1   **Title:** Intranasal inoculation of *Cryptococcus neoformans* in mice produces nasal

2   infection with rapid brain dissemination

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22   Running title: Rapid brain dissemination by *C. neoformans*

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Abstract:

Cryptococcus neoformans is an important fungal pathogen, causing life-threatening pneumonia and meningoencephalitis. Brain dissemination of *C. neoformans* is thought to be a consequence of an active infection in the lung which then extravasates to other sites. Brain invasion results from dissemination via the bloodstream, either by free yeast cells in bloodstream or Trojan horse transport within mononuclear phagocytes. We assessed brain dissemination in three mouse models of infection: intravenous, intratracheal, and intranasal. All three modes of infection resulted in dissemination of *C. neoformans* to the brain in under 3 hours. Further, *C. neoformans* was detected in the entirety of the upper respiratory tract and the ear canals of mice. In recent years, intranasal infection has become a popular mechanism to induce pulmonary infection because it avoids surgery but our findings show that instillation of *C. neoformans* produces cryptococcal nasal infection. These findings imply that immunological studies using intranasal infection should assume the initial sites of infection of infection are brain, lung and upper respiratory tract, including the nasal airways.

Importance

Cryptococcus neoformans causes an estimated 181, 000 deaths each year, mostly associated with untreated HIV/AIDS. *C. neoformans* has a ubiquitous worldwide distribution. Humans become infected from exposure to environmental sources and the

56   fungus lays dormant within the human body. Upon immunosuppression, such as AIDS

57   or therapy-induced as required by organ transplant recipients or autoimmune disease

58   patients, cryptococcal disease reactivates and causes life-threatening meningitis and

59   pneumonia. This study has detected that upon contact with the host, *C. neoformans* can

60   quickly (a few hours) reach the host brain and will also colonize the nose of infected

61   animals. Therefore, this work paves the way to better knowledge of how *C. neoformans*

62   travels through the host body. Understanding how *C. neoformans* infects, disseminates

63   and survives within the host is critically required so that we can prevent infections and

64   the disease caused by this deadly fungus.

65

66   **Introduction:**

67   The genus *Cryptococcus* is populated by environmental fungi, wood-rotting fungi

68   most commonly associated with trees but also with bird guano. Two species of

69   *Cryptococcus* can cause life-threatening disease in humans, characterized mainly by

70   pneumonia and life-threatening meningoenchephalitis. *C. neoformans* var. *grubii* is the

71   most prevalent pathogen of the genus *Cryptococcus*, causing approximately 200,000

72   deaths each year, mostly associated with HIV-positive individuals, while the closely

73   related species *C. gattii* was responsible for an outbreak in British Columbia in

74   apparently immunocompetent individuals (1-3).

75   The current paradigm for the pathogenesis of human cryptococcosis emerged

76   from a series of observations made over several decades. Exposure is due to inhalation

77   of infectious propagules from the environmental niches. Colonization of the lungs by

78   spores, desiccated yeasts or yeast cells is thought to be quickly controlled by the

79   human immune system via granuloma formation (called cryptococcoma). In the 1950s,

80   silent cryptococcal granulomas were reported in lungs, establishing a parallel to latent

81   tuberculosis (4). In support of this notion, serological studies have established that

82   adults have antibodies to *C. neoformans* (5) or had delayed hypersensitivity skin

83   reactions (6), consistent with asymptomatic infection. Both teenagers or children under

84   the age of 5 living in urban areas are immunoreactive against *Cryptococcus* (7, 8).

85   Finally HIV-patients are infected with a serotype most commonly isolated from their

86   place of birth or where they spent their infancy (9). Hence, humans are exposed very

87   early in life to this yeast without developing noticeable disease. Silent or latent infections

88   due to containment in the lungs is further supported by the observation that some

89   recipients of lung transplants develop a cryptococcal infection originated from the donor

90   lung (10-12). Even though most infections of *C. neoformans* in humans are

91   asymptomatic, a significant and unknown proportion of humans become latent carriers

92   of *C. neoformans* which may reactivate as host immunity declines.

