Hemodynamic correlation imaging of the mouse brain for application in unilateral neurodegenerative diseases

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Abstract: We developed a single-camera two-channel hemodynamic imaging system that uses near-infrared light to monitor the mouse brain in vivo with an exposed, un-thinned, and intact skull to explore the effect of Parkinson’s disease on the resting state functional connectivity of the brain. To demonstrate our system’s ability to monitor cerebral hemodynamics, we first performed direct electrical stimulation of an anesthetized healthy mouse brain and detected hemodynamic changes localized to the stimulated area. Subsequently, we developed a unilaterally lesioned 6-hydroxydopamine (hemi-parkinsonian) mouse model and detected the differences in functional connectivity between the normal and hemi-parkinsonian mouse brains by comparing the hemispheric hemodynamic correlations during the resting state. Seed-based correlation for the oxy-hemoglobin channel from the left and right hemispheres of healthy mice was much higher and more symmetric than in hemi-parkinsonian mice. Through a k-means clustering of the hemodynamic signals, the healthy mouse brains were segmented according to brain region, but the hemi-parkinsonian mice did not show a similar segmentation. Overall, this study highlights the development of a spatial multiplexing hemodynamic imaging system that reveals the resting state hemodynamic connectivity in healthy and hemi-parkinsonian mice.

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1. Introduction

Functional connectivity is an important technique for analyzing the relationships between various regions of the brain and is helpful for understanding the organization of complex brain networks. It is an essential tool because it requires no assumptions about brain regionalization but rather detects correlated patterns directly from the acquired neural signals [1,2]. Functional connectivity of the brain can be observed not only in event-based experiments but also during the resting state [3–5]. The study of the brain’s functional connectivity can be valuable for investigating the specific effects of neurodegenerative diseases such as Parkinson’s disease (PD), which is known to disrupt several neural networks. In addition to a general decrease in functional connectivity in the entire brain [6], the motor cortex has been shown to be particularly affected by PD [7–9]. Hemi-parkinsonism is a type of PD that affects only one hemisphere; however, as with many other neurodegenerative diseases, its effects on the functional connectivity of the entire brain has not yet been examined.
Many studies have utilized near-infrared spectroscopy (NIRS) to investigate PD because of its advantages over other imaging modalities. In addition to its greater tolerance of motion artifacts [10], NIRS is capable of imaging deeper tissue regions than those imaged by visible-light-based imaging methods [11]. This is particularly useful in animal studies, allowing the skull to remain intact while hemodynamic data from deeper regions of the brain are acquired [12]. By acquiring hemodynamic data, we can develop functional connectivity measurements of wide areas of the brain. However, not only can near-infrared light be used to develop functional connectivity measurements as OIS (optical intrinsic signal) but visible light has also been used to show functional connectivity measurements [5].

For this study, we developed a wide-field single-camera imaging system using near-infrared light to measure the functional connectivity in healthy and PD mice during the resting state. Our system does not rely on switching between wavelengths but rather uses spatial multiplexing of a single camera to record optical density changes from multiple regions of the mouse brain. We also developed a 6-hydroxydopamine-lesioned hemi-parkinsonian mouse model for use in testing. In our animal model, the skulls of the mice remained intact while hemodynamic signals were acquired for use in locating functionally similar areas of the brain. In most cases, functional connectivity can be quantified by seed-based calculation of the correlation between regions of the brain over a period of time [13]. Other methods, including independent component analysis, have been proposed for quantifying functional connectivity, but in practice, there is little difference between the results of these approaches [14]. In this paper, we use seed-based calculation of functional connectivity to observe the effects of hemi-parkinsonism in mice during the resting state. To further analyze the resting-state brain activity pattern, we used a k-means clustering algorithm [15–18] to segment functional regions of the brain based on hemodynamic signals. These analyses demonstrate the applicability of our system to the study of the effects of various neurodegenerative diseases on global brain activity.

2. Materials and methods

2.1 Imaging system

A uniform bright-field illumination using a fiber ring light guide (2.00-inch, Edmund Optics) was used to deliver light from two LEDs (780 nm (Thorlabs, M780L3) and 850 nm (Thorlabs, M850L3)). A cranial window in a head-fixed mouse was exposed approximately 10 cm below a 100 mm B-coated (650–1050 nm range) achromatic lens (Thorlabs, AC254-100-B-ML). The reflected signal from the cranial window was focused onto the iris with a 75 mm B-coated achromatic lens (Thorlabs, AC254-075-B-ML), allowing the image size to be controlled using the iris (Fig. 1(a)).

