Video Article

Autoradiographic Measurements of [^{14}C]-Iodoantipyrine in Rat Brain Following Central Post-Stroke Pain

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Abstract

Approximately 8% of stroke patients present symptoms of central post-stroke pain (CPSP). CPSP is associated with allodynia and hypersensitivity to nociceptive stimuli. Although some studies have shown that neuropathic pain may involve the dorsolateral prefrontal cortex, rostral anterior cingulate cortex, amygdala, hippocampus, periaqueductal gray, rostral ventromedial medulla, and medial thalamus, the neural substrates and their connections that mediate CPSP remain unclear. [^{14}C]-Iodoantipyrine (IAP) uptake can be measured to evaluate spontaneously active pain. It can be used to assess the activation of neural substrates that may be involved in CPSP in an animal model. The [^{14}C]-IAP method in rats is less expensive to perform compared with other brain mapping techniques. The present [^{14}C]-IAP protocol is used to measure the activation of neural substrates that are involved in CPSP that is induced by lesions of the ventral basal nucleus (VB) of the thalamus in a rodent model.

Video Link

The video component of this article can be found at https://www.jove.com/video/53947/

Introduction

Stroke hemorrhage has been shown to occur in more than 8% of patients who suffer from neuropathic pain, referred to as central post-stroke pain (CPSP).[1-3] CPSP can result from somatosensory dysfunction, thereby inducing hypersensitivity and allodynia.[4] However, the pathophysiological mechanisms of somatosensory dysfunction in CPSP remain uncertain. For example, the loss of somatic sensations results from neuronal deafferentation in the hemorrhagic brain area. Hyperalgesia may be caused by the hyperexcitability of central nociceptive neurons or central disinhibition,[5, 6] but the neural substrates that are involved in CPSP symptoms remain unknown. Some studies have suggested that the dorsolateral prefrontal cortex (dPFC), rostral anterior cingulate cortex (ACC), amygdala, hippocampus, periaqueductal gray (PAG), rostral ventromedial medulla, and their connections with each other mediate nociceptive processing.[7] Additionally, medial prefrontal cortex (mPFC)-amygdala circuits were shown to be involved in pain-related perception.[8] Data on the pathophysiological mechanisms of CPSP are diverse, and the activation of neural substrates in CPSP needs further scrutiny.

[^{14}C]-Iodoantipyrine (IAP) uptake is used to indirectly observe regional cerebral blood flow (rCBF), assuming a relationship between brain activity and CBF. Although [^{14}C]-IAP cannot assess brain activity in real time, such as with functional magnetic resonance imaging (fMRI), it has several advantages. For example, [^{14}C]-IAP is suitable for measuring spontaneously occurring brain events during pathological states.[9] Moreover, [^{14}C]-IAP uptake is measured without anesthesia. It also costs less than other imaging methods, including fMRI and positron emission tomography (PET). The [^{14}C]-IAP method has been suggested to be appropriate for measuring spontaneous pain (*e.g.*, CPSP) that is induced by lesions of the ventral basal nucleus (VB) of the thalamus.[9]

The present protocol describes how to perform the [^{14}C]-IAP method to assess the involvement of neural substrates of CPSP that is induced by lesions of the VB of the thalamus in an animal model. The technique offers a way of determining the pathophysiological mechanisms that underlie CPSP symptoms at the behavioral and neuronal levels.

Protocol

The protocol in the present study received approval from the Academia Sinica Institutional Animal Care and Utilization Committee in Taiwan.

1. Animal Preparations

1. Obtain male Sprague-Dawley rats (approximately 300 - 400 g). Maintain the rats in an air-conditioned room (21 - 22 °C, 50% humidity) under a 12 hr/12 hr light/dark cycle (lights on at 8:00 AM) with free access to food and water.