93   Given that initial human infection is thought to start from the lungs, research in

94   pathogenesis of *C. neoformans* commonly uses pulmonary infection models. The two

95   inoculation routes most commonly used are intratracheal and intranasal infection.

96   Intratracheal infection delivers *C. neoformans* directly to the respiratory tree but requires

97   surgery and significant skill. In contrast, intranasal infection is done by depositing a

98   solution containing *C. neoformans* in the nose of an anesthetized mice, which then

99   inhales it as these animals are obligate nose breathers. Since intranasal infection does

100   not require surgery, it has become a popular mode of inducing *C. neoformans* infection

101   in mice. Intranasal infection has been accepted as a procedure for inducing pulmonary

102   infection without much investigation as to what actually happens after nose deposition

103   of an infective inoculum. In some cases researchers use a third route of infection, the

104   intravenous route, since it allows a rapid dissemination to the brain, presumably by

105   bypassing lung immune response (13, 14). Intravenous model in outbred mice have

106   been shown to be a relevant model when compared to human infection (15-17).

107   In this study we compared intravenous, intratracheal and intranasal infection and

108   observed that all produce rapid brain dissemination. Additionally, we detected that

109   intranasal inoculation leads to presence of yeasts in upper respiratory airways and

110   auditory tract. These observations have important implications for the interpretation of

111   intranasal studies when considering immune responses and aspects of pathogenesis.

112

113   **Materials and Methods:**

114   *C. neoformans* was grown from frozen glycerol stocks into a Yeast Peptone

115   Dextrose (YPD) plate for 2 days, and then cultured overnight at 30 °C in YPD broth with

116   shaking. We used a strain from H99E lineage (available from Lodge laboratory), the H99O

117   strain, a close relative of the original isolate of H99 (18), and the R265 strain of *C. gattii*,

118   obtained from American Type Culture Collection.

119   C57BL/6J mice, aged 8–10 weeks, were obtained from Jackson Laboratories and

120   infected with the indicated $5x10^3$ (low dose) to $5x10^5$ CFU (high dose) in a final volume of

121   40 µl of sterile PBS (19). Intravenous (IV) injections were performed by 40 µl injection in

122   the retroorbital sinus of the animal under isoflurane anesthesia. Intranasal (IN)

123   experiments were performed by placing 40 µl of yeast suspension into the mouse nares

124   while under isoflurane anesthesia (20). Intratracheal (IT) infections were performed under

125   xylazine-ketamine anesthesia. Animal's neck was exposed, the trachea was exposed via

126   midline incision and yeasts inoculated with a 25G gauge syringe directly into the trachea.

127   Incision was closed with Vetbond (3M, St. Paul, MN, USA). Mice were monitored daily for

128   signs of stress and deterioration of health throughout the experiment. All animal

129   experiments were approved by Johns Hopkins University IACUC under protocol number

130   MO18H152.

131   To measure fungal burden, mice were euthanized, exsanguinated via terminal

132   retroorbital bleeding, tissues removed and macerated by passing through a 100 µm mesh

133   into sterile PBS. The tissue homogenate was then plated into YPD agar plates, and colony

134   forming units (CFU) were quantified. For one experiment we rinsed the brains (to remove

135   possible yeast contaminations from the exterior surface of the brain during necropsy). In

136   one experiment we included a non-infected mouse ("sentinel") to test for accidental

137   contamination of tools and materials during necropsy.

138   For histological analysis of the skull, the skin, lower jaw and tongue were removed

139   and remainder of skull was fixed in Formacal and processed for routine histology. A

140   sagittal cut was performed through the middle section of the mouse skull and

141   consecutives 4 µm sections, with 40 µm intervals were cut for Hematoxylin-Eosin,

142   Mucicarmine, Grocott-Methenamine Silver and immunofluorescence. For

143   immunofluorescence 18B7 monoclonal antibody against capsular polysaccharides (21)

144   was used and then detected with anti-mouse IgG_1-Alexa 488 conjugate antibody.

