Behavioral and Neural Resilience to Isolation in a Eusocial Insect

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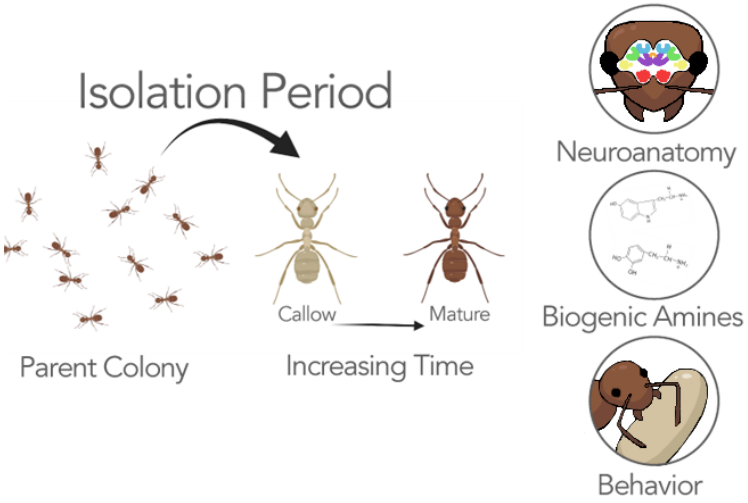
Abstract



Visual Abstract. Newly eclosed (callow) *Camponotus floridanus* minor workers were subjected to variable periods of isolation to determine whether and how diminished social experience and isolation influence the development of the brain, including compartment volumes, biogenic amine levels, and behavioral performance.

Social experience early in life appears to be necessary for the development of species-typical behavior in animals ranging from insects to primates. Isolation during critical periods of maturation has been shown to impact behavior, gene expression, and brain development in vertebrate and invertebrate clades, yet remarkable resilience to social deprivation, senescence, and loss of sensory input have been demonstrated in some ant species. We reared workers of *Camponotus floridanus* under conditions of social isolation for increasing time periods up to 45 days and evaluated their behavioral performance, quantified brain development and biogenic amine levels, and compared results of isolated workers with control workers that had natural social contact during development. We found that brood care and foraging performance by isolated workers were unaffected by a lack of social contact. Antennal lobe volume loss occurred in ants experiencing longer isolation periods, while the size of the mushroom bodies, which function in higher-order sensory processing, increased over time after eclosion and were not different from mature controls. Titers of the neuromodulators serotonin, dopamine, and octopamine remained

stable in isolated workers. Our results indicate that workers of *C. floridanus* are largely robust to the deprivation of social contact early in life.

Introduction

Social experience shapes individual behavior. Exposure to social stimuli is critically important to species-typical behavioral (Taborsky et al., 2012; Lévy et al., 2003), cognitive (Amitai et al., 2014) and neural development (Smith et al., 2010; Wang et al., 2022), as well as neuroimmune function (Donovan et al., 2020; Sallet et al., 2011; Serra et al., 2000; Mashoodh et al., 2012; Hostinar et al., 2012). Across diverse clades, behavioral performance can be negatively impacted if individuals are deprived of social contact when ontogenic trajectories are most sensitive to receiving sensory inputs (Wang et al., 2022; Boyce et al., 2012). A lack of social contact during these critical periods, therefore, can potently disrupt development across multiple biological scales (Rodriguez-Romaguera & Stuber, 2018; Calati et al., 2019; Vora et al., 2022).

Although research on the effects of social isolation has historically focused on vertebrates, eusocial insects have emerged as important models to examine the influences of social deprivation in early adult life on behavior, brain development, and brain gene expression (Boulay and Lenoir, 2001; Chen, 1937; Seid and Junge, 2016; Scharf et al., 2021; Lihoreau et al., 2009; Muscedere et al., 2013; Hewlett et al., 2018; Koto et al., 2015; Wang et al., 2022). Workers are deeply embedded within the social organization of a colony, suggesting a high degree of developmental dependency. Workers socially interact during physical encounters, trophallaxis (oral food exchange), allogrooming, nursing, foraging, and defense. Our understanding of how the complex social fabric of insect societies regulates behavioral and neuroanatomical development is limited (Kronauer and Libbrecht, 2018; Godfrey and Gronenberg, 2019; Wang et al., 2022). Social isolation in ants, for

example, appears to result in significantly altered brain growth relative to that of nestmates developing in typical social environments (Seid and Junge, 2016). Neuromodulators that function in behavioral development may become dysregulated (Boulay et al., 2000) if workers are deprived of social contact, potentially affecting age-related responsiveness to signals and cues that guide the behavior of mature workers (Seid and Traniello, 2005; Muscedere et al., 2013). Neuromodulators and receptor profiles that regulate individual and collective behavior (Kamhi and Traniello, 2013; Kamhi et al., 2017; Friedman et al., 2018, 2020; Maloney, 2021) may also be altered. Nevertheless, the brains of worker ants can be resilient to senescence (Giraldo et al., 2016) and injury (Waxman et al., 2017). This robustness to aging and physical damage in ants raises questions concerning their ability to compensate for stress due to social isolation during periods that are likely critical for behavioral and neural development.

In most ant species, young adult workers (callows) eclose in a socially rich environment of adult nestmates and immatures in their parent colony. Callows are distinguished from their mature siblings by their light coloration due to unsclerotized cuticule and relatively low activity levels (Morel, 1986; Muscedere et al., 2009). Species-typical behavioral maturation is usually incomplete immediately after eclosion, with apparently few exceptions (Traniello, 1978), so manipulations of callow social experiences can be used to experimentally examine the ontogeny of behavior. Social interaction between callows and their siblings influences pheromone responsiveness and temporal behavioral development (Topoff and Mirenda, 1978; McDonald and Topoff, 1985; Jaisson and Fresneau, 1978; Morel, 1986; Richardson et al., 2021). The effects of being deprived of such inputs are not consistently negative, as there is evidence of behavioral and neural robustness. For example, 10-day old *Pheidole dentata* minor workers deprived of brood contact nursed as efficiently as same-aged control workers and did not differ neuroanatomically or neurochemically

98 (Muscedere et al., 2013). The question nevertheless remains as to whether socially isolated ant
99 workers are neurally prepared for behavioral development independent of experience.

