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

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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 remains unclear. Stress significantly suppresses neurogenesis and increases inflammation and apoptosis in the hippocampus, with effects that vary 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. Full cross-fostered pups, regardless of sex, had more microglia in the ventral dentate gyrus compared to controls. Full cross-fostered females had fewer amoeboid microglia compared to female controls. Together, these findings suggest that cross-fostering affects pup hippocampal development, however, the effects depend on type of cross-fostering, sex, and hippocampal region.

Introduction

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

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

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

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

2024). Accordingly, in the current study, we investigated the effects of cross-fostering either the entire litter (full cross-fostering) or a single pup (partial cross-fostering) on hippocampal development in female and male degus. We measured hippocampal development by assessing dentate gyrus volume, postnatal neurogenesis, apoptosis, and microglia prevalence and morphology. We hypothesized that (1) cross-fostering would impact hippocampal development by reducing neurogenesis, increasing cell death and microglial numbers, and altering the microglia morphological profile in both sexes; and (2) partial cross-fostering would be more detrimental to hippocampal development compared to cross-fostering the entire litter.

Methods

Study animals and breeding

Breeding pairs of captive-born *Octodon degus* and their pups were housed in the animal facility at Swarthmore College, in 51 x 25 x 33 cm acrylic terrariums containing corn cob or paper bedding allowing for visual observations. Animals were maintained under standard laboratory conditions (12:12 light/dark cycle, 23 ± 1 ˚C) with *ad libitum* access to water and food. Alfalfa hay was provided three times per week, and cages were cleaned weekly. Degu females (n = 21) were paired with unrelated males (n = 21) for mating and remained together until pup weaning. All experiments were conducted in strict compliance with Institutional Animal Care and Use Committee at Swarthmore College.

Experimental treatment

Pups were cross-fostered at postnatal day (PND) 8, thus allowing for sufficient time for the development of parental and social attachments within the nest (Colonnello et al., 2011;

Guan et al., 2023). The date of parturition was taken into consideration while assigning the treatment groups as previously described (Arusha et al., 2024): control (pups remained with biological parents and siblings), full cross-fostering (all pups from one litter were exchanged with all pups from another) and partial cross-fostering (a single pup was exchanged with one pup of the same sex from another litter). Pups were fostered between litters of similar sizes.

Tissue collection

Euthanasia was preformed at weaning (~5 weeks of age) using an Euthasol injection**.** Brains were immediately dissected and split into the right and left hemispheres. The right hemispheres were placed on dry ice and frozen at –80˚C for future analysis, whereas the left hemispheres (used in the present study) were immersion-fixed in 4% paraformaldehyde for 24h at 4˚C. Following fixation, brains were then transferred to a 30% sucrose solution containing 0.1% sodium azide, and stored at 4˚C until sectioning. Coronal sections of 30 µm thickness were cut using a cryostat at –20˚C. Hippocampal sections were collected in a seven-tube series containing an antifreeze solution (ethylene glycol, glycerol and 0.1M phosphate buffer saline with a pH of 7.4) and stored at –20˚C. All three neural measurements (volume, neurogenesis, and microglia) were performed in parallel series from the same left hemisphere for all animals.

Cresyl violet staining

Brain sections were stained with Cresyl violet to determine the effects of cross-fostering on dentate gyrus volume and to count pyknotic cells. Hippocampal tissue from one of the series was mounted onto Superfrost microscope slides. Cresyl violet staining was performed after the tissue had completely dried on the slides. The staining process started by dipping the slides into distilled water three times to remove residual salts, then submerged in 0.2% Cresyl violet acetate

solution for 4 minutes. Following staining, the slides were placed in a differentiator solution—comprising 70% ethanol and seven drops of glacial acetic acid—for 30 seconds. The slides were then sequentially dehydrated in 95% ethanol (twice, for 1 minute each), followed by 100% ethanol for 1 minute. Finally, slides were cleared in xylene (twice, for 5 minutes each) and cover-slipped using Permount.