2. Experimental Procedure

1. Allow all of the rats to adapt to the environment in their home cages for 1 week before the experiments. During adaptation, perform the von Frey and plantar tests to establish baselines.
2. **von Frey Test**
    1. Place the rat in an acrylic enclosure (30 cm × 30 cm × 80 cm) for 30 min for habitation.
    2. Obtain von Frey filaments (filament no. 11 - 20) that have the same length but varying diameters to provide a range of forces of 2 - 100 g.
    3. To assess the paw withdrawal threshold in the rat's hindpaw, use von Frey filaments to stimulate the center of the hindpaws through a net-like port on the acrylic plate at 5 min intertrial intervals. To record the maximum applied pressure, use the filaments in ascending order, from low to high, until the maximum applied pressure is recorded.[10]
    4. When rats exhibit a paw withdrawal response to the stimulation, record the filament number. Determine the withdrawal response in this trial according to the lowest stimulus.
    5. Repeat the same procedure immediately, for a total of three times in succession on the same rat. Convert the filament number into the corresponding force (in grams) and average the values.
3. **Plantar Test**
    1. Place the rat in a transparent Plexiglas box (divided into four frames, 80 cm × 30 cm × 15 cm) for 30 min for habitation.
    2. Use an infrared beam to stimulate the center of the hindpaw through a glass plate. Adjust the infrared light intensity to obtain an average paw withdrawal response latency of approximately 10 sec. Conduct a trial by depressing a key that turns on the infrared light source and starts a digital solid-state timer. Manipulate the duration of the infrared light beam.
    3. Record the duration of the infrared light when the rats exhibit a paw withdrawal response. The longest duration should not exceed 20 sec in each trial to avoid tissue damage. Use an intertrial interval of at least 5 min to avoid successive stimulation.
    4. Repeat the test with three trials for the left and right hindpaws, and calculate the average for each hindpaw for each rat.
4. **Collagenase Lesion Surgery**
    1. Anesthetize the rat with 4% isoflurane until loss of the toe-pinch response and somatic responses to surgical stimuli occur. Maintain anesthesia with 1.5 - 2% isoflurane for the duration of surgery.
    2. Place the rat in a stereotaxic device with a simple heating pad to maintain body temperature at 36.5 - 37.5 °C. Apply eye cream on the eyes to prevent dryness while under anesthesia.
    3. Shave the fur with sterilized electric clippers, and make a smooth incision (approximately 2-2.5 cm) with a scalpel along the midline of the scalp. Clean the skin and skull with alternating providence iodine and 75% alcohol solution for disinfection. During the surgical phase, use 75% alcohol to sterilize all devices, tools, and the workbench to maintain sterile conditions. Before that, all surgical materials must be sterilized in a steam autoclave.
    4. Use the sterile gloves and instruments, and drill a small hole (3 mm diameter) in the skull with an electric drill over the VB (including ventral posteromedial thalamic nucleus [VPM] and ventral posterolateral thalamic nucleus [VPL]) of the thalamus (3.0-3.5 mm posterior, 3.0-3.5 mm lateral to bregma, and 5.5-5.8 mm depth from skull surface).
    5. Microinject 0.5 µl of normal saline or 0.125 U of type 4 collagenase solution in the control and experimental groups, respectively.
    6. Keep the injection needle in place for an additional 5 min to allow for drug diffusion.
    7. Use dental cement to fill the hole in the skull, and suture the incision. After suture the incision, apply the local analgesic anesthetics (lidocaine ointment) to the wound, and return them to their home cages.
    8. After surgery, singly house the rats in plastic cages until they maintain sternal recumbency, and keeps warm until recovered from anesthesia.
5. **Behavioral Tests after Surgical Recovery**
    1. After 7 days of recovery from surgery, repeat the procedures in sections 2.2 and 2.3 for the test phase. Perform the behavioral tests over 4 weeks while monitoring the health and developmental status of the animals.
    2. Monitor the animals' health conditions (*e.g.*, body weight, feeding amount, and free movement) between the control and experimental groups for the post-surgery phase.
6. **Perform Cannula Implantation with PE-50 Tubing**
    1. Anesthetize the rat with 4% isoflurane, and maintain body temperature at 36.5 - 37.5 °C with a simple heating pad.
    2. With a scalpel, cut two holes (2 cm diameter each) in the midline of the dorsal part of the forelimbs and intersection of the ventral part of the left shoulder and thoracic cavity, respectively.
    3. Dissociate the skin and muscle with a pair of scissors between the two holes.
    4. Connect one end of PE- 50 tubing (20 cm length) to the external jugular vein through the ventral hole. Connect the terminal end of the same PE- 50 tubing to the dorsal hole, and affix to the skin.
    5. Use a syringe to inject saline into the jugular vein to verify that the PE- 50 tubing is not obstructed.
    6. Suture the incision, and inject the rats with 6 mg/kg gentamycin (intraperitoneally).
    7. Flush the tubing every other day after surgery with 0.3 ml of 0.9% saline, followed by 0.1 ml of saline with 20 U/ml heparin.
7. **Final Behavioral Test**
    1. One week after step 2.6, repeat steps 2.2-2.3 to confirm that behavior is stable compared with step 2.5 after surgical recovery.