100 The genus *Camponotus* has served as a model to examine neuroethological impacts of
101 social deprivation in ants. In *C. floridanus* and *C. fellah*, neuroanatomical, neurochemical, and
102 behavioral changes in workers after periods of social isolation have been documented, including
103 decreases in brain volume and behavioral deficits (Seid and Junge, 2016; Boulay and Lenoir, 2001;
104 Boulay et al., 2000; Wada-Katsumata et al., 2011). Here, we integrate analyses of task
105 performance, brain volume, the growth of neuropils specialized for sensorimotor functions, and
106 titers of brain biogenic amine neuromodulators to examine how workers behaviorally and
107 neurobiologically develop during increased periods of isolation and determine if workers are
108 susceptible or resilient to a lack of social contact.

109

110 **Methods**

111 **Colony collection and culture**

112 Three queenright colonies of *C. floridanus* were collected in or near Gainesville, Florida
113 in 2012 and reared in Harris environmental chambers at 25°C and 40-55% relative humidity on a
114 12:12 light cycle. Parent colonies from which workers were sampled had a minimum of 100 minor
115 and 100 major workers. Artificial nests were made from test tubes 75% filled with water and fitted
116 with a tight cotton plug and placed in Fluon®-lined (Bioquip, Rancho Dominguez, CA) plastic
117 nest boxes 16x11x6 cm to 32x22x6 cm depending on colony size. Colonies were fed 1M sucrose
118 on saturated cotton balls and varied protein sources (mealworms, scrambled eggs, and fruit flies)
119 every other day in rotation.

120

121 **Worker social isolation**

122 Five groups of callow minor workers were collected from each of three parent colonies.
123 Randomly selected callows were isolated from nestmates for 2 [2 +/- day], 5 [5 +/- day], 20 [20
124 +/- 2 days], 30 [30 +/- 4 days], or 45 [45 +/- 8 days] days in separate Fluon®-lined boxes (16 x 11
125 x 6 cm). Isolated workers ("isolates") were provided similar conditions in smaller nest boxes and
126 the same diet as parent colonies. After the prescribed isolation period, isolates were assayed for
127 behavioral performance and subsequently sacrificed for immunohistochemical analysis of brain
128 structure or quantification of brain biogenic amine titers. Callow and mature minor workers
129 ("matures") were collected and assayed using the same methods. We calculated a survival rate of
130 63.7% for isolated workers. Because of the mortality of workers experiencing longer periods of
131 isolation, sample sizes for 30- and 45- day isolates were generally smaller. Head width, measured
132 as the widest distance across the eyes, served as a proxy for body size. To control for body size,
133 which may correlate with brain size, we compared the head widths of workers across isolation
134 periods and control groups. We found no significant differences ($p=0.429$, $R^2=0.116$, $F=1.132$,
135 $Z=0.206$, SS type 3, **Supp Fig 1)** and our sampling of workers for assignment to isolation or control
136 treatments was thus unlikely to influence brain mass or volume.

137

138 **Behavioral performance assays**

139 Isolated and control workers (n = 16 [2 day], 19 [5 day], 20 [20 day], 6 [30 day], 5 [45
140 day], 8 [callow] and 13 [mature]) were exposed to a randomized series of assays to determine their
141 ability to recognize and care for brood, and foraging, tasks that affect colony fitness.

142

143

144 *Brood recognition and care*

145 To determine if isolates differ in the ability to recognize and care for immature siblings
146 with similar efficacy as socialized matures and callows, we evaluated their interactions with pupae.
147 Each focal worker was carefully placed in an arena (14 cm diameter Petri dish) beneath a 30 x 10
148 mm red plastic hollow tube that provided a dark area into which workers could move pupae. Three
149 pupae were added at 3 locations equidistant from the darkened chamber before a worker was
150 introduced to the arena. Workers relocated pupae either inside or outside of the dark chamber or
151 otherwise moved alone without interaction. Pupae were considered to be aggregated ("piled") if
152 workers moved two or more together. Workers that moved two or more pupae began doing so
153 immediately. We did not assign lower scores to workers that clustered pupae but did not move
154 them into the darkened area. Worker interactions with pupae were observed directly for 5 minutes,
155 then after an additional 15 minutes the placement of pupae was recorded. This assay for brood
156 recognition and care was measured on a 7-point scale: 1. no interaction or avoidance; 2. olfactory
157 (antennal) investigation in the first 5 minutes; 3. mouthpart (mandibles, palps) contact in the first
158 5 minutes; 4. no pupae moved before 5 minutes but at least one pupa moved during the subsequent
159 15-minute period; 5. one pupa moved in the first 5 minutes; 6. two pupae moved within the entire
160 trial; 7. three pupae moved during the entire trial. Workers received scores of 5-7 independent of
161 where pupae were transported. Higher scores indicate brood recognition and movement of more
162 pupae.

163

164 *Predatory Behavior*

165 A single worker was carefully placed in a Fluon®-lined Petri dish (9cm) and allowed to
166 acclimate for 2 minutes prior to assay initiation. Using Dumont No. 5 fine forceps sanitized with

167 ethanol and dried, a live fly was then offered to the worker and the response was observed for 2
168 minutes. The degree of predatory behavior was scored on a 5-point scale: 1. no response or
169 avoidance; 2. olfactory (antennal) investigation; 3. mandibular flaring; 4. latent attack (delayed
170 and intermittent); and 5. immediate attack (persistent predatory attack from first encounter with
171 prey). The assay arena was also sanitized with ethanol between trials to minimize influence from
172 social signals or prey odors.
173
174 *Locomotion*
175 To examine if isolation affected the physical capability to pile pupae or forage, we used
176 locomotion as a simple and robust proxy to broadly assess neuromuscular impairment (Giraldo et
177 al., 2016). Focal workers were placed in the center of a 10 cm diameter Fluon®-lined Petri dish
178 divided into 4 equal-size quadrants with a crosshair pattern drawn beneath the bottom and
179 acclimatized for 2 minutes. After the acclimation period, worker movement was observed for 5
180 minutes, and the number of times a new quadrant was entered was recorded using a digit counter.
181 Movement between quadrants was only scored when the worker passed the division line by at least
182 one body length.
183
184 **Neuroanatomical and neurochemical measurements**
185 *Brain mass*
186 To quantify isolation-associated changes in brain size and to enable scaling of biogenic
187 amine levels, we recorded and compared brain mass among three isolate groups (n = 9 [2 day], 10
188 [5 day], 12 [20 day], 10 [callows], and 12 [matures]). Brains were dissected in ice-cold insect
189 Ringer's solution after head width was measured. Brains were placed in a tin capsule of known

