Cross-fostering affects microglia and cell death in the hippocampus of female and male degu pups

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Keywords: neurogenesis; dentate gyrus; apoptosis; early life stress; sex differences

Abstract

Parental care is essential for social, behavioural, and neural development of offspring. Disruption of parental care, for example through parental separation, can negatively affect offspring development in rodents. While previous work has focused on maternal and paternal deprivation, the effects of cross-fostering, another form of parental-offspring instability, on brain development remain poorly understood. Such disruptions are thought to induce stress, which in turn can suppress neurogenesis and increases inflammation and apoptosis in the hippocampus, with effects that often differ between sexes. Given that degus (*Octodon degus*) are born precocial and form strong attachments with their parents early in life, this study aimed to investigate the effect of cross-fostering on hippocampal development in female and male degu pups. At postnatal day 8, degus were assigned to either control (pups remained with parents and littermates), partial cross-foster (one pup per litter was cross-fostered), or full cross-foster (the entire litter was cross-fostered) conditions. At weaning (5-weeks-old), offspring brains were collected for immunohistochemistry to examine dentate gyrus volume, density of pyknotic cells, density of immature neurons, and number and morphology of microglia. Both types of cross-fostering reduced hippocampal cell death in both sexes relative to controls, while no significant effects were observed in the dentate gyrus volume or density of immature neurons. Cross-fostering did not affect total microglia density in either sex however full cross-fostered females had fewer amoeboid microglia compared to female controls. Together, these findings indicate that cross-fostering affects hippocampal microglia and cell death in pups, potentially disrupting circuit refinement and plasticity during development, with effects that vary by cross-fostering type, sex, and hippocampal region.

46 **Introduction**

47 Early life stress affects offspring development in a range of mammalian species,
48 including humans (Gimsa et al., 2022; Kentner et al., 2010; Priebe et al., 2005; Raineki et al.,
49 2019). In rodents, there are different paradigms which induce early life stress that include, but
50 are not limited to, changing the parental environment via maternal separation, paternal
51 deprivation, or cross-fostering (Aguggia et al., 2013; Boccia and Pedersen, 2001; George et al.,
52 2010; Madison et al., 2022; Oomen et al., 2011). Any disruption to typical parent-offspring
53 interactions can lead to alterations in offspring neurodevelopment (Champagne and Meaney,
54 2001). During postnatal development, there is high growth and refinement of brain circuits. At a
55 mechanistic level, there are high rates of neurogenesis (i.e. production of new neurons) and
56 regulated cell death (i.e., apoptosis; Hayashi et al., 2008; Loi et al., 2017; Sierra et al., 2014).
57 Moreover, microglia, the neuroimmune cells of the brain, play a very important role in refining
58 circuits during early development, either by removing cells through phagocytosis or by
59 supporting neuronal development and survival (Diaz-Aparicio et al., 2020; Sierra et al., 2014).

60 One of the brain regions that is most sensitive to early life stressors is the hippocampus. It
61 is also one of the few regions where neurogenesis continues during the postnatal period
62 (Kempermann, 2016). Past studies have shown that parental separation affects hippocampal
63 neurogenesis, cell death (apoptosis), and microglia in the offspring, but the effects may depend
64 on age, sex of the offspring, and type of separation (maternal/paternal, acute/chronic). For
65 instance, acute maternal separation (24 h on postnatal day 3) reduces the numbers of immature
66 neurons in the hippocampus of female rats at weaning, while increasing immature neurons in
67 males (Oomen et al., 2009). In contrast, chronic maternal separation has been shown to reduce
68 neurogenesis in male rats (Hulshof et al., 2011). Early life stressors can also affect programmed

69 cell death. For example, maternal separation increases apoptosis in the dentate gyrus of rat pups
70 (Yang et al., 2017). Thus, research suggests a potential link between dysregulated neurogenesis
71 and apoptosis in the dentate gyrus due to early life stress, potentially mediated by microglial
72 activity. Indeed, brief daily maternal separation increases both the number of and phagocytic
73 activity of microglia in the hippocampus of juvenile male mice (Delpech et al., 2016; Johnson
74 and Kaffman, 2018; Walker et al., 2013). In addition to removing cells through phagocytosis,
75 microglia can also promote the production and survival of new neurons (neurogenesis) through
76 the secretion of trophic factors (Diaz-Aparicio et al., 2020). Here we aimed to understand how
77 these three key components—neurogenesis, cell death, and microglia—are regulated following a
78 stressor during development.

79 Most studies to date have focused on the effects of maternal and paternal deprivation on
80 offspring development, whereas the impact of other disruptions of parental-offspring interactions
81 has received much less attention. Cross-fostering disrupts parent-offspring attachment, but unlike
82 maternal separation or paternal deprivation, it does not rely on separation and removing pup's
83 access to a caring adult. Past research has shown that cross-fostering affects offspring body
84 weight, emotional behaviour, cardiovascular and metabolic functions, and the endocrine stress
85 response in different rodents species, including mice, degus, and rats (Arusha et al., 2024;
86 Bartolomucci et al., 2004; Gomez-Serrano et al., 2001; Guan et al., 2023; Lu et al., 2009;
87 Matthews et al., 2011). Degus (*Octodon degus)* are a particularly suitable model for studying the
88 effects of cross-fostering on offspring development because they practice communal care and
89 pups are born precocial and form strong attachments early in life with both parents and
90 littermates (Colonnello et al., 2011; Guan et al., 2023). Importantly, the presence of siblings
91 during cross-fostering may buffer the effects of separation from biological parents (Arusha et al.,

92 2024). Accordingly, in the current study, we investigated the effects of cross-fostering either the
93 entire litter (full cross-fostering) or a single pup (partial cross-fostering) on hippocampal
94 development in female and male degus. We measured hippocampal development by assessing
95 dentate gyrus volume, postnatal neurogenesis, apoptosis, and microglia prevalence and
96 morphology. We hypothesized that (1) cross-fostering would impact hippocampal development
97 by reducing neurogenesis, increasing cell death and microglial numbers, and altering the
98 microglia morphological profile in both sexes; and (2) partial cross-fostering would be more
99 detrimental to hippocampal development compared to cross-fostering the entire litter.
100
101 **Methods**
102 *Study animals and breeding*
103 Breeding pairs of captive-born *Octodon degus* and their pups were housed in the animal
104 facility at Swarthmore College, in 51 x 25 x 33 cm acrylic terrariums containing corn cob or
105 paper bedding allowing for visual observations. Animals were maintained under standard
106 laboratory conditions (12:12 light/dark cycle, 23 ± 1 ˚C) with *ad libitum* access to water and
107 food. Alfalfa hay was provided three times per week, and cages were cleaned weekly.
108 Degu females (n = 21) were paired with unrelated males (n = 21) for mating and remained
109 together until pup weaning. All experiments were conducted in strict compliance with
110 Institutional Animal Care and Use Committee at Swarthmore College and were carried out
111 according to the guidelines of the Association for Assessment and Accreditation of Laboratory
112 Animal Care.
113
114 *Experimental treatment*

