

## A novel approach for locating mice brain regions of *Cryptococcus neoformans* CNS invasion

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### Abstract

Aim of this study was to locate the brain regions where *Cryptococcus* interact with brain cells and invade into brain. After 7 days of intratracheal inoculation of GFP-tagged *Cryptococcus neoformans* strains H99, serial cryosections (10  $\mu$ m) from 3 C57 BL/6 J mice brains were imaged with immunofluorescence microscopy. GFP-tagged H99 were found in some brain regions such as primary motor cortex-secondary motor cortex, caudate putamen, stratum lucidum of hippocampus, field CA1 of hippocampus, dorsal lateral geniculate nucleus, lateral posterior thalamic nucleus, laterorostral part, lateral posterior thalamic nucleus, mediorostral part, retrosplenial agranular cortex, lateral area of secondary visual cortex, and lacunosum molecular layer of the hippocampus. The results will be very useful for further exploring the mechanism of *C. neoformans* infection of brain.

### Introduction

*Cryptococcus neoformans* is an encapsulated fungal pathogen that can cause life-threatening infections of the central nervous system (CNS) in susceptible patients, mainly HIV+ individuals (Day et al., 2013; Eisenman et al., 2007; Hakim et al., 2000; Jarvis et al., 2009; Mitchell and Perfect, 1995; Mwaba et al., 2001; Park et al., 2009; Perfect et al., 2010; Powderly, 1993; Saag et al., 2000). *C. neoformans* are commonly acquired by inhalation (Day et al., 2013; Jarvis et al., 2009).

The mechanism of extrapulmonary dissemination to the CNS are not completely understood. The mouse model of experimental hematogenous meningoencephalitis was commonly used as an *in vivo* mode to investigate the mechanisms of *C. neoformans* penetration into the brain (Maruvada et al., 2012; Noverr et al., 2003).

Understanding the interactions between *C. neoformans*

and brain cells is fundamental in exploring the mechanisms of the *C. neoformans* CNS infection. Brain capillaries can be the sites of the interaction of *Cryptococcus* with brain endothelial cells (Shi et al., 2010). However, brain regions where yeast cells interact with brain cells and invade into brain remain unclear.

Aim of this study was to locate the regions of brain invasion by *Cryptococcus*. We established a novel approach for locating the brain regions of *C. neoformans* CNS invasion in the mouse model of experimental hematogenous meningoencephalitis, which will be very useful for further exploring the mechanism of *C. neoformans* CNS infection.

### Materials and Methods

#### Reagents

PECAM-1 (platelet/endothelial cell adhesion molecule-1) rat monoclonal antibody and p-cPLA<sub>2</sub> rabbit polyclonal antibody were purchased from Santa Cruz

Biotechnology (USA). Dylight 488 goat anti-Rat IgG was purchased from EarthOx Life Sciences (USA). Cy3 goat anti-Rabbit IgG was purchased from Beyotime Biotechnology (China).

#### Yeast strains

*C. neoformans* strains H99 was purchased from ATCC. GFP-tagged H99 was generated by integration of plasmid containing GFP construct (a generous gift of Prof. Robin May, University of Birmingham, UK) into the genome of the H99 with a BioRad PDS-1000/He biolistic particle delivery system (Voelz et al., 2010).

#### Intratracheal inoculation

Mice (female; C57BL/6J mice; 10-12 weeks; 20-25 g) model of experimental meningitis was prepared by intratracheal inoculation of *C. neoformans* strains H99. Mice were anesthetized with intraperitoneal injection of 4% chloral hydrate at 50 mg/kg. A small incision was made in the skin over the trachea. A 30-gauge needle was attached to a tuberculin syringe. The needle was

bent and inserted into the trachea, and 30  $\mu$ L PBS (containing  $10^5$  CFU *Cryptococcus*) was delivered. The skin was sutured with a cyanoacrylate adhesive, and the mice recovered with no visible trauma (Noverr et al., 2003).