190 weight, dried at 55°C, and weighed (+/- 1 microgram) after 48 hours using a Sartorius CP2P
191 microbalance (Sartorius, Goettingen, Germany). Brains were weighed three times and averaged to
192 estimate brain mass. Brain that were statistical outliers (Z>2.5) due to technical difficulties in the
193 process of weighing were excluded from this analysis.
194
195 *Immunohistochemistry and confocal microscopy*
196 Intact brains (n = 9 [2 day], 11 [5 day], 16 [20 day], 3 [30 day], 11 [40 day], 5 [matures],
197 and 9 [callows]) were dissected in ice-cold Ringer's solution, and then immediately placed in ice-
198 cold zinc-formaldehyde fixative with shaking overnight. Fixed brains were then washed in HEPES
199 buffered saline 6 times (10 minutes/wash) and fixed in Dent's Fixative (80% MeOH, 20% DMSO)
200 for 1 hour. Brains were placed in 100% methanol and stored at -17 C° for 1-3 weeks until
201 processed. Brains were next rinsed 6 times (10 minutes/rinse) with 3% Triton X-100 in 1X PBS
202 (3% PBST). Brains were stored overnight in 5% normal goat serum/0.2% PBST/0.005% sodium
203 azide for blocking. After blocking, brains were incubated for 4 days RT with an anti-synapsin
204 antibody to stain neuropils (1:30, Development Studies Hybridoma Bank, Iowa City, Iowa) in
205 constant nutation. Brains were washed with 0.2% PBST (6x10 minutes), then incubated in
206 secondary antibody (1:100 AlexaFluor goat α-mouse) in 5% normal goat
207 serum/0.2%PBST/0.005% sodium azide for 4 days at RT with constant nutation. Brains were
208 washed in 0.2% PBST (6x10 minutes), then dehydrated through an ethanol and PBS series (30,
209 50, 70, 95, 100% ethanol in 1XPBS). Brains were cleared and immersed in methyl salicylate and
210 mounted on stainless steel glass-windowed slides for confocal imaging.
211 Brains were imaged with a Nikon C2 + Si spectral laser scanning confocal microscope
212 (Nikon, Melville, NY, USA) with sections at approximately 1.5 um throughout the entire brain.

213 Images were manually annotated using Amira 6.0 software to quantify neuropil volumes. The
214 individual who annotated brains was blind to brain sample category, thus minimizing human bias.
215 The margins of major functionally specialized neuropils were identified visually or with magic
216 wand tools in Amira. Every eighth frame was annotated manually. Frames without labels were
217 labeled using Biomedisa interpolation (Lösel et al., 2020), then evaluated and edited by the
218 annotator for accuracy. We labeled volumes of the optic lobe (OL), antennal lobe (AL), mushroom
219 body (MB), lateral calyx (MB-LC), and medial calyx (MB-MC), peduncles (MB-P), central
220 complex (CX), subesophogeal zone (SEZ), and rest of the central brain (ROCB). ROCB describes
221 tissue composed of the superior neuropils, ventromedial neuropils, ventrolateral neuropils, and the
222 lateral horn (Ito et al., 2014). For each brain compartment, volumes of only one intact hemisphere
223 were recorded. If both hemispheres were intact, the labeler chose randomly.

224 We compared the relative volumes of brains across all groups and scaling relationships
225 among brain compartments. A detailed description of brain anatomy and development in relation
226 to age and experience in *C. floridanus* is presented in Gronenberg et al. (1996). Relative volumes
227 were calculated by dividing the volume of each focal compartment by the ROCB volume.
228 Damaged compartments were excluded from analysis. If one or more compartments or the ROCB
229 was damaged, the sample was excluded. The MB-LC and MB-MC exhibited similar volumetric
230 trends and thus were combined to one metric (MB-C). As a sensitivity test for using the ROCB as
231 proxy for total brain volume, we ran additional analyses using total brain volume for scaling which
232 indicated that using the ROCB for scaling reflected the same trends as scaling to total brain size
233 (**Supplementary Methods**).

234

235 *Quantification of brain biogenic amines*

236 We quickly dissected workers (n = 11 [2 days], 11 [5 days], 13 [20 days], 3 [30 days], and
237 11 [mature workers]) to quantify levels of serotonin (5HT), dopamine (DA), and octopamine (OA)
238 using isocratic, reversed-phase high-performance liquid chromatography with electrochemical
239 detection (HPLC-ED). Monoamine quantification was performed blind to treatment. The HPLC-
240 ED system (formerly ESA, Inc., Chelmsford, MA, USA) included a model 584 pump, a model
241 MD-150 (3 x 150 mm) reversed-phase analytical column, a model 5011A dual-channel
242 coulometric analytical cell, and a Coulochem III electrochemical detector. Brains were dissected
243 in less than 5 minutes in ice-cold Ringer's solution, homogenized in mobile phase manually with
244 a plastic polypropylene pestle in a centrifuge tube, and centrifuged. The supernatant was then
245 injected into the HPLC column, and monoamine levels were quantified with reference to external
246 standards. Mobile phase was formulated as 50 mM citrate/acetate buffer, 1.5 mM sodium dodecyl
247 sulfate, 0.01% triethylamine, and 25% acetonitrile in MilliQ water (Muscedere et al., 2013).
248 Multiple daily runs of the external standards accounted for any changes in ambient conditions.
249