Two additional 75-mm B-coated achromatic lenses (Thorlabs, AC508-075-B-ML) were also located between the iris and camera. Between these two lenses, the beam was divided into two paths by a dichroic mirror (Semrock, 801 nm long pass). Each beam was filtered using a bandpass filter (Semrock, 780 nm - bandwidth: 12–25 nm and 850 nm - bandwidth: 10–25 nm). The two beams were directed to two different regions of the same camera (Fig. 1). The CCD camera (ORCA Flash 4.0 V2, Hamamatsu, Japan) was set to acquire frames at 10 Hz with a resolution of 1024 x 512 pixels; thus, the image for each of the two wavelength regions occupied 512 x 512 pixels. A 2 x 2 binning was performed for analysis. The camera’s field of view of the mouse brain, including the motor cortex and somatosensory cortex, was approximately 1 cm². The resulting resolution of our acquired image was 0.2 mm. Each mouse was experimented on once; one experiment for electrical stimulation (one healthy mouse) and eight separate experiments (four healthy mice/four hemi-parkinsonian mice) for resting state analyses.
2.2 Image processing and analysis

The two images acquired at different wavelengths were used to calculate oxy-hemoglobin and deoxy-hemoglobin concentration changes. The pixels for the two images were manually aligned, using the bregma as the alignment point. The oxy- and deoxy-hemoglobin changes were evaluated using the Modified Beer Lambert’s Law (MBLL). Details of the MBLL have been described elsewhere [11]. The path length difference for each wavelength was not considered in our analysis. Since our continuous-wave NIRS calculates hemodynamic changes based on baseline intensity values, and assuming the path length difference remains constant, we assumed that the path length difference between wavelengths would not make a large difference in the results. Considering this, the path length according to our system setup is a target of further analysis. As our system depends on optical scattering, the overall effect of spectral variations in optical scattering over time is a point that must be considered, as well as how the spectral variance affects the overall signal sensitivity at each wavelength. The signals from each pixel were time-filtered with a 0.2 Hz first order, low-pass filer in the demonstration experiment (electrical stimulation). The resting state experiment on the other hand was time-filtered with a 0.009–0.08 Hz third-order bandpass filter to remove high-frequency camera noise that could affect the connectivity analysis but also maintain important spontaneous hemodynamic fluctuations according to previous studies [5,19,34]. The images were spatially smoothed with a Gaussian filter and a sigma of 0.6 mm.

The analysis of our images was performed in two stages. First, we used a seed-based correlation calculation to find the spatial differences that may exist during time for all pixels compared to the seeding point. Then, we used a k-means clustering algorithm to analyze brain region functional connectivity without having to select a seed location. The k-means algorithm works by grouping the data into k clusters in a manner that minimizes the summed distances between points within each cluster and the mean of those points [15].

Our system uses NIR light and performs simultaneous measurements over multiple wavelengths. It can quickly image deeper areas of the brain. However, as the measured signal comes from a combination of the deep brain and skull, there exists a possibility that the signal could be partly composed of global noise. To remove the global component from our images, we implemented principle component analysis (PCA), which has been shown to effectively remove global and environmental noise from the NIRS signal [26]. Previous studies have shown that the first principle components are most likely responding to global noise, but in some cases the second principle component may also be noise affected by the environment.
In this study, the analysis for seed-based correlation maps were performed with the first two principle components removed and explained in the results section of this paper [27].

2.3 6-Hydroxydopamine (6-OHDA) lesions

Adult C57BL/6 mice (Charles River Laboratories International, Korea), weighing 20–25 g, were used in this study. All animal experiments were performed in accordance with recommendations for the care and use of laboratory animals by the Ethical Committee of the Korea Institute of Science and Technology (2015-031).