Doublecortin immunohistochemistry

Brain sections were stained with doublecortin (DCX) to assess the effects of cross-fostering on postnatal neurogenesis. All staining procedures were conducted in multi-well plates under gentle shaking. To begin, sections were washed in 0.1M phosphate buffer saline (PBS, pH = 7.4) to remove antifreeze and then incubated in 0.6% hydrogen peroxide in PBS for 30 minutes. Sections were then rinsed with PBS and then incubated for 22h at 4˚C in rabbit anti-DCX primary antibody (1:200 dilution; Cell Signalling, catalogue number: 10124618) diluted in 0.1M PBS containing 3% of normal goat serum (NGS) and 0.4% Triton-X. After incubation, tissue was rinsed in 0.1M PBS, then incubated for 18h at 4˚C in biotinylated goat anti-rabbit secondary antibody (1:500 dilution; Vector laboratories) diluted in 0.1M PBS. This was followed by washes in 0.1M PBS and incubation for 4 hours in an avidin-biotin horseradish peroxidase complex solution (1:1000 dilution; ABC kit, Vector Laboratories). After rinsing in 0.1M PBS, tissue was incubated in diaminobenzidine in the presence of nickel (DAB Peroxidase Substrate Kit, Vector Laboratories) for 3 minutes. Stained sections were mounted on Superfrost microscope slides and allowed to dry for 2–3 days. Slides were then dipped in distilled water three times to remove salts, dehydrated sequentially in 70% ethanol (1 minute), 95% ethanol (1

minute), and 100% ethanol (1 minute), then cleared in xylene (twice, 5 minutes each). Finally, coverslips were applied using Permount.

Iba-1 immunohistochemistry

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

Microscopy, Imaging, and Quantification

All analyses were performed by a researcher blinded to experimental conditions. Brightfield images of the entire left hippocampus stained with Cresyl violet were taken using a

standard light microscope (Olympus BX53) equipped with a camera (ORCA-spark, Hamamatsu) using the 4X objective. The granule cell layer (GCL) and hilus were traced separately using ImageJ to obtain areas in μm^2. Volumes of GCL and hilus were calculated using the Cavalieri's principle: the measured areas were multiplied by the section thickness (30 µm), by 7 (reflecting the 7 sections collect for the hippocampus), and finally by 2 (to present the volume for both hemispheres). The dentate gyrus volume was calculated by adding the volume of the GCL and the hilus. For the pyknotic cell count, two dorsal and two ventral sections of the dentate gyrus were selected for each animal. Ventral sections were defined as those located between interaural-incisor coordinates 2.32 and –0.32 of the degu brain atlas (Kumazawa-Manita et al., 2018). We counted the number of pyknotic cells in the GCL using a 100X oil immersion objective. During counting, the fine focus knob was used to scan through the entire thickness of each section to ensure no cells were missed.

For the analysis of immature neurons, brightfield images were taken from two dorsal and two ventral sections using a 4X objective. Optical density (OD) of DCX labeling was quantified using ImageJ. The GCL was traced in each image, and grey values were obtained. The Rodbard equation was employed to calculate the OD for both the GCL and background region (corpus callosum). For each section, background OD was subtracted from the GCL OD. The average corrected OD for both image densities was used in statistical analysis.

For microglia, brightfield images were taken using an Olympus BX53 microscope with a 20X objective. Two images from the dorsal and two from the ventral dentate gyrus per animal were taken. We used a standardized region of interest (490.4 x 368.5 µm), which included the GCL and hilus regions. We counted the total number of microglia present and categorized them into three morphological types—ameboid, intermediate, and ramified—using ImageJ, as we have

done in the past (Duarte-Guterman et al., 2023). Ramified microglia were defined by a small cell body with at least three processes, each at least twice the length of the cell body diameter. Intermediate microglia were defined by cell bodies with 1–2 processes, each no longer than twice the diameter of the cell body. Ameboid microglia were defined as a dark cell body with very small or no processes. The number of microglia was added to get the total number of microglia per individual and region. A control female brain did not have two ventral section images due to missing tissue sections, therefore, data of only one section was utilized in the analysis.

Statistical Analysis

All statistics were performed in IBM SPSS, Version 28.0.0.0 and the graphs were designed in GraphPad Prism 9. The volumes of the dentate gyrus, hilus, and GCL were analysed using ANOVAs with treatment (control, partial, and full cross-foster) and sex (female, male) as between-subject factors. For OD of DCX, microglia counts, and pyknotic cell density, data were analysed using repeated measures ANOVAs, with treatment and sex as between subject factors and region (dorsal and ventral) as a within-subject factor. For microglia morphology, data were analysed using a repeated measures ANOVA with treatment and sex as between-subject factors and region (dorsal and ventral) and morphology (ramified, intermediate and ameboid) as within-subject factors. Fisher's LSD post-hoc was employed to determine the direction of the effects when ANOVA analysis revealed significant interaction or main effects. For the repeated measures ANOVAs, if the assumption of sphericity was violated, the degrees of freedom were corrected using the Greenhouse-Geisser estimate of sphericity. P-values lower than 0.05 were considered statistically significant.