250 **Statistical analysis and data visualization**

251 All statistics analyses were performed in RStudio (RStudio version 4.2.3, Posit Team
252 2023). For generalized linear mixed models, we used the package '*RRPP'* (Collyer & Adams,
253 2019). We tested for statistical significance using ANOVA with residual randomization in a
254 permutation procedure of 1000 iterations and through the estimation method of ordinary least
255 squares, given combined sample size of 0 or more. Following ANOVAs, *post-hoc* tests were done
256 on pairwise comparisons using t tests with pooled SD with p value adjustment using the Bonferroni
257 method. Depending on the sample size of 30-day and 45-day isolates, we included type III error
258 to account for bias in sample size. Figures were created using the package '*ggplot2*' (Wickham,

259 2016). Color corrections and figure assembly were done in Adobe Illustrator (Adobe Illustrator
260 2023). Illustrations of ants in **Fig 1A** and **Supp Fig 1B** are courtesy of BioRender.com.
261
262 **Results**
263
264 ***Effect of Isolation on Predatory Behavior***
265 Worker predatory behavior was significantly different between isolated groups (p=0.001,
266 R^2=0.294, F=5.540, Z=3.6525, SS type 1, **Fig 1A-B**). Mature workers preyed on fruit flies
267 significantly more than callows, 2-day isolates, 5-day isolates, 20-day isolates, 30-day isolates, but
268 not 45-day isolates (p<0.001, <0.005, <0.001, <0.05, <0.05, respectively; Bonferroni correction).
269 All other pairwise comparisons were not significantly different (p=0.149-1.0). Mature workers
270 moved significantly more than callows and isolates but did not show predatory behaviors
271 significantly different from those of 30- and 45-day isolates **(Fig 1B)**. Therefore, callows and 2-
272 day isolates interacted with prey in a similar manner, showing avoidance or olfactory investigation.
273 In contrast, older isolates (45-day, e.g.) had higher predatory scores resembling those of mature
274 worker controls, scoring on average 2.80 and 3.38, respectively. Workers that experience less than
275 45 days of isolation had significantly lower predatory behavior scores than mature workers, but
276 45-day isolates scored similarly to mature workers, suggesting that increased isolation did not
277 impair predatory behavior.
278 *Effect of Isolation on Brood Recognition and Care*
279 Isolated and control workers interacted with pupae to the same degree and were likely
280 recognized as immature nestmates by both groups. Brood-care performance was significantly
281 different among experimental groups (p=0.009, R^2=0.201, F=3.3518, Z=2.4572, SS type 1, **Fig**
282 **1A, C**), but in *post-hoc* tests, only mature workers and 2-day isolates differed significantly

(p=0.014, Bonferroni correction). All other pairwise comparisons were not significant (p=0.06-1.0). With increased duration of isolation, brood-care scores became similar to those of callows and mature worker controls. Isolated and control workers did not differ in their tendency to move pupae into a darkened location.

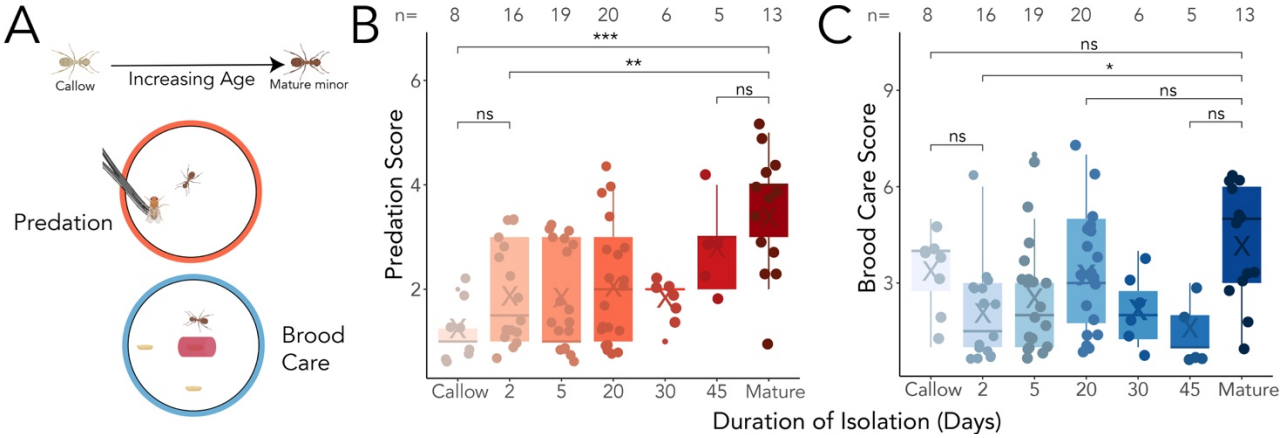


Figure 1. Isolation effects on task performance. (A) Illustration of assays to assess brood-care and predatory behaviors. **(B)** Predation scores of workers isolated for varying time periods compared to callows and mature worker controls. Darkening shades of red correspond with increased time in isolation. **(C)** Brood-care scores of isolates compared to callows and mature worker controls. Darkening shades of blue correspond to increased age and time of isolation. 2-day and mature workers were the only groups that differed significantly in brood care. X = average; horizontal line = median. Sample sizes are above box plots. <0.001 = ***; 0.001-0.01 = **; 0.01-0.05 = *; >0.05 = ns. Socially typical mature workers and callow controls are the darkest and lightest colors, respectively.

Mature workers had significantly higher levels of locomotion than isolate groups and callows, with the exception of 30-day isolates (p=0.001, R^2=0.486, F=12.595, Z= 5.0508 SS type 1, [**Supp Fig 1B-C, Supp Table 1-3**]). Mature workers significantly differed from other experimental groups (callow, 2, 5, 20, 30, 45: all p<0.001, Bonferroni correction) except for 30-day isolates. No other pairwise comparisons were statistically significant (p>0.05).

Brain Size and Compartmental Allometry

Brain mass did not significantly differ between isolated and control workers (p=0.359, R^2=0.092, F=1.09, Z=0.356, SS type 1, **Fig 2A)**. Size trajectories of brain compartments are

illustrated in **Fig 2B**.