#### Immunofluorescence

After 7 days of intratracheal inoculation of *C. neoformans*, mouse chest was cut open, and mouse was perfused with a mammalian Ringer's solution by transcardiac perfusion through a 23-gauge needle inserted into the left ventricle of the heart under the perfusion pressure of about 100 mmHg for 20 min. The brains were removed and put in liquid nitrogen. Serial cryosections (10  $\mu$ m) were incubated overnight with a monoclonal rat anti-PECAM-1 primary antibody and/or a polyclonal rabbit anti-p-cPLA<sub>2</sub> primary antibody. Afterward, sections were incubated with Dylight 488 goat Anti-Rat IgG secondary antibody and/or Cy3 goat anti-Rabbit IgG secondary antibody. Slides were imaged with a confocal microscopy (Leica TCS SP5).

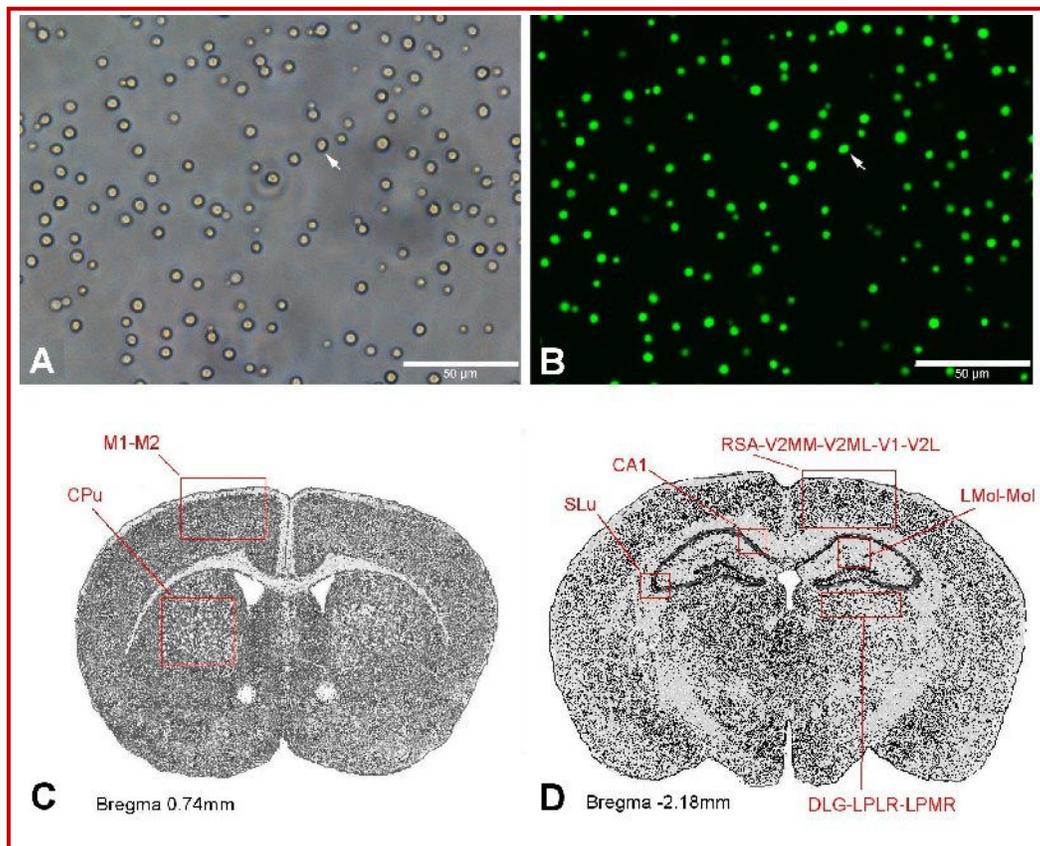


Figure 1: Schematic diagram of mice brain regions of GFP-tagged *Cryptococcus neoformans* invasion

After 7 days of GFP-tagged *C. neoformans* H99 (A, B) intratracheal inoculation, H99 could be found in regions of M1-M2 and CPu (C, Bregma 0.74), and in regions of SLu, CA1, RSA-V2MM-V2ML-V1-V2L, LMol-Mol, and DLG-LPLR-LPMR (D, Bregma -2.18 mm). M1-M2: Primary motor cortex-secondary motor cortex, CPu: Caudate putamen, SLu: Stratum lucidum of hippocampus, CA1: Field CA1 of hippocampus, DLG: Dorsal lateral geniculate nucleus, RSA: Retrosplenial agranular cortex, V2MM: Mediomed of secondary visual cortex, V2ML: Mediolat of secondary visual cortex, V1: Primary visual cortex. Arrows indicate the same H99 under bright-field (A) and fluorescence (B) microscopy. Scale bar, 50  $\mu$ m