115 Pups were cross-fostered at postnatal day (PND) 8, thus allowing for sufficient time for
116 the development of parental and social attachments within the nest (Colonnello et al., 2011;
117 Guan et al., 2023). The date of parturition was taken into consideration while assigning the
118 treatment groups as previously described (Arusha et al., 2024). Litters were assigned to one
119 treatment condition: control (N=5 litters), full cross-fostering (N=4 litters), and partial cross-
120 fostering (N=12 litters). In control litters, pups remained with biological parents and biological
121 siblings for the duration of the experiment. In full cross-foster litters, all pups from one litter
122 were exchanged with all pups from another such that pups were raised with unfamiliar parents
123 but with biological siblings. In partial cross-foster litters, a single pup was exchanged with one
124 pup of the same sex from another litter such that the pup was raised with both unfamiliar parents
125 and unfamiliar siblings. Pups were fostered between litters of similar sizes and ages (<2 days in
126 age difference). Between 1-2 pups per sex per group were used for subsequent analyses, with the
127 exception of the male full cross-foster condition that included 3 male pups from the same litter
128 (out of 4 litters). Sample sizes were decided based on previous work (Arusha et al., 2024; Guan
129 et al., 2023) and are presented in Table 1 and each of the figure captions.
130
131 *Tissue collection*
132 Euthanasia was performed at weaning (~5 weeks of age). Pups received 0.2 mL of
133 undiluted Euthasol (pentobarbital sodium and phenytoin sodium solution), and brains were
134 immediately dissected and split into the right and left hemispheres. The right hemispheres were
135 placed on dry ice and frozen at –80˚C for future analysis, whereas the left hemispheres (used in
136 the present study) were immersion-fixed in 4% paraformaldehyde for 24h at 4˚C. Following
137 fixation, brains were then transferred to a 30% sucrose solution containing 0.1% sodium azide
138 and stored at 4˚C until sectioning. Coronal sections of 30 µm thickness were cut using a cryostat

139 at –20˚C. Hippocampal sections were collected in a seven-tube series containing an antifreeze
140 solution (ethylene glycol, glycerol and 0.1M phosphate buffer saline with a pH of 7.4) and stored
141 at –20˚C. All three neural measurements (volume, neurogenesis, and microglia) were performed
142 in parallel series from the same left hemisphere for all animals.
143
144 *Cresyl violet staining*
145 Brain sections were stained with Cresyl violet to determine the effects of cross-fostering
146 on dentate gyrus volume and to count pyknotic cells. Hippocampal tissue from one of the series
147 was mounted onto Superfrost microscope slides. Cresyl violet staining was performed after the
148 tissue had completely dried on the slides. The staining process started by dipping the slides into
149 distilled water three times to remove residual salts, then submerged in 0.2% Cresyl violet acetate
150 solution for 4 minutes. Following staining, the slides were placed in a differentiator solution,
151 comprising 70% ethanol and seven drops of glacial acetic acid, for 30 seconds. The slides were
152 then sequentially dehydrated in 95% ethanol (twice, for 1 minute each), followed by 100%
153 ethanol for 1 minute. Finally, slides were cleared in xylene (twice, for 5 minutes each) and
154 cover-slipped using Permount.
155
156 *Doublecortin immunohistochemistry*
157 Brain sections were stained with doublecortin (DCX) to assess the effects of cross-
158 fostering on postnatal neurogenesis. All staining procedures were conducted in multi-well plates
159 under gentle shaking. To begin, sections were washed in 0.1M phosphate buffer saline (PBS, pH
160 = 7.4) to remove antifreeze and then incubated in 0.6% hydrogen peroxide in PBS for 30
161 minutes. Sections were then rinsed with PBS and then incubated for 22h at 4˚C in rabbit anti-
162 DCX primary antibody (1:200 dilution; Cell Signalling, catalogue number: 10124618) diluted in

163 0.1M PBS containing 3% of normal goat serum (NGS) and 0.4% Triton-X. After incubation,
164 tissue was rinsed in 0.1M PBS, then incubated for 18h at 4˚C in biotinylated goat anti-rabbit
165 secondary antibody (1:500 dilution; Vector laboratories) diluted in 0.1M PBS. This was followed
166 by washes in 0.1M PBS and incubation for 4 hours in an avidin-biotin horseradish peroxidase
167 complex solution (1:1000 dilution; ABC kit, Vector Laboratories). After rinsing in 0.1M PBS,
168 tissue was incubated in diaminobenzidine in the presence of nickel (DAB Peroxidase Substrate
169 Kit, Vector Laboratories) for 3 minutes. Stained sections were mounted on Superfrost
170 microscope slides and allowed to dry for 2–3 days. Slides were then dipped in distilled water
171 three times to remove salts, dehydrated sequentially in 70% ethanol (1 minute), 95% ethanol (1
172 minute), and 100% ethanol (1 minute), then cleared in xylene (twice, 5 minutes each). Finally,
173 coverslips were applied using Permount.
174
175 *Iba-1 immunohistochemistry*
176 Brain sections were stained for ionized calcium-binding adaptor molecule (Iba-1) to
177 access the effects of cross-fostering on microglia. All staining procedures were conducted in
178 multi-well plates under gentle shaking. Sections were first washed in 0.1M PBS (pH = 7.4) to
179 remove antifreeze, followed by an incubation in 0.6% hydrogen peroxide in PBS for 30 minutes.
180 Tissue was then rinsed with PBS and incubated for 22h at 4˚C in rabbit anti-Iba-1 primary
181 antibody (1:1000 dilution, Wako, catalogue number: 019-19741), diluted in 0.1M PBS
182 containing 3% NGS and 0.4% Triton-X. The following day, sections were rinsed in 0.1M PBS,
183 then incubated for ~18h in biotinylated goat anti-rabbit secondary antibody (1:500 dilution,
184 Vector laboratories) diluted in 0.1M PBS. This was followed by washes in 0.1M PBS and
185 incubation for 4h in an avidin-biotin horseradish peroxidase complex solution (1:1000 dilution;