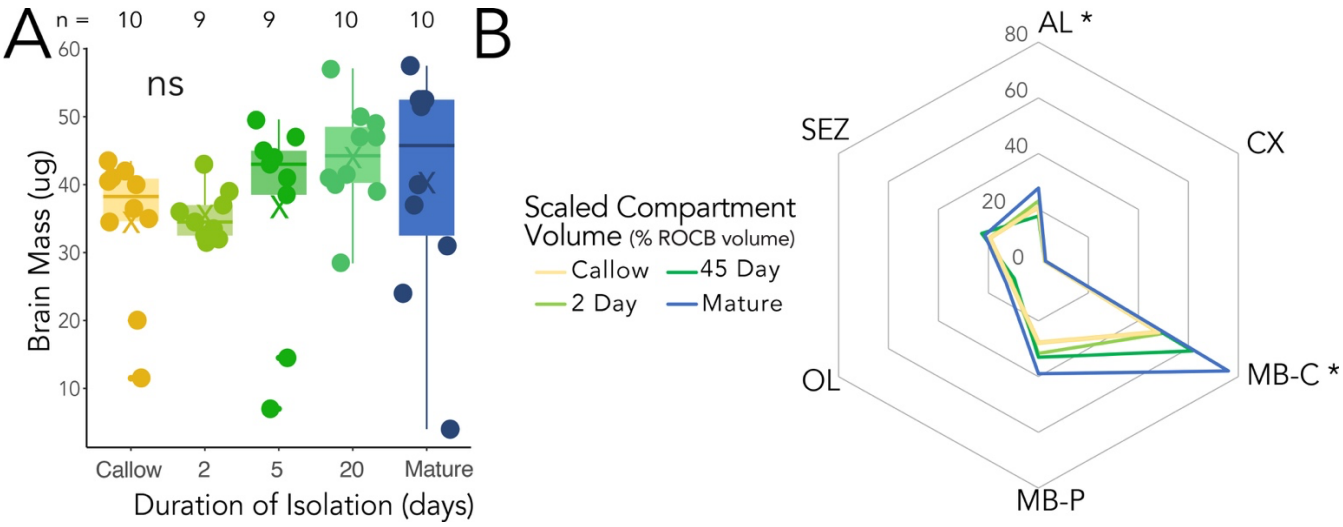


Figure 2. Effects of isolation on brain growth. (A) Brain mass of workers isolated for 2, 5, and 20 days compared to callow and mature worker control groups. Sample sizes of each group are above box plots. X = average; horizontal line = median. **(B)** Spider plot of brain compartment volumes scaled to ROCB volume in callows, 2-day workers, 45-day workers, and mature workers. Numbers on spider plot are proportions relative to ROCB volume. * indicates $p<0.01$; $p>0.05$ = ns.

MB-C increased in volume with age and increased periods of isolation, becoming similar in size to the MB-C of mature workers. However, workers enduring longer periods of isolation had smaller AL volumes compared to those of mature workers. AL volumes were significantly different ($p=0.003$, $R^2=0.323$, $F=4.539$, $Z=3.1128$; **Fig 2A**): mature workers had significantly larger AL volumes relative to the ROCB than 30-day and 45-day isolates ($p=0.017$, 0.00033 respectively, Bonferroni correction). The average relative volume of the MB-P remained consistent throughout isolation periods with no significant changes, indicating that impact on the MB was limited to the calyces ($p=0.295$; $R^2=0.127$, $F=1.2878$, $Z=0.544$; **Fig 3B**), which showed modest growth during isolation ($p=0.001$, $R^2=0.477$, $F=8.0699$, $Z=4.213$; **Fig 3C**). Mature workers had significantly higher relative MB-C volumes than callows, 2-day isolates, and 5-day isolates ($p<0.0001$, <0.0001, <0.004, respectively; Bonferroni correction), but not 45-day isolates ($p=0.0622$). MB-C volumes of 45-day isolates were also significantly larger than callows ($p=0.038$, Bonferroni correction). The MB-C volumes of 20-day isolates were also significantly

larger than 2-day isolates and callows (p=0.0169, p=0.0107, Bonferroni correction). All pairwise analyses for compartmental volumes can be found in **Supplementary Tables.** Other brain compartments did not significantly change with isolation (p=0.228-0.871; **Fig 3D-F**).