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 > 0.532) (Figure 1). Similarly, cross-fostering did not affect the volume of the hilus (all p's> 0.126) or the GCL (all p's > 0.444; Table 1). We did not find any sex differences for any of the volumes (dentate gyrus, hilus, or GCL) (all p's> 0.126; 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 ($F(2,34) = 5.547$, $p < 0.01$). Post hoc analysis indicated that partial cross-fostered females had significantly higher DCX OD than full cross-fostered females in the ventral hippocampus ($p<0.01$; Figure 2). However, there were no significant differences between partial cross-fostered females and control females ($p = 0.123$), or between full cross-fostered females and control females ($p = 0.115$) in the ventral hippocampus. There were no differences between males across treatment conditions in DCX OD in the ventral hippocampus (all p's > 0.076). In the dorsal hippocampus, cross-fostering did not affect DCX OD in either females or males (all p's > 0.118).

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 ($p < 0.05$), whereas full cross-fostered females had lower OD compared to males ($p < 0.05$). In controls, no sex differences in DCX OD were found in either the dorsal or ventral hippocampus (all p's > 0.163).

Cross-fostering altered microglia counts and morphology in the dentate gyrus of degu pups

Cross-fostering affected the number of Iba-1-positive cells in the dentate gyrus of degu pups, irrespective of sex (main effect of sex was not significant: $F(1,33) = 0.386$, $p = 0.538$), but this effect was dependent on region (region by treatment interaction: ($F(2,33) = 3.824$, $p < 0.05$; Figure 3). Full cross-fostered pups had a higher number of Iba-1-positive cells in the ventral hippocampus compared to controls ($p < 0.05$). No differences were found between partial cross-fostered pups and control and between partial and full cross-foster pups (all p's > 0.337). In the dorsal hippocampus, there were no significant differences between any of treatment groups (all p's > 0.200).

Cross-fostering also affected the morphology of Iba-1 cells, but in this case, the effect was sex-specific (sex by morphology by treatment interaction: $F(2.798,46.175) = 4.223$, $p = 0.05$; Figure 4). Full cross-fostered female pups had fewer ameboid microglia compared to female controls ($p < 0.01$) and partial cross-fostered female pups ($p < 0.05$). No other differences were observed in the density of ramified, intermediate, or ameboid microglia across cross-fostering conditions in females (all p's > 0.077). In males, full cross-fostered pups had more intermediate microglia compared to controls ($p < 0.05$; Figure 4). No other differences in microglia morphology density were found across cross-fostering conditions in males (all p's > 0.067).

Cross-fostering differentially altered pyknotic cell density in dorsal and ventral hippocampus regions

Cross-fostering affected pyknotic cell density differently in the dorsal and ventral hippocampus (treatment by region interaction: $F(2, 34) = 9.870$, $p < 0.01$, Figure 5). In the dorsal hippocampus, full cross-fostered pups had fewer pyknotic cells compared to controls ($p<0.001$)

and partial cross-fostered pups ($p<0.05$). Partial cross-fostered pups also had fewer pyknotic cells compared to controls in dorsal hippocampus ($p<0.01$). In the ventral hippocampus, no differences were found across treatment conditions (all p's >.111). No sex differences were found in either dorsal or ventral regions ($F(1, 34) = 2.243$, $p = 0.1431$).

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 (both number and morphology) and pyknotic cell density, but not dentate gyrus volume or neurogenesis. The effects of cross-fostering on microglia and pyknotic cell density were dependent on type of cross-fostering, region, and sex. Pups in the full cross-foster condition had a higher number of microglia compared to controls in the ventral but not the dorsal hippocampus. Full cross-fostering was associated with a reduction in ameboid microglia in female pups and an increase in intermediate microglia in male pups. In dorsal hippocampus, full cross-fostered pups had the lowest density of pyknotic cells, followed by partial cross-fostered pups. Overall, this study demonstrates that cross-fostering affects hippocampal microglia and cell in a region- and sex-specific manner in degu pups.

Cross-fostering did not affect dentate gyrus volume in pups

Cross-fostering did not impact the volume of the dentate gyrus in degu pups. Previous work in other rodents such as rats, mice, and California mice has been mixed. Some studies find

reductions in the dentate gyrus volume of pups exposed to early life stressors such as paternal deprivation (Madison et al., 2022), chronic unpredictable stress (Herpfer et al., 2012), and scarcity-adversity model using low bedding (Raineki et al., 2019). However, other studies find no effect after exposure to chronic unpredictable stress (Huot et al., 2002) or maternal separation (Loi et al., 2017). Overall, our findings highlight the importance of considering both the type of stressor and the species when evaluating the effects of early life stressors on hippocampus development. Notably, maternal and paternal licking and grooming were not affected by cross-fostering in degus (partial or full cross-fostering), although cross-fostered mothers spent more time off the nest compared to control and partial cross-foster groups (Bauer et al., unpublished data). It is possible that having a relatively more stable parental care environment (e.g., compared to separation paradigms) may help protect against the effects of cross-fostering on dentate gyrus volume in pups.