186 ABC kit, Vector Laboratories). Tissue was rinsed in 0.1M PBS followed by incubation in
187 diaminobenzidine in the presence of nickel (DAB Peroxidase Substrate Kit, Vector Laboratories)
188 for three minutes. Stained sections were mounted on Superfrost microscope slides and allowed to
189 dry for 2–3 days. Slides were dipped in distilled water three times to remove salts, dehydrated
190 sequentially in 70% ethanol (1 minute), 95% ethanol (1 minute), and 100% ethanol (1 minute),
191 then cleared in xylene (twice, 5 minutes each). Coverslips were applied using Permount.
192
193 *Microscopy, Imaging, and Quantification*
194 All analyses were performed by a researcher blinded to experimental conditions.
195 Brightfield images of the entire left hippocampus stained with Cresyl violet were taken using a
196 standard light microscope (Olympus BX53) equipped with a camera (ORCA-spark, Hamamatsu)
197 using the 4X objective. The granule cell layer (GCL) and hilus were traced separately using
198 ImageJ to obtain areas in μm^2. Volumes of GCL and hilus were calculated using the Cavalieri's
199 principle: the measured areas were multiplied by the section thickness (30 µm), by 7 (reflecting
200 the 7 sections collect for the hippocampus), and finally by 2 (to present the volume for both
201 hemispheres). The dentate gyrus volume was calculated by adding the volume of the GCL and
202 the hilus. For the pyknotic cell count, two dorsal and two ventral sections of the dentate gyrus
203 were selected for each animal. Ventral sections were defined as those located between interaural-
204 incisor coordinates 2.32 and –0.32 of the degu brain atlas (Kumazawa-Manita et al., 2018). We
205 counted the number of pyknotic cells in the GCL using a 100X oil immersion objective. During
206 counting, the fine focus knob was used to scan through the entire thickness of each section to
207 ensure no cells were missed.
208 For the analysis of immature neurons, pups exhibited a very high expression of DCX,
209 typical for this developmental period, but making it challenging to reliably delineate and count

210 individual immature neurons (Figure 2A and B). Therefore, we used optical density as an index
211 of DCX expression. In addition, although morphological classification of DCX+ cells can
212 provide insight into neuronal maturation, the high expression of DCX also prevented us from
213 reliably scoring individual cell processes. Brightfield images were taken from two dorsal and two
214 ventral sections using an Olympus BX53 microscope with a 4X objective. Optical density (OD)
215 of DCX labeling was quantified using ImageJ. The GCL was traced in each image, and grey
216 values were obtained. The Rodbard equation was employed to calculate the OD for both the
217 GCL and background region (corpus callosum). For each section, background OD was
218 subtracted from the GCL OD. The average corrected OD for both image densities was used in
219 statistical analysis.

220 For microglia, brightfield images were taken using an Olympus BX53 microscope with a
221 20X objective. Two images from the dorsal and two from the ventral dentate gyrus per animal
222 were taken. We used a standardized region of interest (490.4 x 368.5 µm; see placement in
223 Figure 3A), which included the GCL and hilus regions. We counted the total number of
224 microglia present and categorized them into three morphological types—ameboid, intermediate,
225 and ramified—using ImageJ, as we have done in the past (Duarte-Guterman et al., 2023).
226 Ramified microglia were defined by a small cell body with at least three processes, each at least
227 twice the length of the cell body diameter. Intermediate microglia were defined by cell bodies
228 with 1–2 processes, each no longer than twice the diameter of the cell body. Ameboid microglia
229 were defined as a dark cell body with very small or no visible processes. All microglia were fit
230 into the best or closest category by an experimenter that was blinded to the treatment groups. The
231 number of microglia was added to get the total number of microglia per individual and
232 normalised to region of interest to obtain a density measure. For one partial cross-fostered male,

233 due to tissue loss during immunostaining, we did not have dorsal sections for Iba-1 analysis. One
234 control female brain did not have two ventral section images due to missing tissue sections,
235 therefore, data of only one section were utilized in the analysis.
236
237 *Statistical Analysis*
238 All statistics were performed in IBM SPSS, Version 30.0.0.0 and data visualization was
239 performed in R (R Core Team, 2021) using the ggplot2 package (Wickham, 2016). ANOVA
240 assumptions of normality and homogeneity of the variance were checked with visual inspections
241 of QQ plots and residual plots, and Kolmogorov-Smirnov and Levene's tests, respectively. The
242 volumes of the dentate gyrus, hilus, and GCL were analysed using analysis of covariance
243 (ANCOVAs) with treatment (control, partial, and full cross-foster) and sex (female, male) as
244 between-subject factors, and litter size and number of females as covariates. For DCX OD,
245 microglia density, microglia morphology, and pyknotic cell density, data were analysed using
246 repeated measures ANOVAs, with treatment and sex as between subject factors, region (dorsal
247 and ventral) as a within-subject factor, and litter size and number of females as covariates.
248 Including litter size and sex ratio as covariates ensured that treatment-related effects were not
249 confounded by natural variation in litter composition. Including these covariates in the analysis
250 did not affect the main patterns of the results. For all tests, we present the estimated marginal
251 means and the raw data. Post hoc comparisons for the repeated-measures ANOVA were conducted
252 using the Sidak correction. For the repeated measures ANOVAs, if the assumption of sphericity
253 was violated, the degrees of freedom were corrected using the Greenhouse-Geisser estimate of
254 sphericity. Pearson r correlations were performed between cell types, separately for females and
255 males and the different treatment groups. P-values < 0.05 were considered statistically significant.
256

Results

Cross-fostering did not alter dentate gyrus volume or DCX optical density

Cross-fostering did not affect the volume of the dentate gyrus in female and male pups, (all p's > 0.526) (Figure 1). Similarly, cross-fostering did not affect the volume of the hilus (all p's > 0.292) or the GCL (all p's > 0.595; Table 1). We did not find any sex differences for any of the volumes (dentate gyrus, hilus, or GCL) (all p's > 0.292; Table 1).

To examine the effects of cross-fostering on postnatal neurogenesis, we measured the OD of DCX in the dorsal and ventral regions of the hippocampus. We found a significant interaction between treatment, sex, and region ($F(2,32) = 4.212$, $p = 0.024$; $\eta^2 = 0.208$). Post hoc analysis indicated that partial cross-fostered females had a trend for significantly higher DCX OD than full cross-fostered females in the ventral hippocampus (p=0.059; Figure 2). However, this trend was largely driven by a single outlier in the partial cross-fostered group; after winsorising this value to the nearest non-extreme value, the contrast was no longer significant (p = 0.458). There were no significant differences between partial cross-fostered females and control females (p = 0.168), or between full cross-fostered females and control females (p = 0.157) in the ventral hippocampus. There were no differences between males across treatment conditions in DCX OD in the ventral hippocampus (all p's > 0.072). In the dorsal hippocampus, cross-fostering did not affect DCX OD in either females or males (all p's > 0.101).