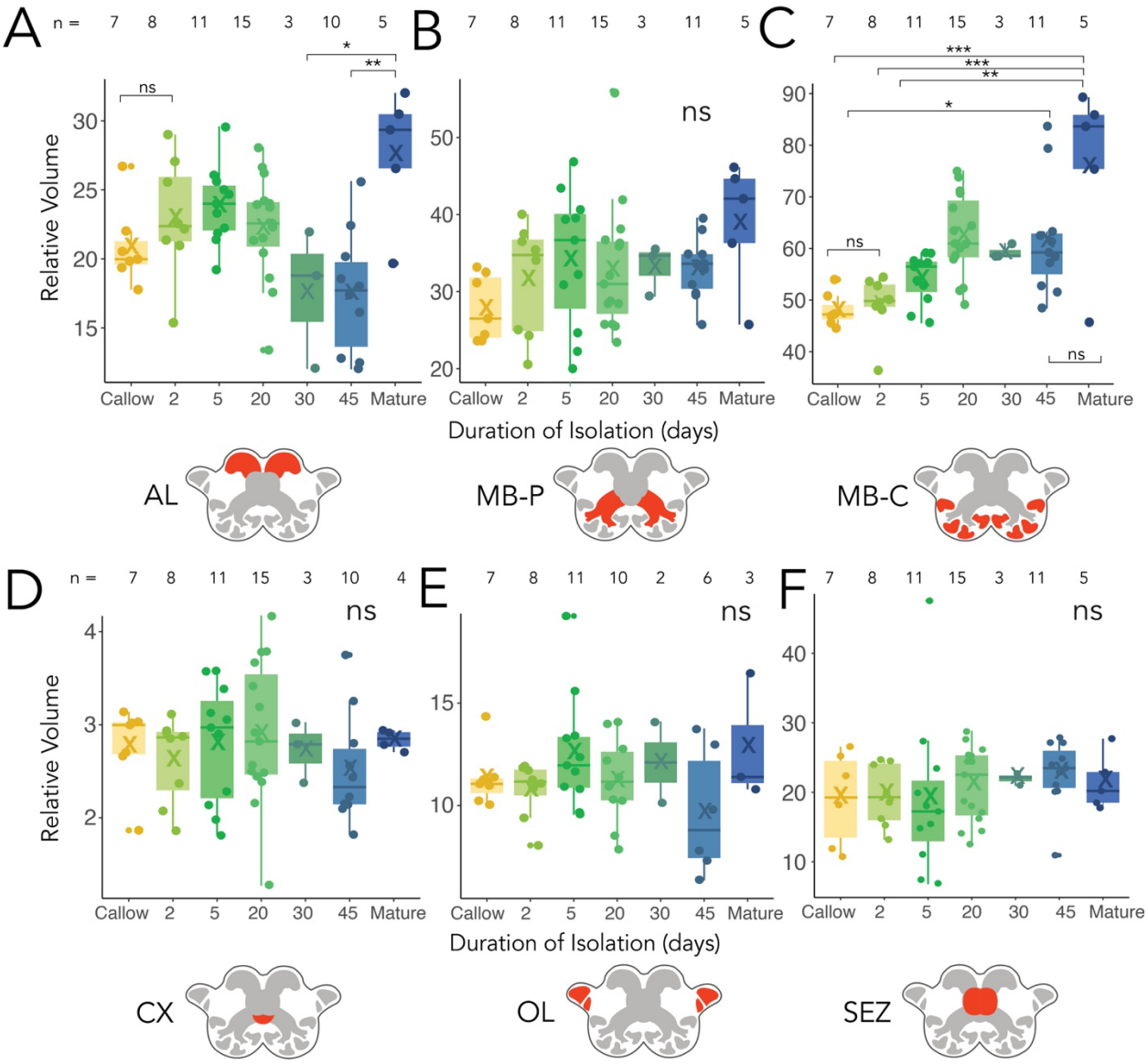


Figure 3. Isolation and growth of brain compartments. A-E: brain compartment volumes (relative to ROCB) for isolated and control workers. Sample sizes of each group are above box plots. X in each box plot represents the average and horizontal line the median of each group **(A)** AL; **(B)** MB-P; **(C)** MB-C; **(D)** CX; **(E)** OL; **(F)** SEZ. p <0.001 = ***; p<0.001-0.01 = **; p<0.01-0.05 = *; p>0.05 = ns.

Biogenic amine levels

338 Brain titers of OA, DA, and 5HT significantly increased with longer periods of isolation
339 (**Fig 4A-C**); levels of 30-day isolates were similar to mature minor workers. DA titers significantly
340 differed across isolation treatments and controls ($p=0.001$, $R^2=0.611$, $F=17.481$, $Z=5.340$, SS type
341 3, **Fig 4A**) and significantly differed between mature worker controls and 2, 5, and 20-day isolates,
342 but not 30-day isolates (mature vs. 2, 5, 20-day isolates, $p<0.00001$, <0.00001, <0.0001,
343 respectively; Bonferroni correction). OA exhibited a similar pattern ($p=0.001$, $R^2=0.723$,
344 $F=42.552$, $Z=7.844$, SS type 3, **Fig 4B**). OA titers significantly increased between isolated worker
345 groups except for 2-day isolates vs. 5-day isolates, 5-day isolates vs. 20-day isolates, and 20-day
346 isolates vs. 30-day isolates. 5HT titers also significantly increased with longer periods of isolation
347 ($p=0.001$, $R^2=0.744$, $F=109.136$, $Z=9.980$, SS type 3, **Fig 4C**), except for 20-day isolates vs. 30-
348 day isolates.

349 While absolute levels of DA, OA, and 5HT increased over time, their ratios increased or
350 decreased compared to those recorded in mature workers (**Fig 4D-F**). OA:5HT significantly
351 decreased with time and extended periods of isolation ($p=0.001$, $R^2=0.326$, $F=7.805$, $Z = 3.672$)
352 with no significant difference between 30-day isolates and mature workers (**Fig 4D**). OA:DA did
353 not significantly change with isolation ($p=0.989$, $R^2=0.00772$, $F=0.0855$, $Z=-2.220$), nor did
354 5HT:DA ($p=0.221$, $R^2=0.102$, $F=1.4831$, $Z=0.755$) except 2-day isolates being having
355 significantly lower 5HT:DA ratios compared to mature workers ($p=0.046$, Bonferroni correction).

356 DA titers relative to total amines levels did not significantly differ across isolation groups
357 and mature workers ($p=0.434$, $R^2=0.0705$, $F=0.968$, $Z=0.164$). Relative OA levels, in contrast,
358 were significantly different ($p=0.015$, $R^2=0.243$, $F=3.371$, $Z=2.126$), including 2-day vs. 20-day
359 and 2-day vs. mature workers were significantly different ($p=0.031$, 0.034, respectively,
360 Bonferroni correction). Relative 5HT quantities were significantly different ($p=0.015$, $R^2=0.194$,

F=3.471, Z=2.083) between 2-day vs. 20-day isolates and 2-day vs. mature workers (p<0.001, <0.005 respectively, Bonferroni correction).

