essentially allowed to move spontaneously during the recording, which could affect the imaging signals in two ways. First, the imaging signal can be influenced by

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the mechanical motion caused by movement of the animals. Second, the signal can be affected by changes in physiological conditions due to excessive exercise. However, no studies have been done to determine the causal connection between animal behavior and brain imaging signals simultaneously. Moreover, human brain functions can be measured with near-infrared spectroscopy (NIRS)[11], where such NIRS studies often report on hemodynamic changes during exercise[12]. However, the relationship between exercise and measurements of CBF are poorly understood.

In the present study, we developed a new method that measures the CBF and behavior of animals simultaneously in fully awake conditions. The measurement system that we developed employs an air-supported spherical treadmill that allows for walking and running, which were originally developed for insect[13,14] and awake mice experiments[7]. We selected mice as measurement subjects in this study because of the potential for applying our findings in further clinical and neuroscientific research with gene-manipulated mice.

Material and Methods

All experiments were conducted in accordance with the guidelines of the Physiological Society of Japan and were approved by the Animal Care and Use Committee of the National Institute of Radiological Sciences, Chiba, Japan. Eight C57BL/6J mice (5-7 weeks) were used to investigate the behavior and regulation of CBF under awake-behaving condition. The animals were housed in our laboratory, and the chamber temperature was maintained at ~25°C and the light/dark cycle was 12 h:12 h.

The mice were anesthetized with a mixture of air and isoflurane (3% for induction, and 2% for surgery) using a facemask. The animals were fixed in a stereotactic frame, and the parietal bone was thinned to translucency at the left somatosensory cortex (an area of 3 x 3 mm, centered at 2.5 mm caudal and 3.0 mm lateral to the bregma). A metal head plate was attached to the skull with dental acrylic. One day after this operation, the mice were again anesthetized with isoflurane (2%) and tethered by screwing the head plate to a metal rod. Isoflurane anesthesia was discontinued after setting mice on the apparatus. For whisker stimulation, the tube for puffing air was placed parallel to the mouse snout at a distance of 20 mm from the whiskers. Whisker stimulation was performed by puffs of air generated by an air tank at a pressure of 15 psi. Stimulus frequency was 10 Hz and stimulus duration was either 10 or 20 seconds. A Master-8 (A.M.P.I) controlled the stimulus delivery.

Beneath the animals, a styrofoam ball (98 mm in outer diameter) supported by a jet of air was set in a metal cup (100 mm in inner diameter). The jet of air was produced by a propeller driven by a DC motor (MABUCHI MOTOR, RS-380PH) so that the styrofoam ball (9 g) with the mouse (20-25 g) who was getting on it was floated. This allowed the styrofoam ball to rotate freely while the mice walked on it. In order to record the walking velocity of the mice, an optical computer mouse was placed on the side of the styrofoam ball and detected the ball rotation at a spatial resolution of 0.3 mm. The XY coordinates were recorded every 0.1 sec, and the mean walking velocity was then calculated by dividing the distance by sampling time.

CBF was measured by using laser-Doppler flowmetry (LDF; FLO-C1, OMEGA FLO, Japan), which measures the Doppler-shift of laser light (with a wavelength of 780 nm) produced mainly by red blood cell movement. The tip diameter of the LDF probe was 1 mm (Probe NS, OMEGA FLO), and the sampling volume of LDF measurement was generally reported to be about 1 mm^3[3,15]. A time constant of 0.1 sec was used. The LDF probe was attached to the thinned parietal bone perpendicular to the brain

surface and positioned over the somatosensory barrel area where the maximum signal change was observed during stimulation. The baseline CBF was computed by averaging the values of LDF signals obtained during either the 4 second or 20 second pre-stimulus periods. The LDF-signal was normalized towards the baseline level as percentage changes from the baseline. Peak amplitude was calculated as the maximum value of the response curve during whisker stimulation.

LDF and motion signals were recorded simultaneously using biopac systems at a sampling rate of 200 Hz. These values were statistically analyzed by Student's t-test and are presented as mean ± SEM. A digital camera was also used to observe the animals when they were whisking, grooming and resting. Throughout the duration of the experiment, we did not observe any of the mice struggling against the head restraint.

Results

Recovery from anesthesia

Under the 2% isoflurane anesthesia condition, the evoked CBF response and exercise were not observed. The intensity of the baseline CBF was approximately 40% higher than it was 20 minutes after anesthesia was discontinued (n=8). The intensity of the baseline CBF decreased immediately after anesthesia was discontinued (Fig. 1), but then increased gradually and reached to a stable level during the subsequent ~16 minute interval. In contrast, the evoked CBF response increased gradually immediately after anesthesia was discontinued. Approximately 14 minutes after anesthesia was discontinued, the evoked CBF became stable. The maximum peak amplitude of evoked CBF (34 ± 18%) was observed within 14 to 16 minutes after anesthesia was discontinued. No exercise was observed for the first 20 minutes post-anesthesia, but the amount of exercise increased significantly during the subsequent 20-minute interval.