Furthermore, we detected sex differences in DCX OD in the partial cross-fostered and full cross-fostered pups. In the ventral hippocampus, partial cross-fostered females had higher DCX OD than males (p = 0.032) although this sex difference disappears when the partial cross fostered female outlier is replaced with the nearest non-extreme value. In contrast, full cross-

279 fostered females had lower OD compared to males ($p = 0.023$). In controls, no sex differences in
280 DCX OD were found in either the dorsal or ventral hippocampus (all p's > 0.429).
281
282 *Cross-fostering altered microglia counts and morphology in the dentate gyrus of degu pups*
283 Cross-fostering did not affect the density of Iba-1-positive cells in the dentate gyrus of
284 degu pups when taking into account litter size and sex ratio in the analysis (all p's > 0.149,
285 including the effect of covariates; Figure 3). Without these covariates, there was a significant
286 treatment by region interaction: ($F(2,33) = 3.824$, $p = 0.033$), however post-hoc analyses did not
287 reveal any significant differences between treatments.
288 While total Iba-1 density was not affected by cross-fostering, we analysed the density of
289 the three morphological states of Iba-1 expressing cells. Cross-fostering also affected the density
290 of ameboid Iba-1-positive cells with the effect being sex-specific but across both dorsal and
291 ventral regions (treatment by sex interaction: $F(2,31) = 4.531$, $p = 0.019$; $\eta^2 = 0.226$, Figure 4).
292 Full cross-fostered female pups had fewer ameboid microglia compared to female controls ($p =$
293 0.026). Partial cross-fostering did not affect the density of ameboid Iba-1 positive cells in female
294 pups compared to control or full cross-fostering conditions (all p's > 0.449). No significant
295 differences were found in the density of ameboid microglia in males (all p's > 0.733). No
296 significant differences were observed in the density of ramified or intermediate microglia across
297 cross-fostering conditions (all p's > 0.089).
298
299 *Cross-fostering differentially altered pyknotic cell density in dorsal and ventral hippocampus*
300 *regions*

Cross-fostering affected pyknotic cell density differently in the dorsal and ventral hippocampus (treatment by region interaction: $F(2,32) = 5.058$, $p = 0.012$, $\eta^2 = 0.240$, Figure 5). In the dorsal and ventral hippocampus, full cross-fostered pups had fewer pyknotic cells compared to controls ($p < 0.001$ and $p = 0.025$, respectively). Partial cross-fostered pups also had fewer pyknotic cells compared to controls but only in the dorsal hippocampus ($p = 0.002$) No sex differences were found in pyknotic cell density in either dorsal or ventral regions (all p's > 0.127).

Correlations between cell types

We examined correlations between microglia density and morphology, DCX OD, and pyknotic cell density within each cross-fostering condition, separately for males and females and hippocampal region. In the dorsal region, we found density of ramified microglia was significantly correlated with DCX OD in partial cross-fostered males only (Pearson $r = 0.914$, $p = 0.03$). No other significant associations between these endpoints in either sex were found in the dorsal region (all p's > 0.10). In the ventral region, intermediate microglia density was correlated with DCX OD in partial cross-fostered females only (Pearson $r = 0.948$, $p = 0.014$). There were no other significant correlations in the ventral region (p's > 0.08).

Discussion

The purpose of this study was to examine the effects of cross-fostering on neural development in female and male degu pups. Degus are born precocial and form strong attachments early in life, making them a great model for studying the effects of early-life adversity and social bonding during development (Arusha et al., 2024; Guan et al., 2023). We found that cross-fostering affected microglia morphology and pyknotic cell density, but not

325 dentate gyrus volume or neurogenesis. Full cross-fostering was associated with a reduction in
326 ameboid microglia in female pups in both dorsal and ventral regions. Full cross-fostered pups,
327 regardless of sex, had a lower density of pyknotic cells compared to controls in the dorsal and
328 ventral regions. However, partial cross-fostering reduced pyknotic cell density in both sexes but
329 only in the dorsal region. Overall, this study demonstrates that cross-fostering affects
330 hippocampal microglia and cell death in a type of cross-fostering-, sex-, and region-specific
331 manner in degu pups.
332
333 *Cross-fostering did not affect dentate gyrus volume in pups*
334 Cross-fostering did not impact the volume of the dentate gyrus in degu pups. Previous
335 work in other rodents such as rats, mice, and California mice has been mixed. Some studies find
336 reductions in the dentate gyrus volume of pups exposed to early life stressors such as paternal
337 deprivation (Madison et al., 2022), chronic unpredictable stress (Herpfer et al., 2012), and
338 scarcity-adversity model using low bedding (Raineki et al., 2019). However, other studies find
339 no effect after exposure to chronic unpredictable stress (Huot et al., 2002) or maternal separation
340 (Loi et al., 2017). Overall, our findings highlight the importance of considering both the type of
341 stressor and the species when evaluating the effects of early life stressors on hippocampus
342 development. Notably, in the current experiment, maternal and paternal licking and grooming
343 were not affected by cross-fostering in degus (partial or full cross-fostering), although cross-
344 fostered mothers spent more time off the nest compared to control and partial cross-foster groups
345 (Bauer et al., in prep). It is possible that having a relatively more stable parental care
346 environment (e.g., compared to separation paradigms) may help protect against the effects of
347 cross-fostering on dentate gyrus volume in pups.

348 Interestingly, we did not find sex differences in dentate gyrus volume in degu pups. This
349 is in contrast with findings in mice, where males have larger hippocampal volume compared to
350 females between PND 10 and PND 17, and again at PND 65 (Qiu et al., 2018). To our
351 knowledge, our study is the first one to examine hippocampal volume in degu pups. Further
352 research is required in both juvenile and adult degus to understand sex differences in
353 hippocampal development.
354
355 *Cross-fostering does not affect neurogenesis in pups*
356 We examined the effects of cross-fostering on postnatal neurogenesis using DCX OD as
357 a marker of immature neurons. We found that cross-fostering did not affect DCX OD compared
358 to controls. Previous work has found that maternal separation leads to a reduction in the number
359 of immature neurons at weaning in female, but not male rats (Oomen et al., 2009). In biparental
360 species, paternal deprivation either has no effect on neurogenesis (cell survival) in adult
361 California mice (Madison et al., 2022), or decreases cell survival in adult females only (Glasper
362 et al., 2018). In mandarin voles, paternal deprivation is associated with a reduction in immature
363 neurons in adulthood compared to biparentally raised pups (He et al., 2018). Overall, these
364 findings suggest that changes in neurogenesis following parental separation are not always
365 observed in all studies and results can vary across sexes and potentially with age. Our
366 observations were limited to a single developmental time point and one neurogenesis marker
367 using optical density. It remains possible that cross-fostering may affect the number of immature
368 neurons, without affecting the density of DCX, or that maturation and survival of new neurons
369 are affected, or that changes are manifested later in adolescence or adulthood.
370

371 *Cross-fostering alters pyknotic cell density in pups*

372 The current study revealed noteworthy alterations in pyknotic cell density in the dentate
373 gyrus depending on the type of cross-fostering and region. We found that full cross-fostered
374 pups, regardless of sex, exhibited fewer pyknotic cells compared to controls in both regions of
375 the hippocampus, while partial cross-fostered pups had reduced pyknotic cell density in the
376 dorsal region only. In contrast, previous studies in rats have shown that maternal separation can
377 increase cell death, although the effects depend on timing of exposure. For example, maternal
378 separation increases hippocampal cell death after an acute 24-hour separation at PND12, but not
379 at PND20 (Zhang et al., 2002). Increases in hippocampal cell death have also been reported
380 following chronic maternal separation in rats (Baek et al., 2012). These discrepancies between
381 previous work and the current study may reflect species differences or differences in the type and
382 severity of the early life stressor, as discussed earlier. Apoptosis is a crucial developmental
383 process that contributes to the formation and refinement of neural circuits, and alterations in this
384 process can influence neuron number and brain function (Forger, 2009; Majcher-Maślanka et al.,
385 2019). Thus, the observed reduction in pyknotic cell density in cross-fostered pups could indicate
386 a decreased level of apoptosis and insufficient cell pruning, potentially resulting in overcrowding
387 of neurons and altered neural circuitry.