## Results

In this study, the mouse model of experimental hematogenous meningoencephalitis was prepared by intratracheal inoculation of GFP-tagged *C. neoformans* H99 (Figure 1A and B). After 7 days of the inoculation, serial cryosections from 3 mice brains were imaged with fluorescence microscopy for locating regions of brain invasion by *C. neoformans*. GFP-tagged H99 were found in some brain regions such as primary motor cortex-secondary motor cortex (M1-M2), caudate putamen (CPu), stratum lucidum of hippocampus (SLu), field CA1 of hippocampus (CA1), dorsal lateral geniculate nucleus (DLG), lateral posterior thalamic nucleus, laterorostral part (LPLR), lateral posterior thalamic nucleus, mediorostral part (LPMR), retrosplenial agranular cortex (RSA), lateral area of secondary visual cortex (V2L), and lacunosum molecular layer of the hippocampus (LMol) (Figure 1C and D). Furthermore, immunofluorescent imaging for specific regions in Figure 1C and D are showed respectively in Figure 2 (CPu, M1-M2, RSA-V2L), Figure 3 (DLG, LMol, SLu), and Figure 4 (CA1).

Some evidence from human cases and experimental animal models of *C. neoformans* meningoencephalitis indicate that cerebral capillaries are the portal of *C. neoformans* invasion into the brain (Chang et al., 2004 ; Chretien et al., 2002; Lee et al., 1996; Neuville et al., 2002; Shi et al., 2010). In this study, PECAM-1 (platelet/endothelial cell adhesion molecule-1), a marker of brain vessel endothelial cells, was stained with immunofluorescent antibody to show microvasculature, which enable us to locate both *C. neoformans* and vasculature.

Our results have showed that *C. neoformans* invaded into hippocampal regions such as LMol and SLu (Figure 3) after 7 days of intratracheal inoculation. Our previous work showed that host cytosolic phospholipase A2 $\alpha$  (cPLA<sub>2</sub>) contributes to group B *Streptococcus* penetration of the blood-brain barrier (BBB) (Maruvada et al., 2011). However, the role of cPLA<sub>2</sub> in *C. neoformans* CNS infection remains unclear. cPLA<sub>2</sub> had been showed to be involved in A $\beta$ -dependent deficits of learning and memory in a mouse model of Alzheimer's disease (Sanchez-Mejia et al., 2008). Therefore, it will be very interesting to examine whether the cPLA<sub>2</sub> in hippocampus is involved in *C. neoformans* invasion and resulting memory impairment of cryptococcal meningoencephalitis. We have found that cPLA<sub>2</sub> is extensively active in hippocampus CA1 (Figure 4). The disappearance of p-cPLA<sub>2</sub> positive cells in infected areas indicated that *C. neoformans* invasion into CA1 cause the damage of the tissue in this region (Figure 4). Although more evidence need for clarifying the role of cPLA<sub>2</sub> in *C. neoformans* CNS infection, the preliminary experiment has showed that this approach will be very useful for future studies of *C. neoformans* CNS infection.

## Discussion

Approaches of traditional histology had been used to study the CNS invasion by *C. neoformans*. Experiments were designed to characterize the anatomical site where *C. neoformans* crossed the BBB after intravenous injection of *Cryptococcus*. However, during a natural infection *Cryptococcus* are gradually released from the lung and enter the blood stream in limited numbers (Shi et al., 2012). Previous study showed that yeasts were observed in or next to the cortical capillaries of the brain and cerebellum and that no yeast cells were observed in the choroid plexus after intravenous inoculation. In this study, mice model of CNS infection was prepared by intratracheal inoculation, which is more close to natural infection as compared with intravenous inoculation. We provided the first evidence that several important brain regions were infected by *C. neoformans* after 7 days of intratracheal inoculation.