8. **Radiotracer Injection**
    1. Place the rat in a resting cage for 5 - 10 min for adaptation.
    2. Using a splitter, connect PE- 50 tubing to two 1 ml syringes. Fill one syringe with normal saline and fill the other with [^{14}C]-IAP solution (125 µCi/kg in a volume of 0.3 - 0.5 ml).
    3. Inject the radiotracer into the external jugular vein, and replace the syringe with another syringe filled with 3 M potassium chloride.
    4. Ten seconds after the radiotracer injection, inject 3 M potassium chloride under an overdose of isoflurane, and sacrifice the animals according to standard euthanasia methods (*i.e.*, isoflurane from a vaporizer for 50 min) based on the guidelines of the Academia Sinica Institutional Animal Care and Utilization Committee in Taiwan.
    5. After 1 min, expose the skull, and trim off the remaining muscle. Using rongeurs, peel away the dorsal surface of the skull from the brain. Trim away the sides of the skull using rongeurs. Next, using a spatula, cut the olfactory bulbs and nerve connections along the ventral surface of the brain, and remove the brain.
    6. Use optimal cutting temperature (OCT) compound to freeze the brain in dry ice and methylbutane (approximately -55 °C). Store the brain tissue in a freezer.[11, 12]

9. **Brain Slicing**
    1. Orient the tissue in a microtome, with the hindbrain facing down. Use a cryostat to slice the brain into 20 µm-thick sections.
    2. Place the brain slices on microscope slides in a cryostat at -20 °C, with a 240 µm interval between each slice.
    3. Place the microscope slides and five standard filter papers with graded radioactivity into exposure cassettes for 3 days at -20 °C. According to the sequence of the brain slices, arrange the microscope slides from top to bottom. Finally, place the filter papers in the bottom of the cassettes.[12]
    4. Remove the phosphor screen from the exposure cassettes, and use a variable-mode imager to read the phosphor screen and generate images to show [^{14}C]-IAP uptake for the brain slices.

3. Data Analysis

1. After step 2.9.4, adjust the images using Statistical Parametric Mapping (SPM) and ImageJ software. Reconstruct all of the images using serial coronal sections. Smooth and normalize the images according to a reference rat brain model.[12, 13]
2. For quantitative assessments, measure the region-of-interest (ROI) of the brain images using ImageJ software to determine the pixel signal intensity, and use statistical software for the analysis.[12, 13]
3. To investigate the connections between different brain nuclei, use MATLAB correlation analysis software to display radioactivity ratio in an interregional correlation matrix, and visualize the matrices as color maps. Finally, use Pajek software for network analysis.[12, 13]
4. Use 2 × 5 two-way mixed analysis of variance (ANOVA), with group and weeks as factors, to compare the duration of heat tolerance in the plantar test and mechanical force in the von Frey test in the sham and CPSP groups at baseline and weeks 1 - 5. Use a 2 × 31 two-way ANOVA to measure the radioactivity ratio according to group and brain area. When appropriate, conduct Tukey's Honestly Significant Difference (HSD) *post hoc* tests. Calculate Pearson correlation coefficients to assess correlations among all of the selected brain areas in the sham and CPSP groups.