145   Hematoxylin & eosin sections were scanned using a slide scanner at the Oncology Tissue

146   Services Core (School of Medicine, Johns Hopkins University). Remaining sections were

147   imaged using an Olympus AX70 microscope (Olympus America, NY, USA). Image

148   cropping and annotation were performed using ImageJ (22).

Results:

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150   We compared 3 routes of infection (IV, IN, and IT) to ascertain the kinetics of *C.*

151 *neoformans* dissemination to the brain (Fig.1). We had previously verified the infectious

152 dose of $5x10^5$ CFU induces 100% mortality in 22 days for H99E. We quantified fungal

153 burden in blood, lung and brains. In two mice, we detected fungi in blood although in

154 these we also detected bacteria in blood (data not shown). This suggests that animals

155 with *C. neoformans* in the blood had severe systemic infection that may have

156 predisposed to concomitant bacterial infection. We conclude that the majority of animals

157 clear *C. neoformans* from the bloodstream. After 7 days of infection, we measured

158 increased numbers of yeast cells in the brain after IV inoculation (more than 10^5, upper

159 limit of detection) than after IN or IT inoculation (below 10^4 CFU). In the lung there was a

160 slight increase in fungal burden when the IT route is used, as compared to the IV route.

161 We did not detect differences in brain fungal burden between IN and IT inoculation.

162 Remarkably, we noted there was a substantial amount of yeast in mouse brains as early

163 as day 3 after inoculation of yeast by both IN or IT, which prompted us to ask how

164 quickly *C. neoformans* disseminates to the mouse brain.

165   We focused on the intranasal model since this procedure is non-invasive,

166 requiring only brief anesthesia and no surgery. We found that 3 h post infection with

167 $5x10^5$ yeast inoculum, the fungal burden in the brain was in the order of hundreds of

168 CFU for most animals (Fig. 2A). The surprising finding that yeasts were detectable in

169 the brain within hours after IN inoculation prompted us to perform additional controls.

170 For one experiment we rinsed the brains (to remove possible yeast contaminations from

171   the exterior surface of the brain during necropsy) but we got comparable results to the

172   first experiment (data pooled from both experiments is shown). We also culled one non-

173   infected mouse ("sentinel") at the end of the experiment to measure eventual

174   contamination of tools and materials during necropsy that could cross-contaminate the

175   media plates. We recovered no CFU from the sentinel mouse which gave us

176   confidence that the fungal burden we detect are result from brain infection and not

177   accidental contamination (data not shown). To investigate if early dissemination could

178   occur at lower doses we infected animals with $5x10^3$ CFU (at this dose *C. neoformans*

179   causes 80% mortality at 40 days). We still observed quick dissemination to the brain,

180   indicating that this was not a consequence of exposure to overwhelmingly high numbers

181   of yeasts. Finally, we observed quick dissemination to the brain with strain H99O, a

182   strain closely related to the original H99 clinical isolate of *C. neoformans*, as well as the

183   R265 strain of *C. gattti*, the strain associated with the Vancouver outbreak (Fig. 2B). It is

184   possible that the detected yeast burden is arrested in the small capillaries (23-26), or in

185   the post-capillary venules (27) and has not yet invaded the brain parenchyma. However,

186   this is consistent with invasion of the brain since upon arrest in capillaries, *C.*

187   *neoformans* quickly crosses endothelial barriers (24) and possibly the blood brain-

188   barrier. These experiments show that after intranasal infection, pathogenic strains of *C.*

189   *neoformans* and *C. gattii* disseminate to the mouse brain in as little as 3 h.