For intravital imaging of *C. neoformans* CNS invasion, the yeast cells can be labeled with fluorescent dyes (Shi et al., 2010). However, the yeast cell will lose the fluorescent label when it multiplies. Consequently, it will be hard to track the *C. neoformans* if it begins to replicate. To overcome this disadvantage, we generated GFP-tagged H99 by integration of plasmid containing GFP construct into the genome of the H99. In *in vitro* models, GFP-tagged *Cryptococcus* had been used for automated analysis of cryptococcal macrophage parasitism (Voelz et al., 2010) and for the study of interaction between yeast and human brain microvascular endothelial cell (Huang et al., 2011). In our *in vivo* model of this study, using of GFP-tagged H99 enable us to track and locate the regions of brain invasion by *C. neoformans* after 7 days of intratracheal inoculation of *Cryptococcus*.

In addition, this approach also provides some other advantages: Firstly, GFP-tagged *Cryptococcus* in serial cryosections are readily visualized via fluorescence microscopy. Secondly, brain structures such as vasculature that are morphologically distinguishable from *Cryptococcus* can both be stained as the same color in fluorescence imaging, which save one light path for fluorescence imaging of other molecular. Thirdly, all cell nucleus DNA can be stained with DAPI as blue color, and any tissue damage (disappearance of blue) is readily detected.

As an example of using this approach in study of CNS infection, we have showed that the molecular such as cPLA<sub>2</sub> can be stained with fluorescence dyes and can be visualized by fluorescence microscopy to examine the role of this molecular in CNS infection. In fact, most fluorescence microscopes can have four light path used for fluorescence imaging. In this approach, GFP-tagged H99 and PECAM-1 (green) and DAPI (blue) occupy two light path, and there are two other light path leave