Interestingly, we did not find sex differences in dentate gyrus volume in degu pups. This is in contrast with findings in mice, where males have larger hippocampal volume compared to females between PND 10 and PND 17, and again at PND 65 (Qiu et al., 2018). To our knowledge, our study is the first one to examine hippocampal volume in degu pups. Further research is required in both juvenile and adult degus to understand sex differences in hippocampal development.

Cross-fostering does not affect neurogenesis in pups

We examined the effects of cross-fostering on postnatal neurogenesis using DCX OD as a marker of immature neurons. We found that cross-fostering did not affect the DCX OD compared to controls. However, within the ventral hippocampus, DCX OD was decreased in full

cross-fostered females compared to partial cross-fostered females. Previous work has found that maternal separation leads to a reduction in the number of immature neurons at weaning in female, but not male rats (Oomen et al., 2009). In biparental species, paternal deprivation did not affect the survival of new neurons in adult California mice (Madison et al., 2022), however paternal deprivation was associated with a reduction in immature neurons in adult mandarin voles compared to biparentally raised pups (He et al., 2018). Overall, these findings suggest that changes in neurogenesis following parental separation are not always observed in all studies and species. Our observations were limited to a single developmental time point and one neurogenesis marker. It remains possible that cross-fostering may affects survival of new neurons or that these changes are manifested later in adulthood.

Cross-fostering alters pyknotic cell density in pups

The current study revealed noteworthy alterations in pyknotic cell density in the dentate gyrus. We found that full cross-fostered pups, regardless of sex, exhibited fewer pyknotic cells compared to controls. In contrast, previous studies in rats have shown that maternal separation can increase cell death, although the effects depend on timing of exposure. For example, maternal separation increases hippocampal cell death after an acute 24-hour separation at PND12, but not at PND20 (Zhang et al., 2002). Increases in hippocampal cell death have also been reported following chronic maternal separation in rats (Baek et al., 2012). These discrepancies between previous work and the current study may reflect species differences or differences in the type and severity of the early life stressor, as discussed earlier. Apoptosis is a crucial developmental process that contributes to the formation and refinement of neural circuits, and alterations in this process can influence neuron number and brain function (Forger, 2009;

Majcher-Maślanka et al., 2019). Thus, the observed reduction in pyknotic cell density in full cross-fostered pups could indicate a decreased level of apoptosis and insufficient cell pruning, potentially resulting in overcrowding of neurons and altered neural circuitry.

Cross-fostering altered microglia numbers and morphology in pups

We found that cross-fostering altered both the number and morphology of microglia in the hippocampus. Full cross-fostered pups had more microglia in the ventral hippocampus than control pups, irrespective of sex. These findings are consistent with previous work showing that adverse early life environment due to a combination of minimal bedding, frequent cage changes, and a Western diet increase microglial numbers in the mouse hippocampus at weaning (Cohen et al., 2016)(Cohen et al., 2016). Similarly, maternal separation in rats (Banqueri et al., 2019) and paternal deprivation in California mice (Madison et al., 2022) have also been associated with increased microglia in the offspring hippocampus. Microglia play a crucial role in the central nervous system, contributing to synaptic remodeling through pruning, as well as supporting neuronal survival via the release of neurotrophic factors (Cornell et al., 2022; Sierra et al., 2014). In our study, the increase in in microglial numbers was driven by males and was restricted to the ventral hippocampus, which is known for its role in stress and anxiety regulation (Bannerman et al., 2004; Fanselow and Dong, 2010; Kheirbek and Hen, 2011). Overall, this finding suggests that cross-fostering may influence neural circuits involved in stress regulation and emotional processing. Further research is needed to determine whether cross-fostering-related increases in microglial number during development translate into changes in mood and behaviour in degus.