Representative Results

Figure 1A depicts the experimental timeline. Rats were assigned to the sham and CPSP groups for the behavioral tests (*i.e.*, von Frey test and plantar test). The first day of the experiment served as baseline, and tests were repeated at weeks 1 - 5. PE-50 catheterization was performed in the external jugular vein at week 4. Heparin (20 U/ml, 0.1 ml/day) was injected during weeks 4 and 5. Five minutes after the heparin injection, [^{14}C]-IAP was injected, followed 10 sec later by an overdose of anesthetic for sacrifice. One minute later, the rats were decapitated, and OCT-embedded brain slices were made. **Figure 1B** depicts the rats in a surgical stereotaxic device, cannula implantation sites, and histological map of the brain slices based on a rat brain atlas. **Figure 1C** shows the right hole of the external jugular vein in red, the location of the PE- 50 tubing, and final experimental setup.

Figure 2A shows brain slices that were exposed in the cassettes. The phosphor screen was analyzed using a variable-mode imager. Sample and standard data for the brain slices were then analyzed. **Figure 2B** shows the standard autoradiographic curves. The left panel shows the relationship between image intensity (pixel/mm^2) and radioactivity counts per minute (CPM), thus yielding the following predicted linear equation: $Y = 44.542X + 196.24$. The right panel shows that the resolution (pixel/mm^2) was enhanced as the exposure time increased (in days). The optimal resolution was observed on Day 4.

Figure 3A shows the experimental setup for the plantar test, which assesses thermal pain. The CPSP group exhibited a significant decrease in the paw withdrawal threshold *(i.e.*, less heat tolerance) compared with the sham group at baseline and weeks 1 - 5 (all $p < 0.05$). **Figure 3B** shows the experimental setup for the von Frey test, which assesses mechanical pain. The CPSP group exhibited a significant decrease in mechanical force (gw) at baseline and weeks 1 - 5 (all $p < 0.05$).

Figure 4A shows the ROIs in an anatomical atlas. The ROI analysis showed that activation of the infralimbic cortex (IL), prelimbic cortex (PrL), and cingulate cortex area 1 (Cg1) was significantly higher in the CPSP group in the right hemisphere, with the exception of the VB (**Figure 4B**).

Differences in inter-regional correlations of rCBF were observed between the CPSP and sham groups in the right hemisphere (**Figure 4C**). The matrix (Fisher's Z-statistics) of all of the regions was analyzed using Pearson's correlation. **Figure 4C** shows differences in inter-regional correlations of the involvement of neural substrates in the CPSP group. Pain-related neural substrates were determined by analyzing differences in inter-regional correlations of rCBF. The red lines in **Figure 4D** indicate significant positive correlations, and blue lines indicate significant negative correlations.