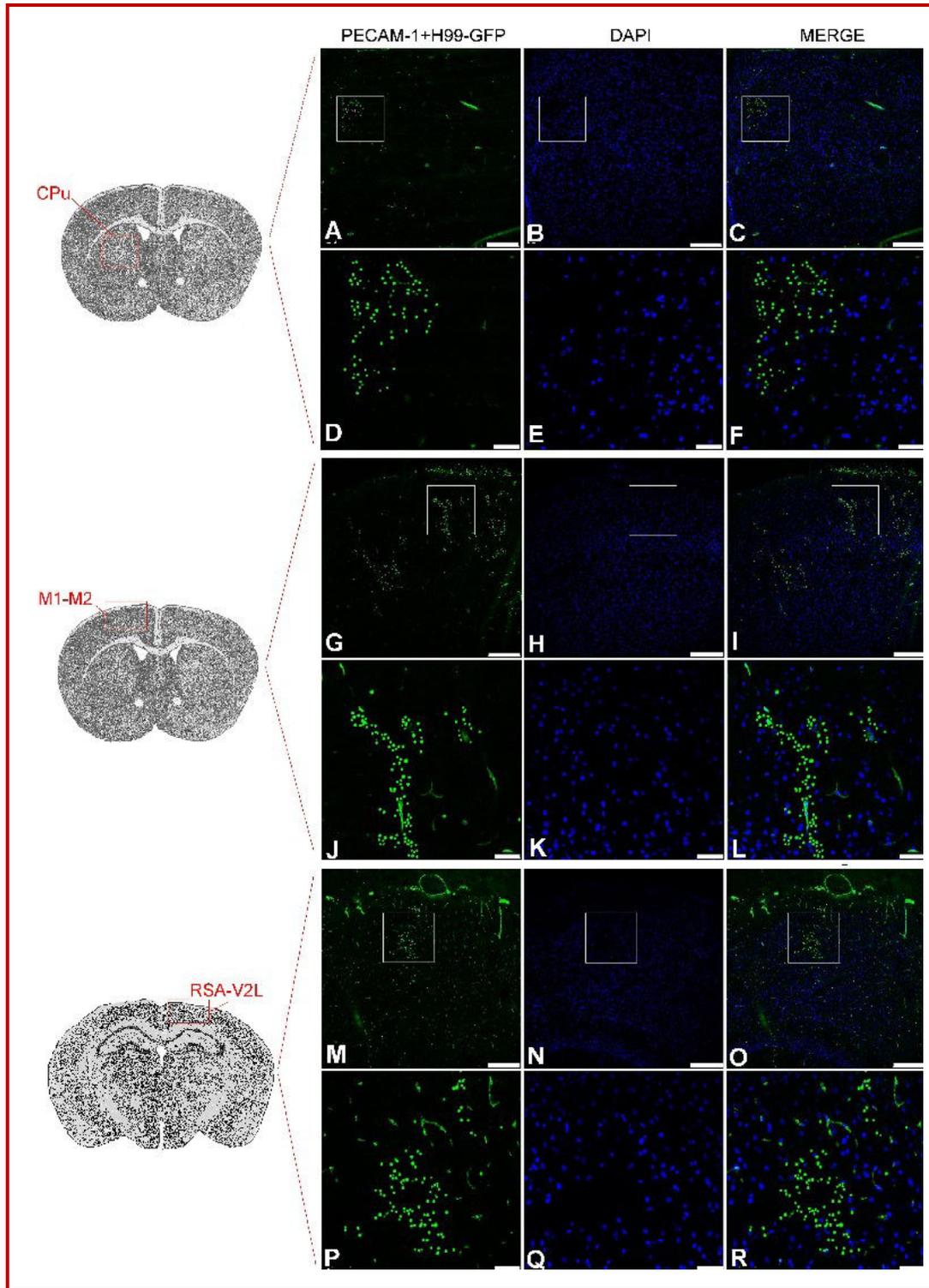


Figure 2: *C. neoformans* invasion into caudate putamen (CPu), primary motor cortex-secondary motor cortex (M1-M2), and the regions from retrosplenial agranular cortex (RSA) to lateral area of secondary visual cortex (V2L)

Many GFP-tagged *Cryptococcus* (H99-GFP, green and round) were found in CPu, M1-M2, and RSA-V2L after 7 days of intratracheal inoculation. PECAM-1 of brain vessel endothelial cells was stained as green, microvessels (green but not round) are distinguishable from green and round H99. DAPI stains cell nucleus. A-C, G-I, M-O: 10X, bar=250 µm; D-F, J-L, P-R: Higher magnification (40X) of the area enclosed within the box in A-C, G-I, M-O, respectively, bar = 50 µm