For unilateral 6-OHDA lesions, the mice were deeply anesthetized using a 3:1 cocktail of diluted Zoletil® (140.9 mg of zolazepam mixed with 145.5 mg of tiletamine in 5 ml of sterilized injection solution, Virbac, France) and xylazine (23.32 mg/ml), at a final volume of 0.1 ml/100 g (intraperitoneal injection), and placed in a stereotaxic frame (David Kopf Instruments, USA) to receive 6-OHDA injections. Solutions of 6-OHDA (Sigma-Aldrich, USA) at a concentration of 4 µg/µl were prepared in saline with 0.1% ascorbic acid, and a total volume of 1 µl was manually injected with a micropositioner into the right medial forebrain bundle (−1.2 mm AP, +1.2 mm ML, and −5.0 mm DV) [28] of each mouse in the PD group using a 5 µl Hamilton microsyringe, at a rate of 0.5 µl/min. The entire process of injection and disease progression was extensively described previously [29]. Mice in the sham control group were injected with 1 µl of 0.1% ascorbate saline. The needle was left in situ for an additional 5 min before being slowly retracted. The wound was then cleansed with saline and sutured with non-absorbable black silk (AILEE Co., Korea).

2.4 Stereotaxic surgery procedures

A Zoletil/Rompun mixture (0.1 ml/100 g) was administered to the mice, which were then placed in a stereotaxic frame and secured with a nose cone and ear bars [20,21]. Stereotaxic coordinates were determined with respect to the bregma (Fig. 2). An incision was made on the top of the head and the flaps were fixed to the side with forceps so that the skull was visible. The cranial surface was carefully cleaned with 4% alcohol and scraped to expose approximately 1 cm² of the skull. The camera was focused so that the motor cortex could be clearly observed, but the limbic, somatosensory, and retrosplenial cortices were also well imaged. Parcellation of cortical regions was defined based on previous studies [5,37].

![Fig. 2. (a) Schematic of cortical regions of the mouse brain. (b) In vivo image of cranial window. Red circle is the field of view and the black dot is the bregma. (c) Field of view of our imaging system. (B: bregma, L/S: lambdoidal suture).](image-url)
2.5 Experimental procedure

Two separate experiments were performed in this study. For the first experiment, we directly stimulated one healthy mouse brain using a bipolar electrode (A320, Isostim) positioned into a hole drilled through the skull, approximately over the motor cortex, for direct brain stimulation. The approximate positioning of the electrode can be seen in Fig. 3(a). Stimulation was initiated after 10 seconds of baseline measurement, at an intensity of 0.1 mA, for a fixed duration of 15 seconds. The stimulation was repeated five times, but only the first trial was taken for analysis due to excessive vasomotion resulting from the first trial. The effect of vasomotion will be discussed in Section 3. The purpose of this test was to provide an example of the system’s ability to monitor hemodynamic changes in the mouse brain. For the second experiment, we observed resting state cerebral hemodynamic changes over a period of 7 minutes, in four healthy and four hemi-parkinsonian mice. Resting state means that no other external stimulation was applied to the mouse and it remained in its stereotaxic frame (DJ-308, Daejong Instrument) under constant anesthetic conditions.

3. Results

3.1 System demonstration - hemodynamic changes during electrical stimulation

Electrical stimulation was delivered for 15 s to one of the motor cortex regions, and the hemodynamic response was analyzed for a pixel near the electrode (Fig. 3(a)). Immediately after the stimulus onset, a small rise in deoxy-hemoglobin and a small decline in oxy-hemoglobin, known as the “initial dip,” were clearly observable. A large influx of blood followed, and a typical hemodynamic signal was then observed for increasing blood flow [30]. A single trial hemodynamic response is shown in Fig. 3(b). Although the figure does not show a complete return to baseline, it is trending towards baseline. After 50 s, there is continuous fluctuation around baseline levels, indicating additional vasomotion due to the direct stimulation. This continuous fluctuation after the first trial made the analysis of successive trials difficult, and we assume that the exaggerated vasomotion came from the high power of the electrode [31].
Fig. 3. (a) Placement of the stimulating electrode and measurement window. (b) Hemoglobin (oxy, deoxy, and total) concentration changes over time measured near the electrode during electrical brain stimulation. The dotted black lines indicate the stimulation period. The onset delay of the expected rise in oxy-hemoglobin is the signature of the initial dip related to an immediate increase in cerebral metabolism. (c) Hemodynamic changes in the healthy mouse brain before, during, and after electrical stimulation. The shaded portion of the time scale indicates when stimulation was applied.