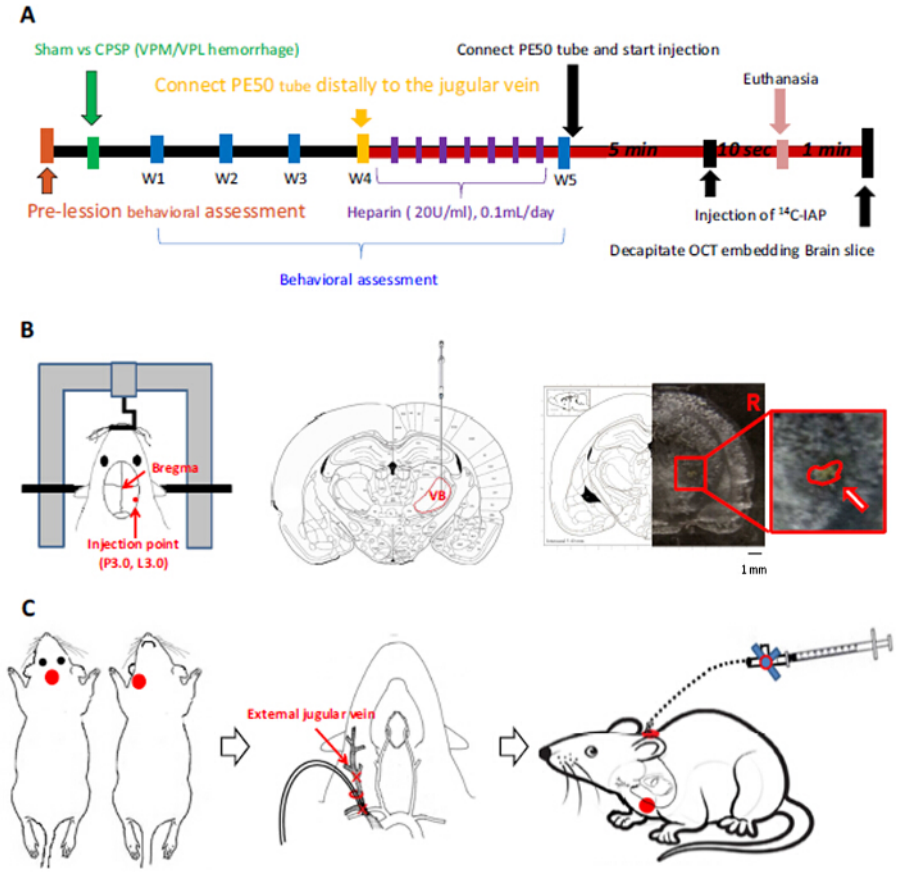


Figure 1. Experimental Timeline of Lesioning the Ventral Basal Nucleus (VB) of the Thalamus to Induce Central Post-Stroke Pain (CPSP) and Injecting [^{14}C]-IAP. (A) Ventral basal nuclei lesions to induce CPSP for the behavioral assessments and injections of [^{14}C]-IAP to measure the activation of neural substrates that are involved in CPSP. **(B)** Location of VB. **(C)** [^{14}C]-IAP injections. Scale bar = 1 mm. Please click here to view a larger version of this figure.

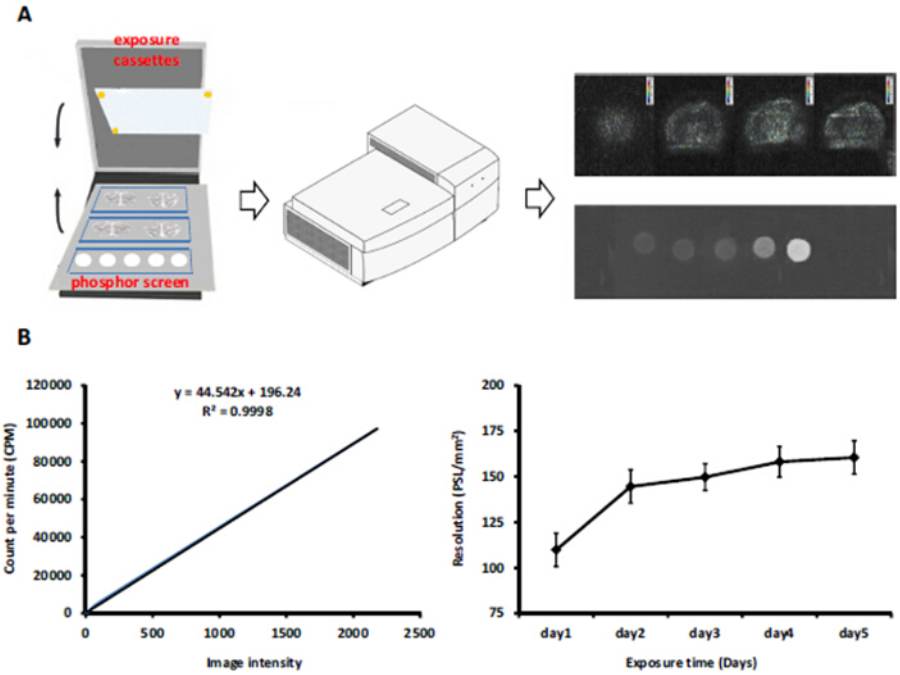
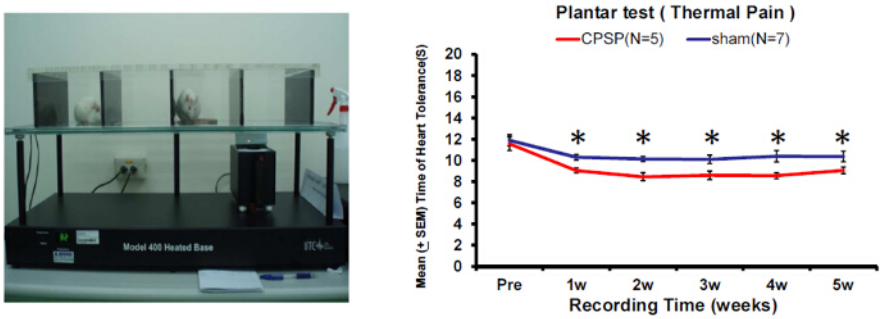