190   To confirm presence of yeast in the brain, we performed immunofluorescence in

191   skulls of infected mice, using a monoclonal antibody against *C. neoformans* capsular

192   GXM (21). In agreement with the results of CFU quantification, we detected yeast cells

193   (positive immunostaining and with typical morphology) in the mouse brain at 24 h post

194   infection (Fig. 3A, inset). We wondered if it was possible for *C. neoformans* could

195   provoke damage to the nose mucosa and from there access the brain through

196   damaging the olfactory mucosa and using the olfactory nerves to traverse the cribriform

197   plate (as shown for bacterial pathogens (28, 29) or if after damage the mucosa access

198   the host bloodstream for dissemination. We reasoned that if dissemination occurred

199   through the olfactory system, then the olfactory bulb would contain the majority of

200   yeasts, compared to the remainder of the brain. We infected mice and separated their

201   olfactory bulb from the remainder of the brain (Fig. 3B). We found that the olfactory bulb

202   contains similar amounts of yeasts to the remainder of the brain, despite its small size

203   compared to the rest of the brain.

204   To investigate the interaction of *C. neoformans* with the nose, and possible

205   damage to the nose mucosa that could facilitate dissemination, we performed

206   histological studies using a combination of histology, immunofluorescence, Grocott-

207   Merwald-Silver, and mucicarmine (Fig. 4). We found yeasts scattered throughout the

208   entire upper respiratory tract, particularly in the turbinates of the nose, and some yeasts

209   close to the cribriform plate (Fig.4A-C), as well as in the ear canals of mice (Fig. 4D).

210   Yeasts were abundant in airways, surrounded with a material that is likely mucus

211   secretions from the host, but that stained abundantly with capsular GXM antibody,

212   indicating a component of secreted polysaccharide in the airways (Fig. 4B). Budding

213   forms rested on the respiratory epithelium cilia and olfactory epithelium layer, and yeast

214   numbers increased in number from 3 h to the 24 h post infection and increased

215   immunostaining of GXM (Fig. 4F). We noted at 24 h post infection there were rare

216   enlarged yeast cells within the nose turbinates and ears (Fig. 4C-G) whose cell body

217   diameter is above 10 μm, and therefore can be consider titan cells (30-32). The finding

218   of titan cells in the nose of mice is concordant with Lima *et al.*, (33) which found

219   enlarged cells in noses of guinea pigs after some days of infection. Histopathology

220   analysis detected no damage to the host epithelial layers as well as no inflammatory

221   infiltrate from 3 h to 24 h post infection; it seems that either yeasts go undetected or

222   they do not cause enough tissue damage to trigger inflammation in murine host in the

223   early stages of infection. We conclude that, up to 24 h post-infection, presence of *C.*

224   *neoformans* in nose of mice causes minimal, if any, damage to nose mucosa and the

225   nose shows no sign of inflammation at this stage of infection.

Discussion:

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227   Animal models of infection are critical tools for understanding the pathogenesis of

228   infectious diseases. In the cryptococcal field, mice are the most commonly used

229   mammalian species to study pathogenesis and immune responses. We compared the

230   kinetics of brain dissemination in three models of cryptococcal infection that are

231   commonly used: IV, IN and IT. We were particularly interested in IN infection since this

232   approach has become increasingly popular in the field. We found early dissemination to

233   the brain and colonization of upper respiratory tract by *C. neoformans* and *C. gattii*. Our

234   data suggest early invasion of the brain and a colonization of the nose by both

235   cryptococcal species in mouse models.

236   Quick dissemination to the brain observed in our mouse model is compatible with

237   an hematogenous route of dissemination. Recent work has proposed that escape from

238   the lungs occurs due to phagocyte drainage through the lymphatic system (34). It has

239   not yet been shown how *C. neoformans* escapes from the lymph nodes into the

240   bloodstream (35-37), but this will likely be elucidated in the future. We detected no

241   yeasts in the circulating blood of mice, which raises a question of how the bloodstream

242   is so quickly invaded and quickly cleared. Other groups observed that in IV infection

243   fungi are cleared from the bloodstream in hours or the day after infection (27, 35), with

244   fungemia only resurfacing very late in the infection. These observations, together with

245   ours, imply that yeast transit time in blood is very short (25). The short transit time is

246   seemingly due to arrest of yeasts within small capillaries, as can occur in the ears of

247   mice (24) and in the lumen of leptomeningeal capillaries (24, 27). Yeasts arrested in