A time series of hemodynamic images for the entire cranial window is shown in Fig. 3(c). The rapid metabolic changes following stimulus onset can be seen in specific regions (e.g., motor cortex) of the cranial window, with the largest changes in deoxy-hemoglobin occurring near the electrode. By approximately the 5th second after the start of stimulation, the first signs of a large increase in oxy-hemoglobin were observed. Correlations of hemodynamic changes were calculated over 5-s intervals using a seed-based method with the stimulation point as the seed (Fig. 4) [22,23]. The correlation results during electrical stimulation showed significant increases 0-15s seconds after the onset of stimulation, particularly in the motor cortex, indicating a high connectivity state during electrical stimulation. Figure 4 shows that even for direct brain stimulation for normal mice, a correlation between both motor cortices can be seen, indicating some connectivity between the two functional areas.
3.2 Functional connectivity during resting state

Data of the hemodynamic changes during the resting state were collected for 7 minutes at an acquisition rate of 10 Hz. The overall monitoring time of 7 minutes was set based on the memory limit of the camera; however, it was sufficient to reveal connectivity differences between a group of healthy and hemi-parkinsonian mice. Each mouse was anesthetized by injection of a Zoletil/Rompun mixture and placed in the stereotaxic frame for the entire period. As we mentioned in 2.2, global and environmental noise was removed using PCA before calculating seed-based correlations. Typically, the first principle component (PC1) is global noise, but in our data, the second principle component (PC2) also exhibited a similar pattern. Figure 5(a) shows a trend of strong low frequency oscillations in not only PC1, but also PC2 for all mice, except for N3, where strong low frequency oscillation was only observed in PC1. In particular, the frequency power spectrum density less than 0.005 Hz, which is not an important frequency band [35,36], includes a high PC1 and PC2 peak (Fig. 5(b)). Also, the difference between PC1 and PC2 is not statistically significant, in contrast to PC3–PC5 (Fig. 5(c)).

Each original image was divided into 20 principle components and was reconstructed with PC1 and PC2 removed. Pearson’s correlation coefficients were calculated over time using a reconstruction image for left and right seeds at corresponding points on the motor cortex, to create connectivity maps [4,22]. The approximate seed locations can be seen in. The motor cortex was chosen because it is the area that is most affected by PD. Seed-based correlation maps with reconstruction images for each mouse show different patterns depending on the number of the principle component. For example, in Fig. 6(b), the left seed-based correlation image with PC1 and PC2 removed in mouse N2 shows a more clearly segmented motor cortex area than the image produced by only removing PC1. In the case of mouse PD3, the entire brain signal was affected by PC2 and in the case of mouse N3, not much changed because PC2 did not have a strong power spectrum density in the low frequency band (Fig. 5(a)).
Fig. 5. PCA result in normal and hemi-parkinsonian mice during a 7-minute resting period. (a) Time course corresponding to PC1–PC5 of each mouse. (b) Averaged power spectrum density of each group less than 0.05Hz. (c) Averaged power spectrum density of each principle component less than 0.005 Hz (*p<0.05, **p<0.01).
Fig. 6. Example of left motor cortex seed correlation map during a 7 minute depends on removal number of PC. N2 shows more clearly segment motor cortex with remove PC1 and PC2 than only remove PC1. In case of PD3, PC2 effect entire brain signal and N3 shows not much change between remove PC1 or PC1 and PC2.

The correlation maps for oxy-hemoglobin concentration in healthy mice showed a symmetric pattern between the left and right hemispheres. In hemi-parkinsonian mice, a less symmetrical pattern was observed (Fig. 7).
To quantify the loss of symmetrical connectivity during the resting state, a new correlation coefficient value was calculated for the correlation maps that were obtained from the seeds in the right and left hemisphere. This procedure was implemented for both the healthy and hemi-parkinsonian mice for the oxy- and deoxy-hemoglobin channels (Fig. 8). The individual correlation values for the samples show a clear distinction between the normal mice and hemi-parkinsonian mice. Small-to-moderate positive correlations were found for the hemi-parkinsonian mice (i.e., asymmetrical resting state connectivity), as opposed to the healthy mice, which showed much stronger correlations.
3.3 K-means clustering for hemodynamic changes

To prevent a bias with regard to seed selection in the image, we used k-means clustering to segment the image based on similar temporal hemodynamic fluctuations. K-means clustering is an algorithm to group similar data points into a predetermined number of clusters. However, successful clustering is highly dependent on the number of clusters chosen. In order to identify the proper number of clusters, we employed the elbow method which calculates the sum of squared errors (SSE) corresponding to different number of clusters [32,33]. The individual results of the elbow method for the four healthy mice (N 1–N 4) and four PD mice (PD 1–PD 4) are as follows, N 1 = 19, N 2 = 17, N 3 = 18, N 4 = 19, PD 1 = 23, PD 2 = 20, PD 3 = 17, and PD 4 = 21, which produce an average value of 19.25 ± 1.9 clusters. Therefore, we chose 20 clusters for our clustering algorithm.