Figure 2. Standard Autoradiographic Curves. (A) Sample and standard curves were obtained for different exposure times and image resolutions. **(B)** Standard curve of image intensity and CPM and standard curve of exposure time and resolution. Please click here to view a larger version of this figure.

A



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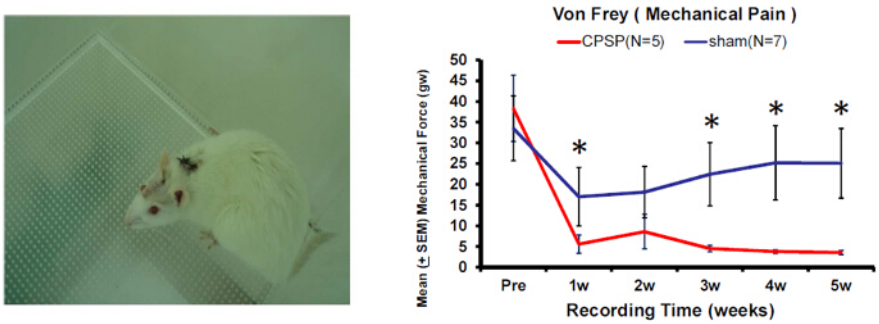

Figure 3. The Plantar Test (Thermal Pain) and von Frey Test (Mechanical Pain) were Conducted in the Sham and CPSP Groups at Baseline and Weeks 1 - 5. (A) CPSP rats exhibited a lower paw withdrawal threshold in the plantar test (*i.e.*, less heat tolerance) compared with sham rats, indicating greater thermal pain. **(B)** CPSP rats exhibited a lower paw withdrawal threshold in the von Frey test compared with sham rats, indicating greater mechanical pain. SEM, standard error of the mean. Green asterisks (*) indicate a significant difference compared with the sham group. Please click here to view a larger version of this figure.