388 Partial cross-fostering affected the dorsal and ventral regions differently in pups. The
389 dorsal hippocampus is primarily involved in spatial learning and other cognitive aspects of
390 memory, whereas the ventral hippocampus is associated with stress and anxiety regulation
391 (Bannerman et al., 2004; Fanselow and Dong, 2010; Kheirbek and Hen, 2011). Further research
392 is needed to determine whether cross-fostering-related changes in cell death during

393 development translate into changes in cognition, mood or other hippocampal-related behaviours
394 in degus.
395
396 *Cross-fostering altered microglia numbers and morphology in pups*
397 We found that cross-fostering altered the morphology of microglia in the hippocampus
398 but not overall microglia density. Microglia play a crucial role in the central nervous system,
399 contributing to synaptic remodeling through pruning, as well as supporting neuronal survival
400 via the release of neurotrophic factors (Cornell et al., 2022; Sierra et al., 2014). Although we did
401 not observe changes in overall microglia numbers, previous work has shown that an adverse
402 early life environment due to a combination of minimal bedding, frequent cage changes, and a
403 Western diet increases microglial numbers in the mouse hippocampus at weaning (Cohen et al.,
404 2016). Similarly, maternal separation in rats (Banqueri et al., 2019) and paternal deprivation in
405 California mice (Madison et al., 2022) have also been associated with increased microglia in the
406 offspring hippocampus.
407 Instead, in the current study, we found that cross-fostering affected the morphology of
408 microglia in a sex-dependent manner. Full cross-fostered female pups had fewer ameboid
409 microglia compared to control females regardless of hippocampal region. Ameboid microglia are
410 typically associated with phagocytosis, synaptic pruning, and the secretion of pro-inflammatory
411 cytokines and are characterized by a rounded morphology lacking the long processes often
412 observed in ramified microglia (Burke et al., 2016; Colonna and Butovsky, 2017; Franco and
413 Fernández-Suárez, 2015; Paolicelli et al., 2022). The current literature agrees that microglia vary
414 from ameboid to ramified states, but each morphology does not have exclusive functions. The
415 ability to transition between different stages allows microglia to maintain a balanced immune

416 response (Paolicelli et al., 2022). One possible interpretation of our results is that the reduction in
417 ameboid microglia in full cross-fostered female pups may reflect lower levels of phagocytosis,
418 potentially contributing to the reduced pyknotic cell density observed. However, this may not be
419 the only contributing factor because cross-fostering also decreased pyknotic cell density in males
420 with no changes in microglia morphology. In addition, there were no significant correlations
421 between microglia (numbers and morphology) and pyknotic cell density in our samples, although
422 the results may be limited by the small sample size. Overall, while cross-fostering clearly
423 affected microglial morphology in the female hippocampus, whether these changes directly lead
424 to apoptosis remain to be elucidated.
425
426 *Brain measures depend on type of cross-fostering*
427 Our results indicate that full cross-fostered pups showed greater effects on cell death and
428 microglia morphology, and to some extent, neurogenesis compared to partial cross-fostered pups.
429 These findings were contrary to our hypothesis and previous work demonstrating that presence
430 of a familiar sibling (when the entire litter is transferred) reduces the effect of cross-fostering on
431 circulating cortisol levels and weight gain, compared to pups that were cross-fostered on their
432 own (Arusha et al., 2024). In adult rats, the effects of corticosterone on hippocampal
433 neurogenesis vary depending on the dose, with studies showing increases, decreases, or no effect
434 (reviewed in Duarte-Guterman et al., 2017). It is possible that a lower-stress condition (such as
435 full cross-fostering based on cortisol levels and weight gain; Arusha et al., 2024) may
436 paradoxically produce greater effects on the hippocampal development compared to a moderate
437 stressor (such as partial cross-fostering). Further work is needed to investigate the impact of
438 stress hormones on hippocampal development in degus.

Conclusion

Because degus are precocial and fathers, in addition to mothers, may also contribute to offspring care, studies examining cross-fostering in this species may provide a more translationally relevant model for understanding how early-life caregiving environments, such as those experienced in adoption and foster care, shape developmental outcomes. In this context, our results demonstrated that cross-fostering did not affect volume or postnatal neurogenesis in the hippocampus. However, cross-fostering affected pyknotic cell density and microglia morphology, in a type of cross-fostering-, region- and sex-specific manner. Our findings complement earlier work with the same cross-foster degu model to examine its effects on HPA axis regulation (Guan et al., 2023; Arusha et al., 2024) and they further advance our understanding of how this early life stressor influences brain development. Finally, the results also highlight the importance of including both sexes and diverse forms of early-life stressors, such as cross-fostering paradigms. Further work is needed to determine the long-term effects of cross-fostering on brain function and potential behavioural consequences.

Acknowledgments

This research was supported by the Canada Research Chair Program to PDG and Swarthmore College to CMB. GA was funded by Ontario Graduate Scholarship and Faculty of Social Sciences at Brock University. SE was funded by Women's Science Research Fellowship and KB, DK, and KA were funded by Swarthmore College Summer Opportunities and Research Fellowships. We thank the animal care staff at Swarthmore College for technical assistance.

460 **Table 1**. Sample size for each group, and volume (mm^3) of the dentate gyrus, hilus, and granule cell

461 layer (GCL) in female and male degu pups in control, partial, and full cross-fostering conditions.

462 Estimated marginal means are presented ± SEM.

463

	Sample Size		**Dentate Gyrus**		**Hilus**		**GCL**	
	Females	Males	Females	Males	Females	Males	Females	Males
Control	7	7	6.32±0.42	6.26±0.40	3.41±0.30	3.51±0.28	2.91±0.19	2.75±0.17
Partial	5	6	6.14±0.52	6.38±0.44	3.55±0.37	3.61±0.31	2.59±0.23	2.77±0.19
Full	6	9	6.05±0.45	6.81±0.39	3.26±0.32	3.89±0.27	2.80±0.20	2.91±0.17

464

465

466 **FIGURE CAPTIONS**

467 **Figure 1.** Representative image of the dorsal (A) and ventral (B) hippocampus in degu pups. The
468 granule cell layer (GCL) and hilus are marked. Dentate gyrus volume (C) was not affected by partial
469 or full cross-fostering in male and female pups. Individual data points are shown with the estimated
470 marginal means (±SEM). Sample sizes were the following: control n=7 females, n=7 males; partial
471 cross-foster n=5 females, n=6 males; full cross-foster n=6 females, n=9 males.