248   capillaries would not be detected by our methodology. In contrast, others found that

249   blood contains both free yeast cells as well as yeast cells in the buffy coat, i.e., engulfed

250   by phagocytic immune cells, for up to 20 days post-inoculation. Depletion of

251   mononuclear phagocytes decreases the number of yeasts brain detected in the brain

252   (23, 26, 34, 35). Regardless of the short transit time, there is sufficient experimental

253   evidence to support that dissemination to the brain occurs via the hematogenous route,

254   by direct crossing of the blood brain barrier by free yeast cells, and by indirect carriage

255   within host mononuclear phagocytes, the Trojan-horse transport.

256   In models of intravenous (IV) inoculation, yeast dissemination to the brain occurs

257   as early as 90 min after inoculation (36) (38). The speed of *C. neoformans*

258   dissemination to the brain was attributed to the intravenous inoculation model that can

259   simulate fungemia, an event that occurs very late in natural infections. Our findings

260   confirm that IV infection disseminated more efficiently to the brain, but all infection

261   routes lead to presence of viable yeasts within the brain as early as 3 h post infection.

262   Others had detected yeasts in the brain after intranasal infection as early as 3 d (39).

263   We did not experimentally confirm the same quick dissemination to the brain occurs

264   with intratracheal infection, but we expect the intratracheal route of infection to be

265   similar to the intranasal route for 2 reasons: i) at day 3 the fungal burden was similar for

266   both routes; ii) due to sneezing (and possibly coughing reflexes), inoculation into the

267   trachea would quickly spread *C. neoformans* throughout the entire upper respiratory

268   tract. It is our view that once the lungs are infected, the entire upper respiratory tract

269   becomes infected. Therefore, we believe that brain dissemination occurs very rapidly

270   and possibly in a matter of hours, irrespective of the infection route used. This finding

271   and others (34, 39) establish that cryptococcal brain dissemination occurs early (a

272   matter of few hours) and simultaneously with lung infection.

273   After noting early dissemination post-intranasal inoculation we wondered if a

274   route of dissemination through a nasal-olfactory system could be used by *C.*

275   *neoformans*. Both *Burkholderia pseudomallei* and *Listeria monocytogenes* can reach

276   the brain via damage to the olfactory mucosa and travelling upwards through the

277   olfactory nerve tracts as well as other cranial nerves (28, 29). *Neisseria meningitidis*

278   invades mouse brains by damaging the olfactory epithelium and travelling along the

279   olfactory tract, through the cribriform plate, to invade the brain (40). The filamentous

280   fungus *Mucor* invades the facial blood vessels from the sinus to reach the eye, the

281   cranial nerves and the brain. In the case of *C. neoformans*, the possibility of brain

282   dissemination through the nose has been pursued previously (41). In mice, yeast cells

283   were observed along the olfactory nerve and the meninges starting at day 3 post

284   intranasal instillation (41). Our study differs from this previous study because we

285   focused on the earliest stages of infection and found no evidence of an upward invasion

286   of the brain. We observed no signs of mucosal damage and no inflammation in the nose

287   cavity, despite the presence of abundant yeasts and secreted GXM. In interpreting

288   these results, we relied on the terminology of the damage-response framework that

289   defines infection as the acquisition of the microbe by the host and colonization as a

290   state where the damage resulting from the host-microbe interaction is insufficient to

291   affect homeostasis (42). From this perspective, the lack of visible damage suggests that

292   infection leads simply to nasal colonization and not overt disease. Given the lack of

293   damage and inflammatory infiltrate our findings do not support the notion that invasion

294   of nasal structures is involved in dissemination of *C. neoformans*. Nevertheless,

295   intranasal inoculation results in nasal colonization where fungal cells may later interact

296   with local immune defenses and potentially affect the development of the immune

297   response.