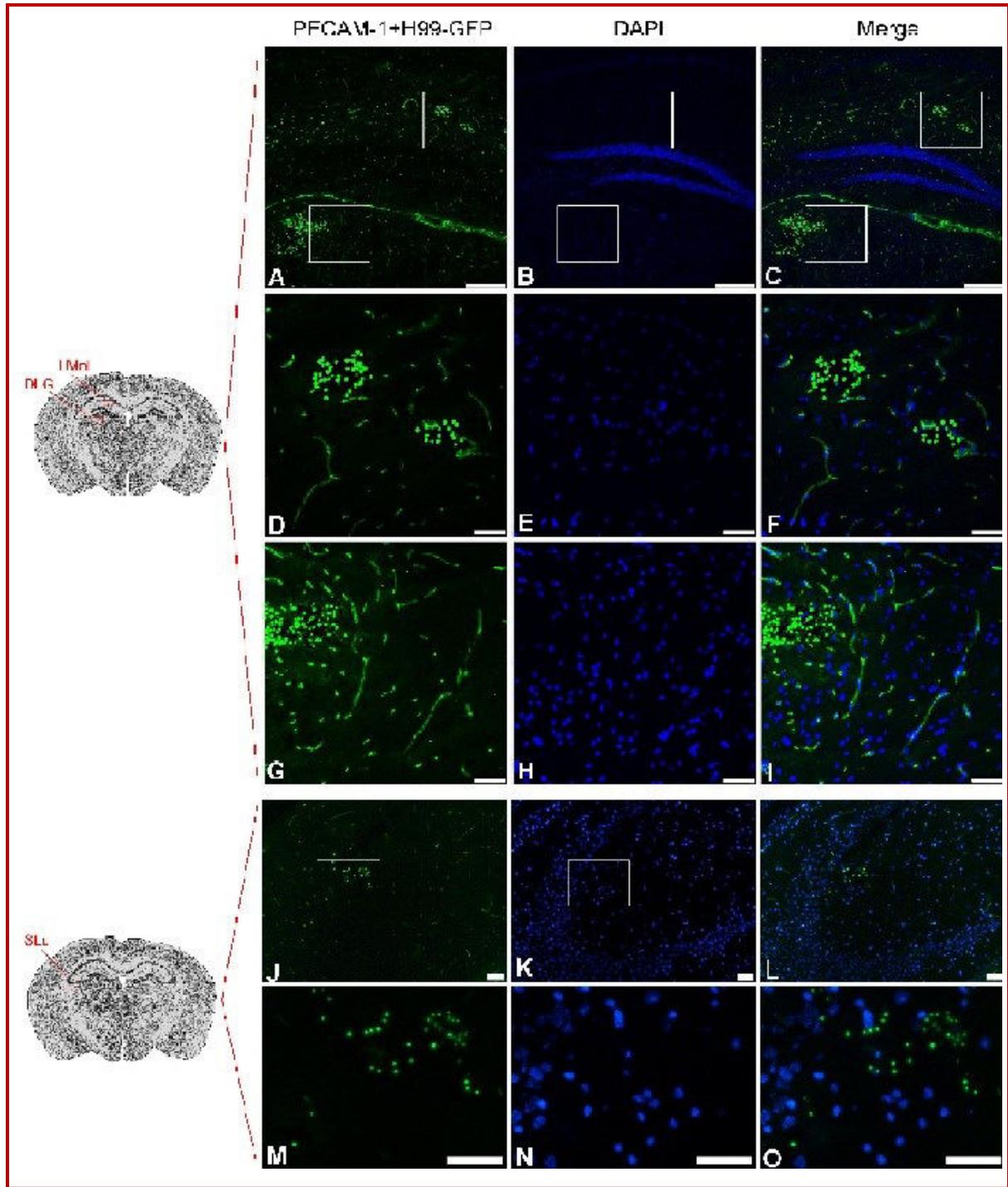


Figure 3: *C. neoformans* invasion into lacunosum molecular layer of the hippocampus (LMol), dorsal lateral geniculate nucleus (DLG), or stratum lucidum of hippocampus (SLu)

Many GFP-tagged *Cryptococcus* (H99-GFP, green and round) were found in LMol, DLG, or SLu after 7 days of intratracheal inoculation. PECAM-1 of brain vessel endothelial cells was stained as green, microvessels (green but not round) are distinguishable from green and round H99. DAPI stains cell nucleus. A-C, J-L: 10X, bar=250  $\mu$ m; D-F: Higher magnification (40X) of the area enclosed within the upper right box in A-C, bar = 50  $\mu$ m. G-I: Higher magnification (40X) of the area enclosed within the left down box in A-C, bar =50  $\mu$ m. M-O: Higher magnification (40X) of the area enclosed within the box in J-L, bar = 50  $\mu$ m