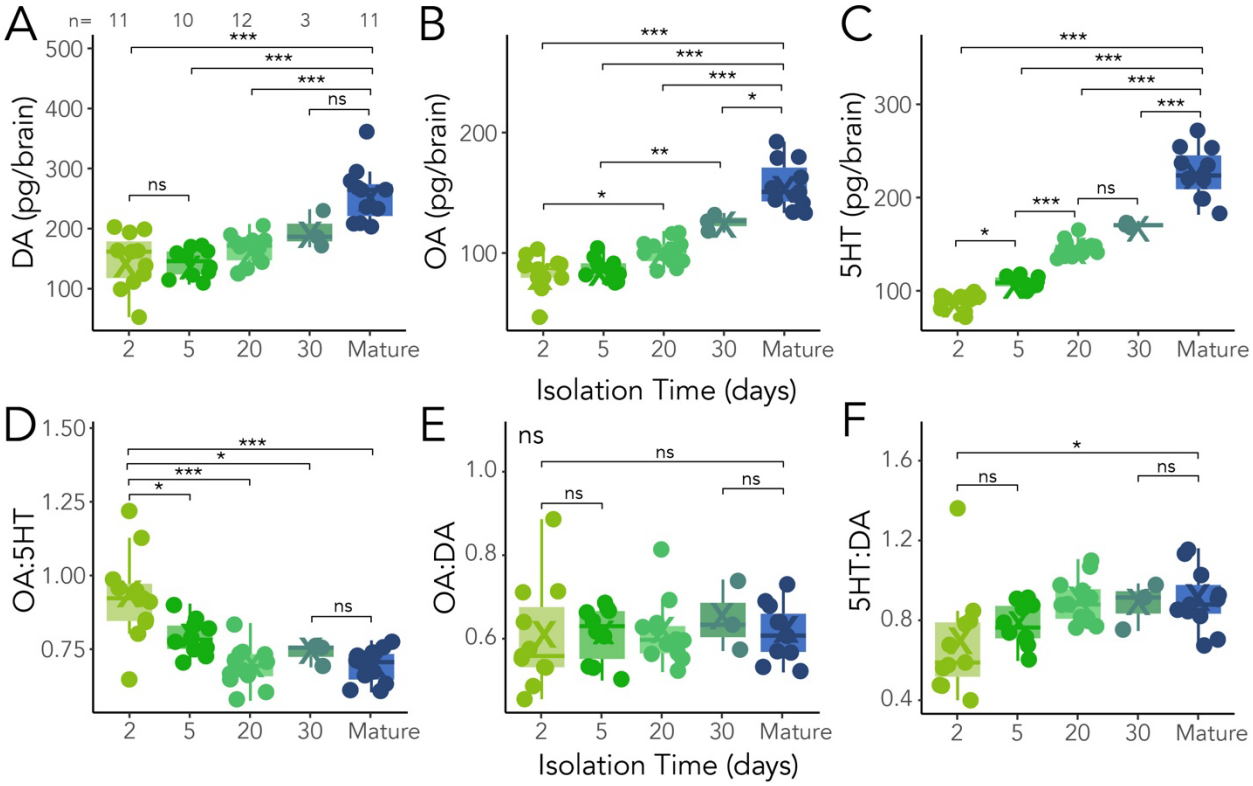


Figure 4. Isolation effects on monoamine levels (A-C) and ratios of DA, OA, and 5HT (D-E). The transition from green to blue corresponds to increasing isolation time periods. The deepest blue boxplot corresponds to mature worker controls. Averages, medians, and samples sizes as in Figure 3. p <0.001 = ***; p<0.001-0.01 = **; p< 0.01-0.05 = *; p>0.05 = ns.

Discussion

Overall, the behavior of *C. floridanus* workers isolated for varying durations after eclosion was comparable to that of control workers that developed in a natural social context. Two-day isolates showed predatory behavior similar to that of callow control workers, and predatory behavior by 45-day isolates was similar to that of mature control workers, suggesting foraging was not impaired by isolation. Social isolation resulted in a significant difference in brood care – as measured in our assay – only between 2-day isolates and mature control workers, but otherwise had no effect. This result may reflect the development of behavioral competencies associated with

377 biomechanical and physiological maturation (Muscedere et al., 2011). Workers in all isolation
378 treatment groups showed significantly decreased locomotion relative to mature workers except for
379 30-day isolates (**Supp Fig 1B-C)**, consistent with Wang et al. (2022) for isolated bumblebees but
380 different from *Cataglyphis niger* ant workers (increased movement following isolation and then
381 decreased movement [Ahronberg and Scharf 2021]) and *Camponotus fellah* workers (increased
382 movement after isolation [Koto et al., 2015]). This decline in movement did not appear to influence
383 brood care or predatory behavior in isolated *C. floridanus* workers and thus did not appear to be a
384 cost of isolation.

385 *C. floridanus* worker brains normally increase in size during a 10-month period after adult
386 eclosion, the greatest growth rate (nearly 100%) occurring in the MBs and ALs (Gronenberg et al.,
387 1996). We found no significant difference in mass between isolates, control callows, and mature
388 workers, indicating that isolation did not significantly affect brain size during the 45-day
389 assessment period**.** Furthermore, older isolates and socially typical mature workers generally had
390 larger relative MB-C volumes compared to workers isolated for shorter time periods and socially
391 typical callows. MB-C size in 45-day isolates was not significantly different from that of mature
392 controls and 2-day isolates, suggesting gross neuroanatomical development remained stable under
393 conditions of isolation. These findings differ from the results of Seid et al. (2016), which showed
394 that MBs of isolated *C. floridanus* workers do not change in volume relative to total brain size,
395 whereas MBs increased in our study. MB-C resilience through 71 days of isolation has been found
396 in paper wasps (Jernigan et al., 2021). Using bumblebees as a model, Wang et al. (2022) associated
397 expression differences in juvenile hormone-related genes and transcriptomic network
398 dysregulation with isolation-induced variation in brain volume. Bumblebees isolated after eclosion
399 for 9 days showed significant differences in the variance of MB volume rather than average volume

compared to socially typical bees, suggesting growth destabilization in some, but not all, workers. Our study identified resilience in brain growth, possibly attributable to different mechanisms of developmental programming in *C. floridanus*. We note our findings do not demonstrate a complete lack of influence of isolation on brain size and structure because we did not determine temporal patterns of growth that could indicate the development of the MBs or other compartments accelerated or slowed differentially over time in isolates and/or socially typical workers. Our results nevertheless support the hypothesis that MB development is resilient to social isolation.

AL volume decreased with periods of isolation approximately 5 days or longer; 45-day isolates had, on average, smaller ALs than control callows and mature workers. This decline in AL size is similar to the findings of Wang et al. (2022) on bumblebees. Although AL investment decreased with isolation duration in *C. floridanus,* workers still maintained olfactory abilities necessary for performing the tasks we assayed. Isolation did not impact OL volume in our study of *C. floridanus*, although it did reduce OL volume in paper wasps, which integrate visual information used in conspecific facial recognition (Jernigan et al., 2021; Pardo-Sanchez, 2022). The CX, which functions in sensorimotor processing in insects (Turner-Evans and Jayaraman, 2016; Pisokas et al., 2020), did not change significantly in volume in isolated workers. These results also differ with those of Wang et al. (2022) for isolated bumblebees. The volume of the SEZ, which regulates mouthparts (Miroschnikow et al., 2020) and is likely involved in the control of brood-care motor functions, did not significantly change with isolation.