472

473 **Figure 2.** A) Representative image of DCX expression in the dorsal (A) and ventral (B)
474 hippocampus of degu pups. Scale bars equal to 200 µm or 50 µm. C) DCX optical density (OD) in
475 the dorsal (D) and ventral (C) regions in female and male degus. Cross-fostering did not affect DCX
476 OD in either region in both sexes. Individual data points are shown with the estimated marginal
477 means (±SEM). Sample sizes were the following: control n=7 females, n=7 males; partial cross-
478 foster n=5 females, n=6 males; full cross-foster n=6 females, n=9 males.

479

480 **Figure 3**. (A) A depiction of Iba-1 expression within the region of interest (black rectangle box) in
481 the dentate gyrus of the hippocampus. Total number of microglia in the dorsal (B) and ventral (C)
482 hippocampus. There were no significant differences across cross-fostering groups in either region.
483 Individual data points are shown with the estimated marginal means (±SEM). Sample sizes were the
484 following: control n=7 females, n=7 males; partial cross-foster n=5 females, n=6 males (one male
485 sample was damaged and did not have dorsal sections); full cross-foster n=6 females, n=9 males.

486 **Figure 4.** Representative images of microglia morphology showing (A) ameboid, (B) intermediate,
487 and (C) ramified microglia (scale bar = 20 µm). (D) Full cross-fostered female pups had a lower
488 ameboid microglia density than control (* $p < 0.05$). Cross-fostering did not affect the density of
489 intermediate (E) or ramified microglia (F). Individual data points are shown with the estimated
490 marginal means (±SEM). Sample sizes were the following: control n=7 females, n=7 males; partial
491 cross-foster n=5 females, n=6 males (one male sample was damaged and did not have dorsal
492 sections); full cross-foster n=6 females, n=9 males.
493
494 **Figure 5. (**A) Representative image of pyknotic cells (arrows) in the granule cell layer in degu pups
495 (scale bar = 20 µm). Pyknotic cell density was affected by cross-fostering in the dorsal (B) and
496 ventral hippocampus (C). In the dorsal region, full cross-fostered pups had the lowest density of
497 pyknotic cells, followed by partial cross-fostered pups when compared to controls (** $p < 0.01$, ***
498 $p < 0.001$). In the ventral region, full cross-fostered pups had a lower density of pyknotic cells
499 compared to controls (* $p<0.05$). Individual data points are shown with the estimated marginal
500 means (±SEM). Sample sizes were the following: control n=7 females, n=7 males; partial cross-
501 foster n=5 females, n=6 males; full cross-foster n=6 females, n=9 males.

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689

Figure 1

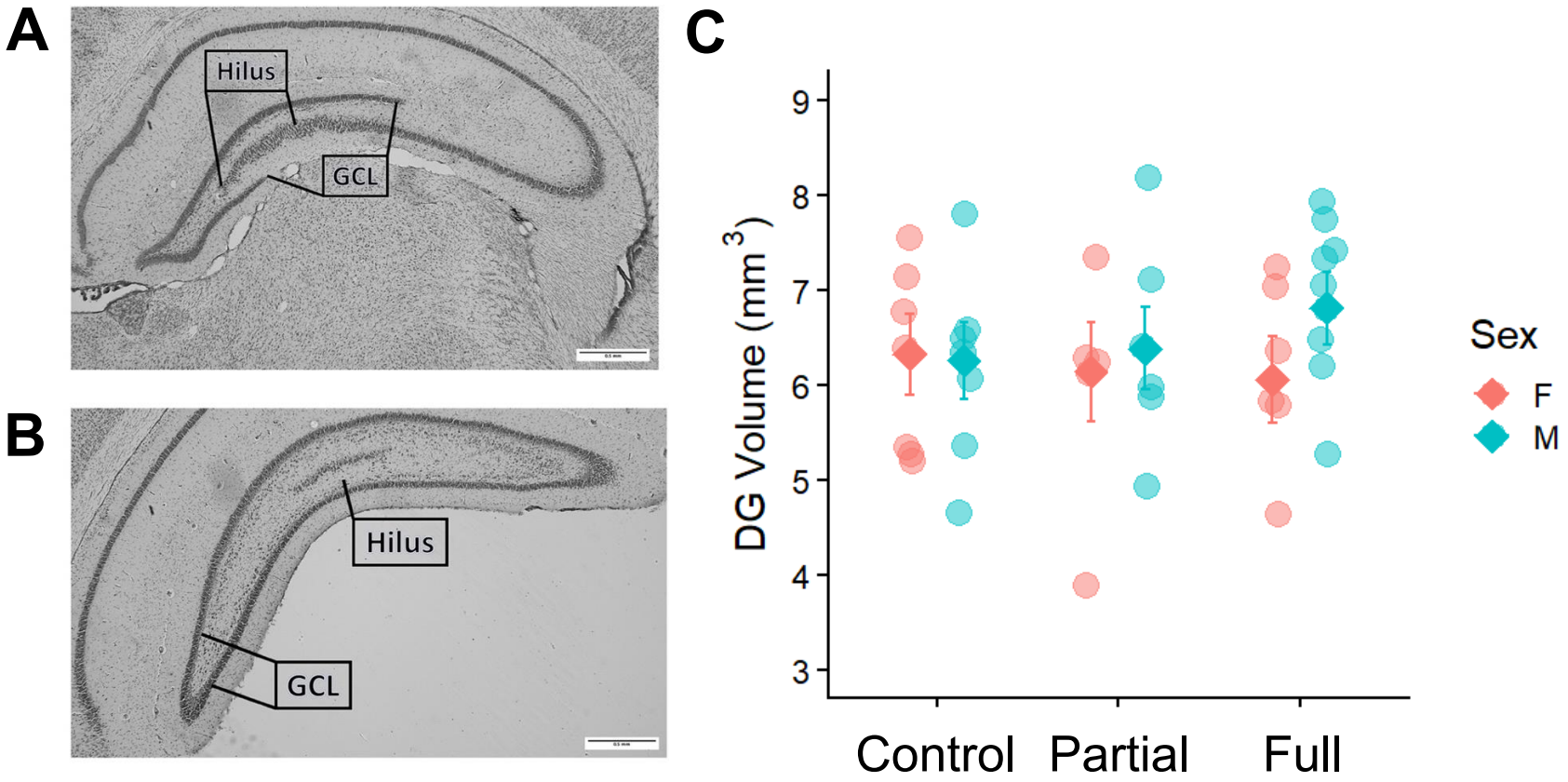
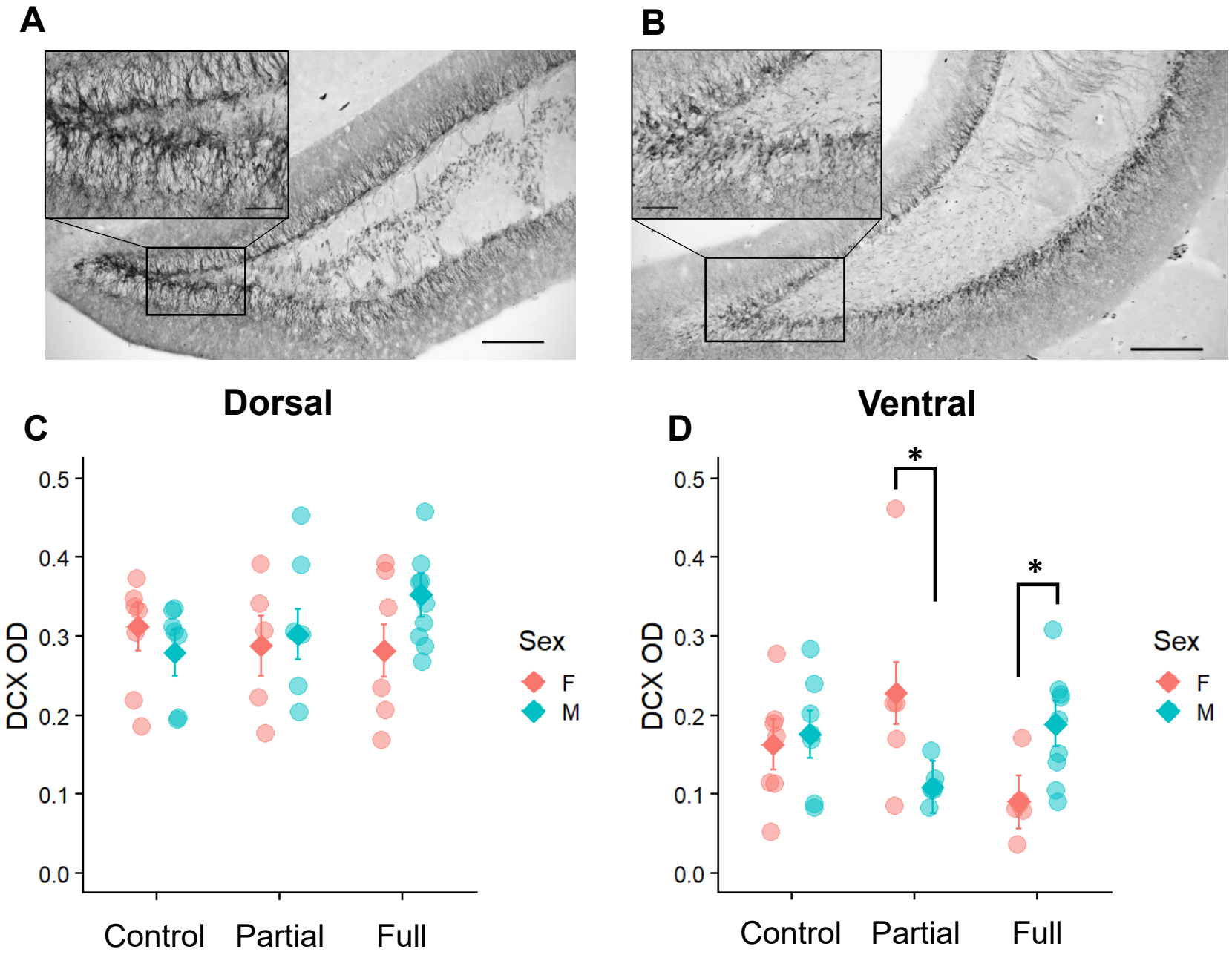