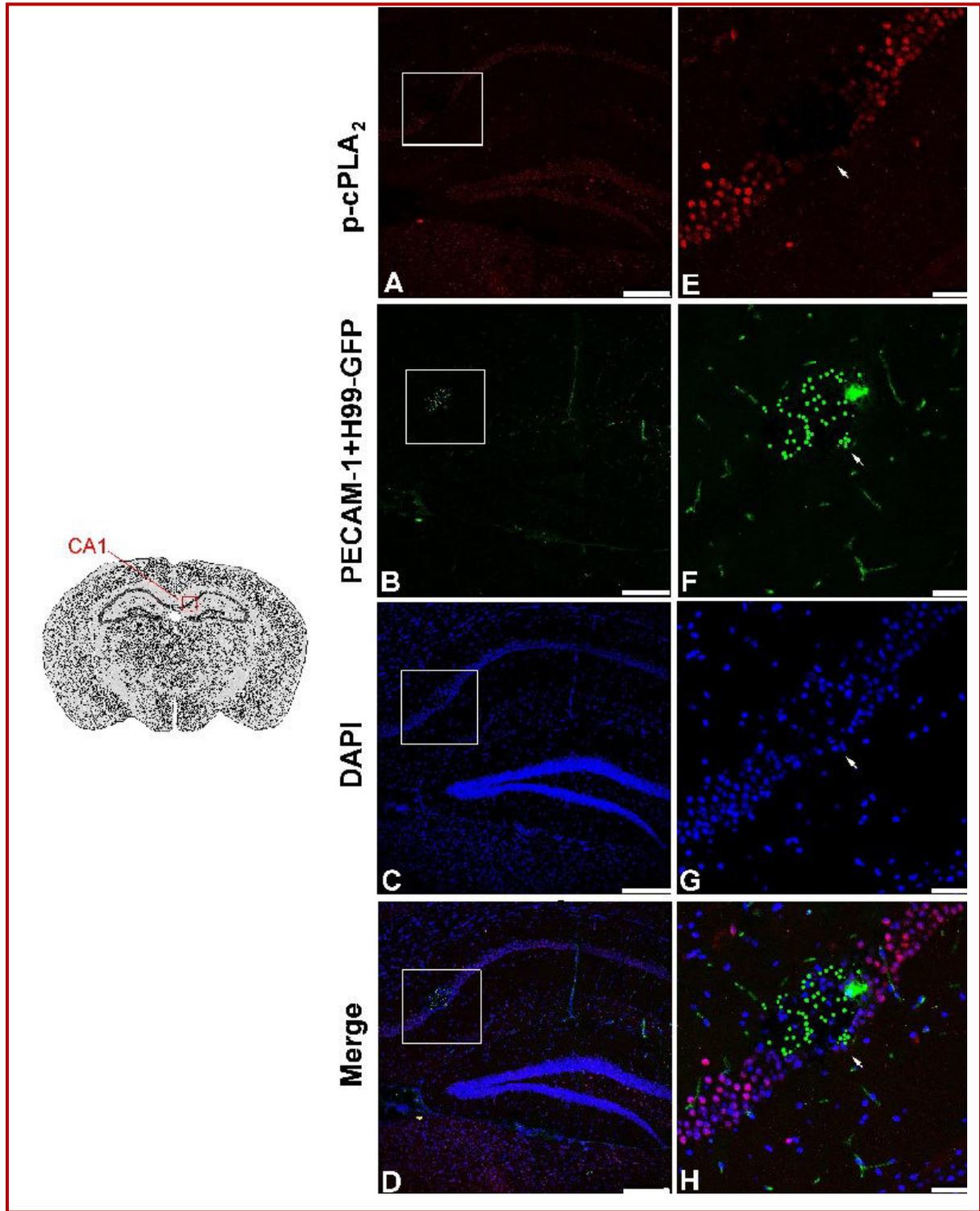


Figure 4: *C. neoformans* invasion into field CA1 of hippocampus (CA1)

Many GFP-tagged *Cryptococcus* (H99-GFP, green and round) were found in CA1 after 7 days of intratracheal inoculation. PECAM-1 of brain vessel endothelial cells was stained as green, microvessels (green but not round) are distinguishable from green and round H99. p-cPLA2, a Ser 505 phosphorylated cPLA2, is the active form of cPLA2. Cells in CA1 highly express p-cPLA2 (red). DAPI stains cell nucleus. Arrow indicates that H99 caused tissues damage in infected regions of CA1. A-D: 10X, bar = 250  $\mu$ m; E-H: Higher magnification (40X) of the area enclosed within box in A-D, bar = 50  $\mu$ m.

for fluorescence imaging of two other molecular. If gene knockout or transgene mice are used in the model, up to three or four molecular that may be involved in CNS infection can be examined in the same experimental model, which will make this approach a valuable research tool in the field of *C. neoformans* CNS infection.

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## Conclusion

We provided the first evidence that several important brain regions were infected by *C. neoformans* after 7 days of intratracheal inoculation, which will be very useful for further exploring the mechanism of *C. neoformans* CNS infection.

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## Ethical issue

All animal experiments were approved by the Ethics Committee of Fujian Medical University, China.

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## Conflict of Interest

We declare that we have no conflict of interest.

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