Worker behavioral maturation, including repertoire expansion and a corresponding increase in brain size, correlates with increasing monoamine levels and the development of serotonergic circuitry in some ants (Seid and Traniello, 2005; Seid et al., 2008; Muscedere and Traniello 2012; Wnuk et al., 2011). Our *C. floridanus* data indicate that levels of OA, DA, and

423 5HT increase with age in the brains of isolated workers, despite a lack of social contact. Indeed,
424 30-day isolates had monoamine levels most like those of mature workers, whereas 2-day isolates
425 were least like mature workers. Ratios of biogenic amines can significantly change with age in
426 ants and may influence the development of age-related behavior (Seid and Traniello, 2005) by
427 modifying response thresholds. However, we found no significant differences between older
428 isolates and mature controls, indicating that isolation does not significantly alter neuromodulator
429 ratios. Boulay et al. (2000) and Wada-Katsumata (2011) found that 5HT and DA levels were robust
430 to social isolation. Boulay et al. (2000) hypothesized OA may reduce the effects of social
431 deprivation, acting homeostatically to maintain social cohesion. Whether octopaminergic
432 signaling has a stimulatory or inhibitory effect on prosocial behaviors such as trophallaxis, which
433 may be affected by periods of social isolation, remains unclear.

434 Although it may be generally assumed that smaller size reduces competence, we do not
435 know how whole-brain or compartmental volumes affect behavioral ability. Indeed, the
436 relationship between brain size and behavioral capacity is actively debated (e.g., Chittka and
437 Nevin, 2009). In ants, elaboration of the MB appears to represent increased demands for higher-
438 order processing (Kamhi et al., 2016; Godfrey and Gronenberg, 2019; Muratore et al., 2022) and
439 larger OLs are correlated with more complex visual ecologies (Sheehan et al., 2019; Arganda et
440 al., 2020). Larger ALs have been associated with age-related olfactory ability (Muscedere et al.,
441 2012), although Hart et al. (2023) showed that ant alarm pheromone response is encoded by less
442 than 10 of a total of approximately 500 AL glomeruli, a result that suggests that the relationship
443 of neuropil size and behavior is unclear. We also do not understand how circuit configuration,
444 activation, synaptic strength, or other neural processes in the ant brain connectome are influenced
445 by worker isolation.

446 Neuromodulation can restore sensorimotor functions following injury (Zhang et al., 2021).
447 Neuromodulators can alter synaptic functions to significantly change circuitry (Marder, 2012;
448 Sillar et al., 2014), increase the robustness of network functions (Haddad and Marder, 2018) and
449 their ability to recover from disruption and/or stress (Golowasch, 2019; Thuma et al., 2013). In the
450 ant *Pheidole dentata*, monoamine levels continue to increase in titer in aging workers, which do
451 not appear to decline in task performance capability (Giraldo et al., 2016). Given that DA, OA,
452 and 5HT can flexibly reshape circuits, neuromodulators may reduce isolation-induced
453 neuroanatomical deficits in ants, stabilizing circuit dynamics or returning them to a stable state if
454 disrupted. Although we did not identify an increase in monoamine levels in isolates, this does not
455 preclude neuromodulatory compensation to maintain circuitry and behavioral functions under
456 conditions of social deprivation.
457
458 *Conclusion*
459 The results of the present study and prior research on the impacts of senescence on
460 neurochemistry, neuroanatomy, apoptosis, and behavior (Giraldo et al., 2016), deprivation of
461 experience (Muscedere et al., 2013), and reduced olfactory processing ability (Waxman et al.,
462 2017), suggest that ant brains have an unusual capacity to buffer insults of aging, social isolation,
463 and physical damage to sensory appendages. The 36% mortality of isolated *C. floridanus* workers
464 we found in the present study indicates a significant negative effect that may, however, be due
465 more to social processes affecting nutrition and energy availability (Koto et al., 2015; Ahronberg
466 and Scharf 2021) than neurobiological deficits resulting from isolation. Workers that survive
467 neurobiological and physiological bottlenecks appear able to recover from challenges to brain
468 development, which in *C. floridanus* are dependent on age as well as experience (Gronenberg et

469 al., 1996). Age-related increases in brain size in ants appear to be due to stable programmed axonal
470 and dendritic growth processes. If sensory experience is lacking or temporally atypical, brain
471 development may still proceed in part in the absence of inputs or may be delayed (e.g.,
472 Mackevicius et al., 2023). In our study, the effect of social isolation in *C. floridanus* appears to
473 reflect such robust programming, perhaps due in part to stabilizing effects of neuromodulators.

474 The resilience of worker ants to challenges during development may functionally resemble
475 differences that enable some individuals – perhaps those with adaptive synaptic phenotypes
476 (Sakurai et al., 2014) or ion channel properties (Goaillard and Marder, 2021) – to withstand neural
477 damage and preserve behavior. We hypothesize that the ability to buffer stressors may involve
478 such variation in circuitry that characterizes clade-wide neuroarchitectures of the miniaturized
479 brains of ants, acknowledging that few species have been studied. Analyses of temporal patterns
480 of brain gene expression in *C. floridanus* isolated workers can identify transcriptomic changes
481 linked to brain development and influenced by social experience (Bonasio et al., 2010; Simola et
482 al., 2013) and possibly identify genetic mechanisms of resilience. Genomic studies of
483 phylogenetically and sociobiologically diverse ant species can also contribute to our understanding
484 of how workers are susceptible to and/or recover from social deprivation or are resilient to
485 isolation.

486

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Author Contributions

BCG, IBM, and JFAT designed the study. BCG recorded behavior. BCG and IBM immunohistologically prepared and imaged brains. EJS quantified brain volume. BCG, ZC, and MM measured monoamine titers. BCG statistically analyzed volumetric data. BCG drafted the manuscript. BCG, EJS, IBM, ZC, MM, and JFAT developed and edited the manuscript. JFAT secured funding.

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