A
Hilus
GCL
B
Hilus
GCL
C
DG Volume (mm^3)
9
8
7
6
5
4
3
Control
Partial
Full
Sex
F
M

Figure 2


A
B
Dorsal
Ventral
C
D
DCX OD
0.5
0.4
0.3
0.2
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Control
Partial
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Sex
F
M
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Figure 3

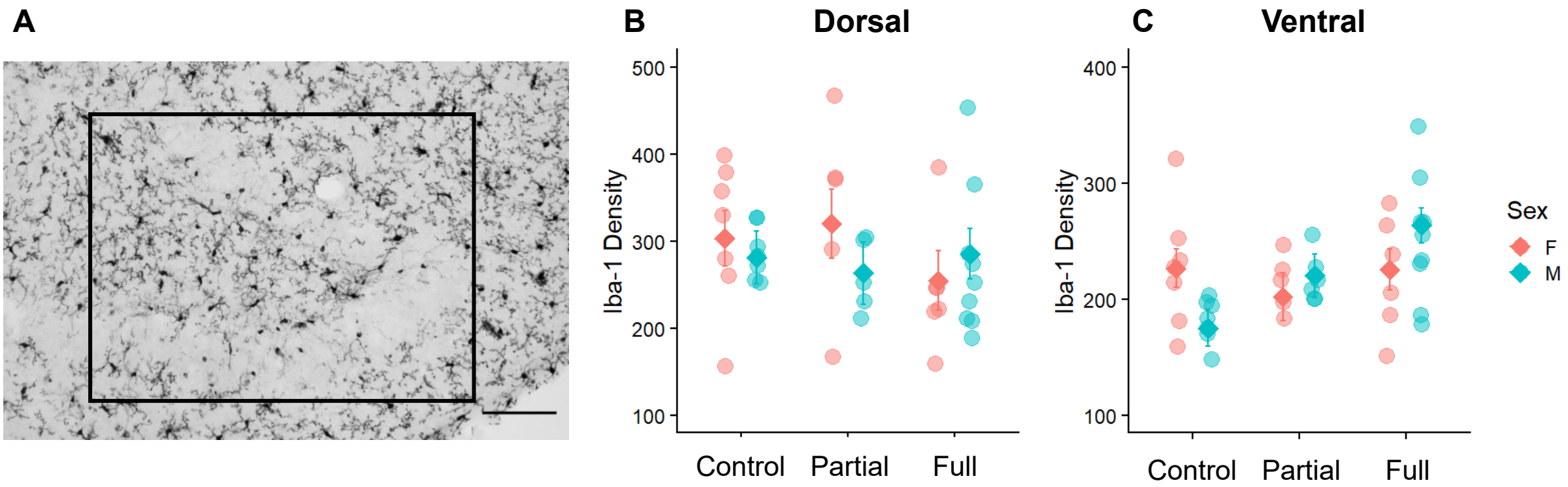

A
B
Dorsal
C
Ventral
Iba-1 Density
500
400
300
200
100
Control
Partial
Full
Iba-1 Density
400
300
200
100
Control
Partial
Full
Sex
F
M