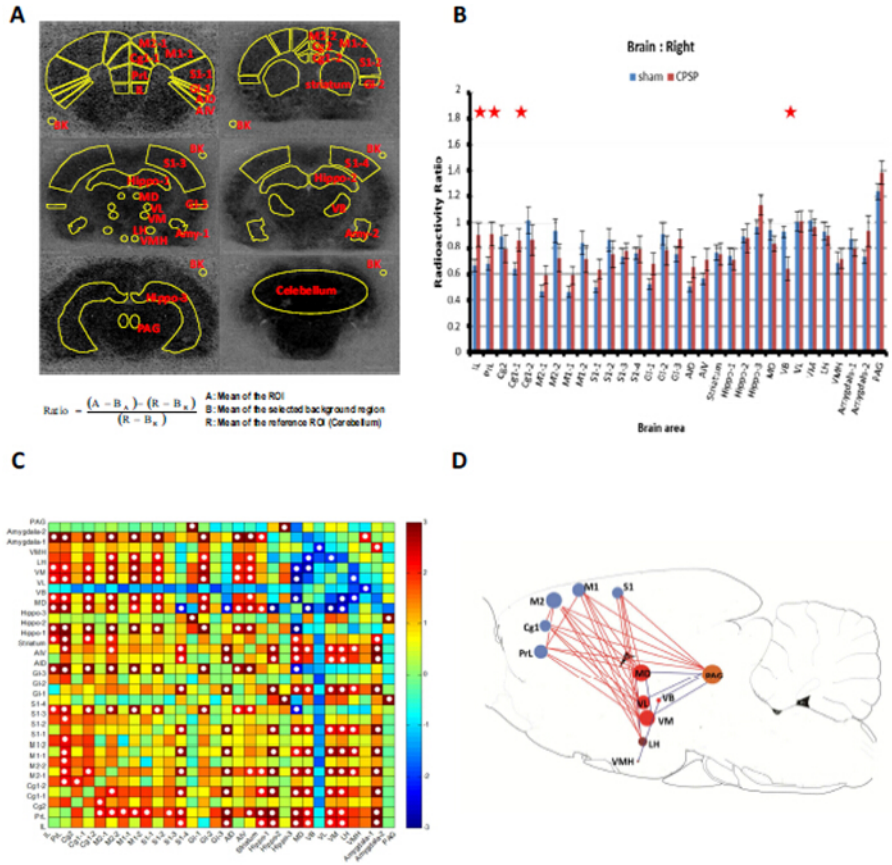

Figure 4. ROI Analysis and Relationship among Inter-Regional Correlations Between Neural Substrates Involved in CPSP. (A) Brain areas were determined and analyzed by the ratio formulation. **(B)** The ROIs in the IL, PrL, Cg1, and VB were significantly different in the right hemisphere. **(C)** Analysis of rCBF in the selected brain areas. **(D)** Inter-regional correlations between brain areas. Please click here to view a larger version of this figure.

Discussion

In the behavioral tests, the CPSP group exhibited reductions of the paw withdrawal threshold in the thermal pain test and mechanical force in the von Frey test at baseline and weeks 1 - 5. The findings were consistent with a previous study.[14]

The [^{14}C]-IAP method relies on the pixel intensity of brain images for the quantitative analysis of different brain slices. To evaluate the data in the brain images, the pixel signal intensity was defined. In the present study, the environmental background signal was defined as 25.811 - 46.979 CPM. The signal of the [^{14}C]-IAP 0.001 μCi filter paper was defined as 42 CPM. The pixel signal intensity was < 0.001 μCi, serving as the background intensity. The pixel intensity of five filter papers was determined for 0.001, 0.01, 0.1, and 10 μCi, serving as the pixel gray scale for each of the brain images. [^{14}C]-IAP radioactivity showed a positive correlation with pixel intensity and radioactivity count on a logarithmic scale. Therefore, the above procedure can be followed for the calibration of [^{14}C]-IAP radioactivity to pixel intensity.

When performing the [^{14}C]-IAP experimental protocol, some points need to be considered. For example, the external jugular vein can become blocked, and experimenters need to ensure the patency of the PE- 50 tubing with heparin every day. Additionally, the location of the lesion sites can sometimes be misplaced, resulting in nonsignificant CPSP symptoms. Before the injections, the accuracy of the injection sites and locations relative to bregma must be confirmed. The angle and volume of each injection must also be accurately determined.

Limitations of brain images also need to be considered. Distortions of the brain images can occur after exposing brain slices in the cassettes to a phosphor screen. The brain images need to be normalized to a standard brain atlas using an image analysis program to avoid potential distortions in the brain images. Furthermore, different isotopes can yield different results because of their different mechanisms and action. For example, the metabolite and mechanism of action of [^{18}F]-fludeoxyglucose (FDG) are similar to glucose. Therefore, the [^{18}F]-FDG images were shown to be similar to the pathway of glucose metabolism. Additionally, the half-life of [^{18}F]-FDG is short; therefore, it must be combined with PET to generate the images. [^{201}Tl] is suitable for assessing myocardial blood flow perfusion using single-photon emission computed tomography. Therefore, choosing a suitable isotope for assessing brain images is important.

The application of the [^{14}C]-IAP method to assess brain activation in CPSP is less costly than other brain mapping techniques (*e.g.*, PET and fMRI). The [^{14}C]-IAP method is suitable for spontaneously occurring events, but it cannot be used for brain mapping in real time. The method is different from other brain mapping techniques, such as PET and fMRI. Furthermore, the present [^{14}C]-IAP protocol can measure subtle changes in rCBF in any pathological condition.

The [^{14}C]-IAP method can be used to test conventional pain pathways, such as the spinothalamic tract (STT), medial thalamus (MT)-ACC, and mPFC-amygdala neural circuits. The activation of each of these pathways impacts the others. The activation of these pathways in CPSP has been detailed in our previous paper.[12]

Disclosures

The authors have nothing to disclose.

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