298   Animal models show that some animals can harbor *C. neoformans* in nose for

299   extended periods of time. Intranasal instillation resulted in yeast cells detected in the

300   nasal cavities for periods up to 1 month of both mice and rats (43) and this infection is

301   large enough to be detected by whole-animal non-invasive imaging as early as 1.5

302   weeks after infection (39). In immunocompetent laboratory-mice infected intranasally,

303   yeasts can be detected up to 90 d after instillation (44), and guinea pigs carried *C.*

304   *neoformans* in nose for several weeks (33). Some strains of *C. neoformans* are

305   rhinotropic, with the onset of nasal lesions occurring very late in the disease in

306   laboratory infected mice (45). Further, *C. neoformans* is frequently detected in nose of

307   animals outside of the laboratory setting. Asymptomatic nasal carriage of *C.*

308   *neoformans* has been reported in cats, dogs and koalas (46, 47). The percentage of

309   positive animals can reach as high as 95%, as reported for feral cats in Italy (48). *C.*

310   *gattii* is also quite frequent in animals, with British Columbia region having positive nasal

311   swabs in 4% and 7% of the cat and dog population, respectively (49). In conclusion,

312   there is evidence that *C. neoformans* can survive and even colonize upper respiratory

313   tract of some felines and rodents, including mice, a common experimental model. This

314   frequent colonization is associated with disease since cryptococosis is infrequent in

315   species such as cats (50) but relatively prevalent in koalas (47). Frequent detection of

316   *C. neoformans* in wild animals and the prolonged detection of *C. neoformans* in noses

317   of laboratory animals shows that *C. neoformans* (and perhaps other *Cryptococcus*

318   species) colonizes the upper airways of animals.

319   Serological studies indicate that humans are exposed to *C. neoformans* at an

320   early age (7), and that *C. neoformans* can reside in the lungs in a latent form (9). Non-

321   pathogenic species of *Cryptococcus* spp. were identified in several body sites: healthy

322   scalps (51), in mouths of 20% of the healthy population (52), in skin of children (53), and

323   in breast milk (54). Lung transplant patients or bronchiectasis patients frequently identify

324   the presence of *Cryptococcus*, but no pathogenic species (55, 56). Indeed, pathogenic

325   species of *Cryptococcus* spp. are rarely found in microbiome studies. In nose and upper

326   respiratory tract the majority of studies (57-62) reported no *Cryptococcus* spp isolates

327   from nasal cultures, while one study found rare *Cryptococcus* spp. (and no *C.*

328   *neoformans*) by culturing nasal cavity lavages (63). There is one notable exception:

329   high-throughput sequencing identified *Cryptococcus neoformans* as the most abundant

330   fungal species in the middle meatus in 60% of healthy patients and 90% of chronic

331   rhinosinusitis patients in St. Louis, Missouri, USA (64). The explanation for this striking

332   exception is unknown and potentially may be a technical problem. Overall, *C.*

333   *neoformans* is rarely recovered from healthy humans. In contrast, *C. neoformans* is

334   frequently isolated from upper respiratory tract of some felines. This discrepancy is

335   likely due to a combination of increased exposure of animals to environmental

336   reservoirs of *C. neoformans* as well as immunological differences in host-species.

337   However this is a surprising finding since animal disease has so far recapitulated

338   human disease (13, 15, 65).

339   In summary, our study shows the presence of the colonization of the upper

340   respiratory tract of *C. neoformans* in mouse models with rapid dissemination to the

341   brain, independently of the infection route. The rapid appearance of yeast cells in

342   different body compartments where they initiate local immune responses suggests

343   caution in associating a particular systemic response with a specific tissue. At the very

344   least, our findings suggest the need to revisit long held views on cryptococcal

345   pathogenesis in animal models of infection using the most modern cellular and

346   immunological tools.