Figure 4

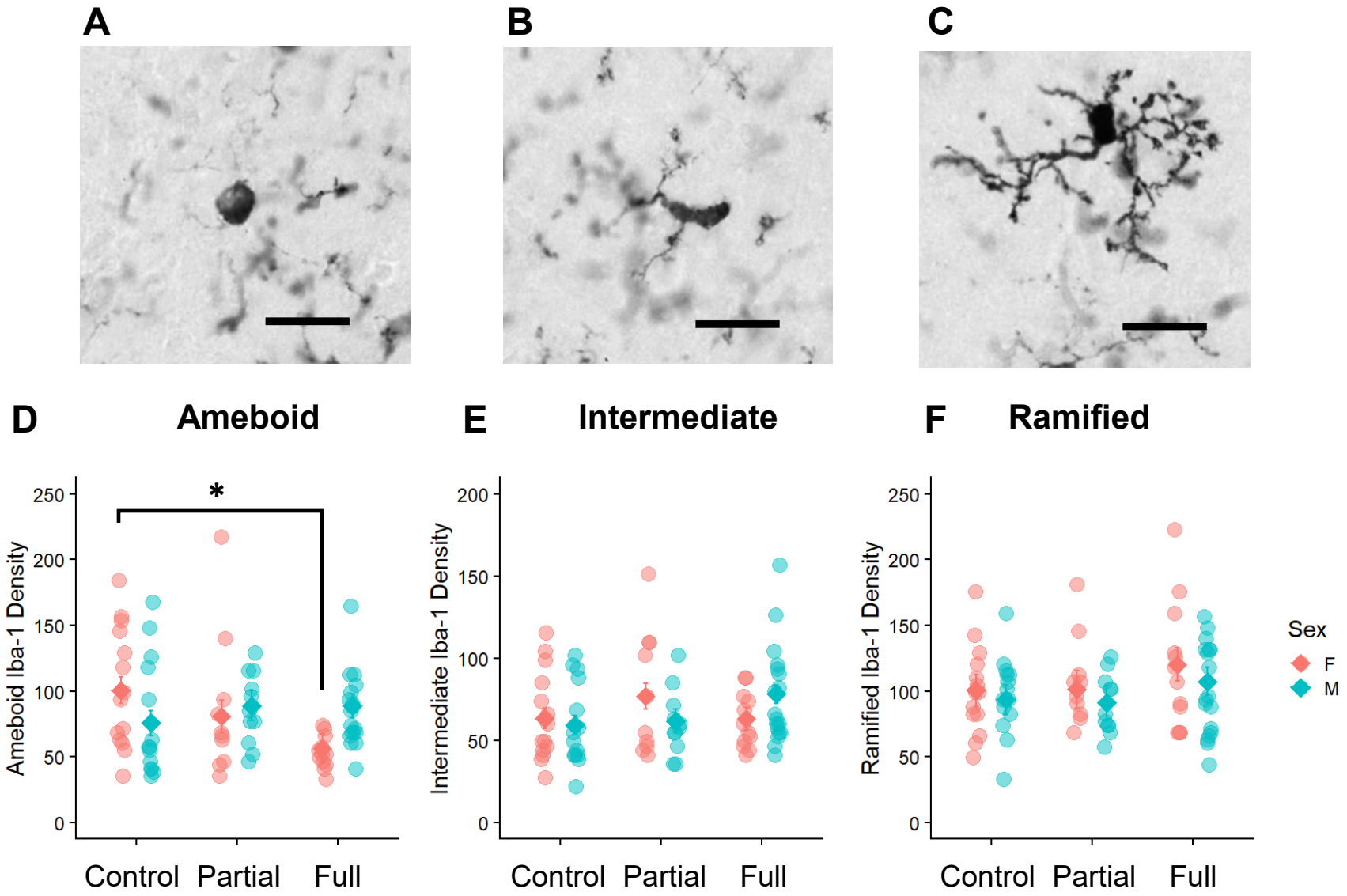
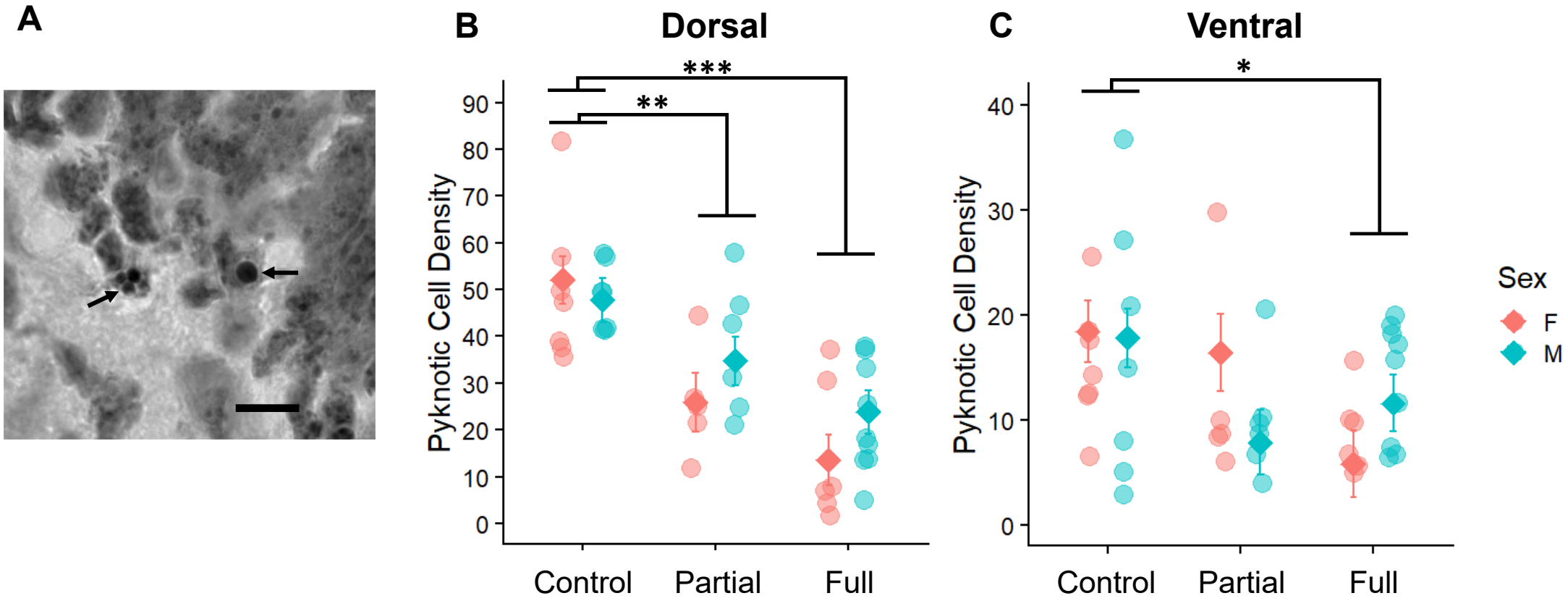

A
B
C
D
Ameboid
E
Intermediate
F
Ramified
*
Ameboid Iba-1 Density
Intermediate Iba-1 Density
Ramified Iba-1 Density
250
200
150
100
50
0
Control
Partial
Full
Sex
F
M

Figure 5


A
B
Dorsal
C
Ventral
***
**
*
Pyknotic Cell Density
90
80
70
60
50
40
30
20
10
0
Control
Partial
Full
Sex
F
M