Figure 9 shows the results of the clustering analysis (k = 20). Although the deoxy-hemoglobin concentration changes exhibit symmetric left- and right-seed correlation maps (Fig. 8), the motor cortex region is not clearly seen. For this reason, we selected only the oxy-hemoglobin concentration. We chose the clusters that corresponded with physiologically known lobes in the mouse. The healthy mouse brain can be segmented into four clusters corresponding to the limbic, motor, somatosensory, and retrosplenial cortical areas. The functional clustering of the left and right hemispheres appears symmetrical across the cortex. However, in the case of PD mice, the motor cortex is split into two different clusters, as shown in PD 1, PD 3, and PD 4, for a total of five clusters. The clustering patterns in the left and right hemispheres are either highly asymmetrical (PD 1, PD 3), organized in an abnormal way (PD 2), or fuzzily distributed (PD 4). This result suggests a loss of functional organization in the hemi-parkinsonian model.
4. Discussion and conclusions

In the present study, we developed a wide field single-camera hemodynamic imaging system that utilizes the minimal absorbance of NIR light and performed a correlation analysis of the resulting hemodynamic signals with the goal of visualizing the effects of unilateral neurodegenerative diseases. The results of the electrical stimulation experiment demonstrated the system’s sensitivity to rapid metabolic changes in the brain by clearly showing an “initial dip” at the onset of stimulation. This initial dip is marked by a small decrease in oxy-hemoglobin and increase in deoxy-hemoglobin and is indicative of an initial uptick in cerebral metabolism before the delivery of oxygen [24,25]. This shows our system’s ability to measure rapid changes in the hemodynamics that can be used for further investigation of direct brain stimulation.

We then compared healthy mice with hemi-parkinsonian mice and showed a trend of asymmetrical resting-state functional connectivity pattern in the hemi-parkinsonian mice. This result supports the notion that PD greatly affects the functional connectivity of the mouse brain, which is consistent with previous research [3]. The clustering results for hemi-parkinsonism indicate a loss of symmetrical functional organization compared to that seen in healthy mice, especially in the motor cortex region. This hemodynamic monitoring and correlation analysis shows the potential of our system to be used as a monitoring tool for understanding the effects of neurodegenerative diseases on functional connectivity.

Compared with other imaging methods for functional connectivity in animal studies (e.g., fMRI), our single-camera system is relatively sensitive and easily adaptable. We chose to implement a PCA algorithm to remove global and environmental noise and observe seed-based correlations. However, further investigation of the sources of the global noise, whether it be skull or white matter, should be performed. The depth information from our system still needs to be better analyzed in comparison with visual light OIS systems. However, by utilizing our current setup, we were able to more clearly distinguish between healthy and PD mice. Since the STN(subthalamic nucleus) of PD mice brain is abnormal, we thought that NIR could detect signals not only in the cortex, but also in deeps structures of the brain. In future works, if we perform experiments using both NIR and visual light simultaneously, we can more thoroughly research the causes of PD. In addition, we should investigate the effects

Fig. 9. K-means clustering image of oxy-hemoglobin concentration change patterns during the resting state. Four healthy (N 1–N 4; top row) mice and four hemi-parkinsonian (PD 1 – PD 4; bottom row) mice were analyzed. The healthy mice showed clustering corresponding to functionally connected bilateral areas of the brain; however, the hemodynamic signal from the PD mice was not clustered in a similar way.
that an injection anesthesia compared to inhalation anesthesia has on resting stated connectivity measurements.

There are several future possibilities for this study that are worth mentioning, especially concerning its implications for further PD research. For example, another experiment could be performed with our imaging system to examine functional connectivity changes during treatment (e.g., deep brain stimulation) for PD. In addition, future work will be devoted to longitudinal studies to investigate the connection of cerebral hemodynamics with disease progression. Histological data from the mice should be obtained to measure the severity of PD, as some hemi-parkinsonian mice demonstrated higher correlations than others (Fig. 7). Although the severity of the disease may have differed between the animals, our results support the notion that hemi-parkinsonian animals have a loss of functional connectivity, especially in the motor cortex, in comparison with the healthy group.

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Disclosures

The authors declare that there are no conflicts of interest related to this article.

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