347

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599

600   **Figure Legends:**

601   **Figure 1. *C. neoformans* dissemination to the brain and lung after different**

602   **inoculation routes.** Mice were infected with 5 x 10^5 H99E strain of *C. neoformans* via

603   intravenous (IV), intranasal (IN) and intratracheal (IT) route. Colony forming units (CFU)

604   analyzed at A) 3 days post infection (dpi) and, B) 7 dpi. Each data point represents one

605   individual mouse and bars represent mean and SD. Numbers represent P-values

606   calculated from two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with

607   Q = 5%, as compared to IV infection.

608

609   **Figure 2. *C. neoformans* is detected in the brain as early as 3 h post infection (hpi)**

610   **after IN infection.** A) Mice were infected with either 5 x 10^5 CFU (High dose) or 5 x 10^3

611   CFU (Low dose) of H99E via intranasal instillation and euthanized at the indicated time to

612   measure yeast tissue burden. B) Mice were infected with H99O strain of *C. neoformans* or

613   R265 strain of *C. gattii*. Each data point represents one individual mouse and bars

614   represent mean and SD.

615

616   **Figure 3. *C. neoformans* is detected in the brain and the olfactory bulb as early as 3**

617   **h post infection (hpi) after IN infection.** A) Mice were infected with 5 x 10^5 CFU of *C.*

618   *neoformans* via intranasal instillation, euthanized at the indicated time, and processed for

619   histology. Yeast cells could be observed in the olfactory bulb of the brain of mice at 24

620   hpi (top panel, arrows). Non-infected animals were processed under the same

621   conditions to show specificity of staining of yeast capsular immunofluorescence (bottom

622   panel). B) Mice were infected with high dose of *C. neoformans*. At the indicated hpi,

623   olfactory bulb was separated from the remainder of the brain (illustrated in right panel) and

624   CFU were counted (left panel). Each data point represents one individual mouse and bars

625   represent mean and SD. Numbers represent P-values calculated from two-stage linear

626   step-up procedure of Benjamini, Krieger and Yekutieli, with Q = 5%.

627

628   **Figure 4. Histopathology of mouse nasal cavities upon *C. neoformans* infection.**

629   Colonization and secretion of GXM in airways of mice. Enlarged yeasts could be

630   observed in airways and in ears. Mice were infected with 5 x 10^5 CFU of H99E or H99O

631   strains (as indicated) of *C. neoformans* via intranasal instillation, euthanized at the indicated

632   time and processed for histology. Immunofluorescence staining of medial sagittal cuts of

633   mouse skulls after staining with DAPI nuclear counterstain and anti-GXM-monoclonal

634   antibody 18B7. A) Aspects of the cribriform plate and the nose turbinates showing

635   yeasts in the airway. B) Accumulation of GXM in the nose mucosa. Specificity of

636   staining was confirmed by performing immunostaining in a non-infected mouse (not

637   shown); C) Grocott-Methenamine Silver staining (left, middle) and immunofluorescence

638   (right panels) showing enlarged yeast cells in nose airways (arrowheads). D) Grocott-

639   Methenamine Silver staining showing enlarged yeast cells in auditory canal (arrowhead,

640   left panel) as well as abundant yeasts in auditory canals (right panel). Scale bars 25 μm.

641   E) Hematoxylin-eosin staining of entire skull (left) (3 h post-infection). F) quantification

642   of yeasts in nose and yeasts in airways. G) Aspect of nose mucosa, close to cribriform

643   plate (24h post-infection), displaying lack of inflammation and showing enlarged yeast

644   cells (inset).

A

⬤ **Brain** ■ **Lung**

3 days p.i.

B **7 days p.i.**

A

H99E, Nose, 24hpi

DAPI GXM- mAb 18B7 DAPI + GXM

Brain - Olfactory bulb
Cribriform plate
Olfactory mucosa

Non-infected, Nose, 24hpi

B

Olfactory bulb

A H99E, Nose, 3hpi
DAPI   GXM- mAb 18B7   DAPI + GXM

B H99E, nose, 24hpi
DAPI   GXM- mAb 18B7   DAPI + GXM

C H99E, Nose, 24 hpi   H99O, Nose, 24 hpi   H99O, Nose, 24 hpi   **D** H99O, Ear, 24 hpi
DAPI   DAPI + GXM
GXM

E

F Number of yeasts per section
Lumen   Mucosa
3h 6h 24h   3h 6h 24h

G H99O, Nose, 24 hpi