Awake-baseline condition

Since we observed that baseline CBF became stable ~16 minutes after the anesthesia was discontinued (Fig. 1), the following comparisons of the resting and exercise conditions were made for data obtained 16 minutes after the anesthesia was discontinued. Resting state was defined as the period when the walking velocity is near zero. During the resting state, the spontaneous fluctuation (standard deviation) of the baseline CBF was ±2.7% relative to the mean level. Baseline CBF level was also observed to be stable. During the walking periods, the spontaneous fluctuation of the baseline CBF

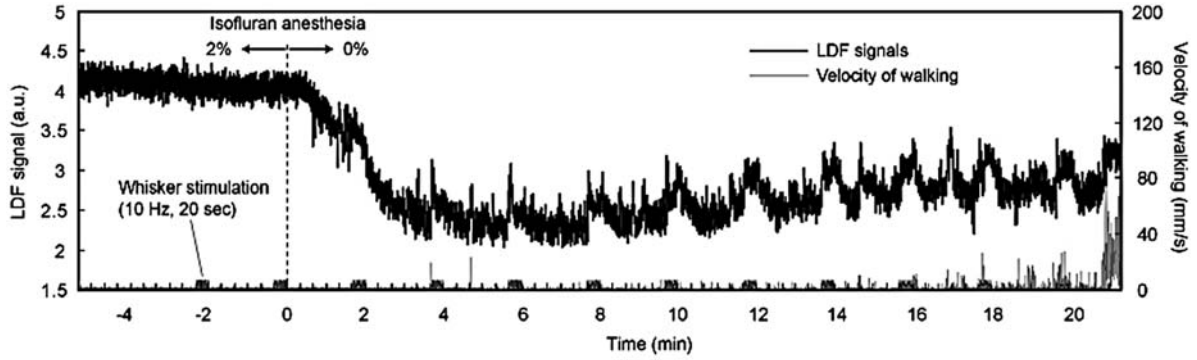

Figure 1 Amount of exercise and CBF in mice before and after isofluran anesthesia was terminated. The data are from one representative animal. Note that the baseline CBF under 2% isoflurane anesthesia was higher than under unanesthetized condition (0% isoflurane). Evoked CBF and the exercise were not observed during 2% isoflurane anesthesia.

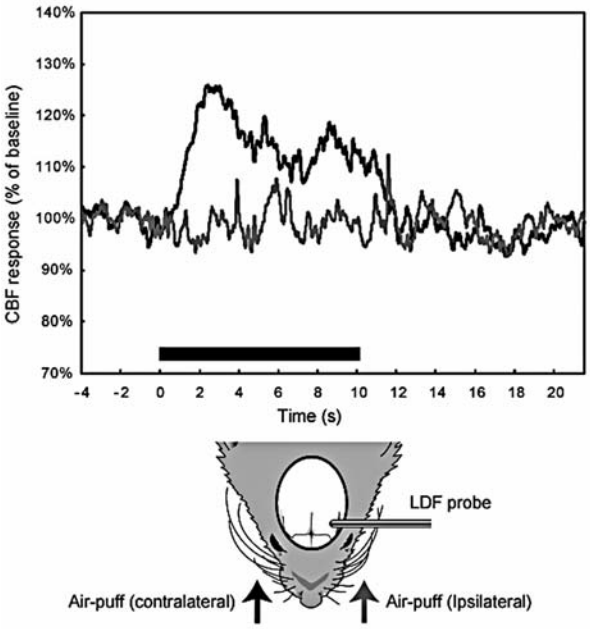

Figure 2 A comparison of evoked CBF responses to the contralateral and ipsilateral whisker stimulation. CBF was measured at the somatosensory barrel cortex using LDF. Baseline CBF obtained for 4 seconds immediately before the onset of whisker stimulation was considered to be 100%. The gray line was obtained during ipsilateral air-puff stimulation, and the black line was obtained during contralateral air-puff stimulation. An increase in CBF was observed during contralateral stimulation (maximum amplitude is 27.6%); however, there was no detectable increase in CBF during ipsilateral stimulation.

was ±3.5% and baseline CBF level was slightly increased.

Evoked responses to whisker stimulation

An increase in LDF signal was observed in response to contralateral air-puff stimulation when the animal was recovered (Fig. 2). In one representative animal, evoked CBF amplitude peaked at 27.6% (n=1) for contralateral stimulation, whereas no detectable change in CBF was observed for ipsilateral stimulation. The ipsilateral stimulation data also showed that the LDF signal was not influenced by mechanical motion due to the induction of whisker stimulation.

Discussion

In this study, we attained simultaneous recordings of CBF and animal behavior in animals that were awake without any significant influence of mechanical motion noise. Since LDF measures CBF signals based on the Doppler effect, this measurement is sensitive to the vibration noise, particularly in awake-behaving animals. In this study, LDF signals showed no detectable change during ipsilateral air-puff stimulation (Fig. 2), which suggests that vibration noise caused by whisker stimulation does not significantly affect LDF signals. We also showed that spontaneous fluctuation of baseline CBF was similar during the resting and walking state. Although the baseline CBF showed a slight increase during periods of exercise, changes in LDF signals were not coincident with changes in animal motion. These results indicate that the observed signal change in LDF during exercise represents physiologic changes in CBF but not attributable to vibration noise.

We also observed that baseline CBF under isoflurane anesthesia is higher than when the animals are awake (Fig. 1). In addition, the evoked CBF response was not observed under 2% isoflurane anesthesia. In isoflurane-anesthetized rats, it was shown that the CBF response to neural stimulation increased in a dose-dependent manner, although the measurement has not performed under awake condition[16]. Since the rats and mice have different sensitivity to the isoflurane concentration (e.g., 1.3% for rats and 1.7% for mice at a minimum alveolar concentration), it is possible that the effect of anesthesia on the CBF response may differ among the species. In the present study, we further observed that the onset of recovery in CBF baseline and evoked response from the condition of the anesthesia was faster than that of the animal behavior recovery. In addition, the stabilization of CBF nearly followed the

recovery of the animal exercise. Further studies are needed to elucidate the relationship between CBF stabilization and behavioral states of animals (e.g., wakefulness).

Acknowledgment

We are thankful to Mr. Joonas Autio for discussion and valuable comments on this study.

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