In addition to the changes in microglia numbers, cross-fostering also affected the morphology of microglia in a sex-dependent manner. Full cross-fostered female pups had fewer ameboid microglia compared to control females, whereas full cross-fostering male pups exhibited an increase in intermediate-type microglia. Ameboid microglia are typically associated with phagocytosis, synaptic pruning, and the secretion of pro-inflammatory cytokines and are characterized by a rounded morphology lacking the long processes often observed in ramified microglia (Burke et al., 2016; Colonna and Butovsky, 2017; Franco and Fernández-Suárez, 2015; Paolicelli et al., 2022). The current literature agrees that microglia vary from ameboid to ramified states, but each morphology does not have exclusive functions. The ability to transition between different stages allows microglia to maintain a balanced immune response (Paolicelli et al., 2022). One possible interpretation of our results is that the reduction in ameboid microglia in full cross-fostered female pups may reflect lower levels of phagocytosis, potentially contributing to the reduced pyknotic cell density observed, however this effect was localized to the dorsal hippocampus. Overall, while cross-fostering clearly affected microglial number and morphology in the hippocampus, whether these changes directly lead to apoptosis remain to be elucidated.

Brain outcomes depend on type of cross-fostering

Our results indicate that full cross-fostered pups showed greater effects on microglia, cell death, and to some extent, neurogenesis compared to partial cross-fostered pups. These findings were contrary to our hypothesis and previous work demonstrating that presence of a familiar sibling (when the entire litter is transferred) reduces the effect of cross-fostering on circulating cortisol levels and weight gain, compared to pups that were cross-fostered on their own (Arusha et al., 2024). In adult rats, the effects of corticosterone on hippocampal neurogenesis vary

depending on the dose, with studies showing increases, decreases, or no effect (reviewed in (Duarte-Guterman et al., 2017). It is possible that a lower-stress condition (such as full cross-fostering based on cortisol levels and weight gain; Arusha et al., 2024) may paradoxically produce greater effects on the hippocampal development compared to a moderate stressor (such as partial cross-fostering). Further work is needed to investigate the impact of stress hormones on hippocampal development in degus.

Conclusion

In conclusion, cross-fostering did not affect volume or postnatal neurogenesis in the hippocampus. However, full cross-fostering affected microglial number and morphology, as well as pyknotic cells density, in a region- and sex-specific manner. This study advances our understanding of how early-life environmental factors impact brain development. More importantly, it highlights the importance of including both sexes and other forms of early-life stressors, such as cross-fostering paradigms. Further work is needed to investigate the long-term effects of cross-fostering on brain function and potential behavioural consequences.

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Table 1. Effects of cross-fostering on the volume (mm^3) of the dentate gyrus, hilus, and granule cell layer GCL in female and male degu pups. Data are presented as mean ± SD.

	Dentate Gyrus		**Hilus**		**GCL**	
	Females	Males	Females	Males	Females	Males
Control	6.28 ±1.01	6.19±0.99	3.29±0.62	3.44±0.75	2.99±0.71	2.75±0.33
Partial cross-fostering	5.98±1.27	6.42±1.12	3.38±0.74	3.68±0.82	2.60±0.59	2.74±0.35
Full cross-fostering	6.15±0.95	6.92±0.84	3.38±0.62	4.00±0.70	2.77±0.37	2.92±0.19

FIGURE CAPTIONS

Figure 1. Representative image of the dorsal (A) and ventral (B) hippocampus in degu pups. The granule cell layer (GCL) and hilus are marked. Dentate gyrus volume (C) was not affected by partial or full cross-fostering in male and female pups.

Figure 2. A) Representative image of DCX expression in hippocampus of degu pups. B) DCX optical density in the dorsal (B) and ventral (C) hippocampus of female and male degu. Cross-fostering did not affect DCX optical density with respect to controls, but full cross-fostered pups had lower DCX optical density compared to partial cross-fostered pups in the ventral hippocampus. Mean is shown as line. * $p<0.05$, ** $p < 0.01$.

Figure 3. (A) A depiction of Iba-1 expression within the region of interest in the dentate gyrus the dorsal hippocampus. Total number of microglia in the dorsal (B) and ventral (C) hippocampus. In the dorsal hippocampus, there were no significant differences found across cross-fostering groups. In the ventral hippocampus, full cross-fostered pups had a higher number of microglia compared to control ($p < 0.05$). Mean is shown as line. *** $p < 0.001$, *$p < 0.05$

Figure 4. Representative images of microglia morphology showing (A) ameboid, (B) intermediate, and (C) ramified microglia. (D) Full cross-fostered female pups had a lower ameboid microglia density than control ($p < 0.01$) and partial cross-foster females ($p<0.05$). (E) Full cross-fostered male pups had a higher intermediate microglia density than male control group ($p < 0.05$). Mean is shown as line, *$p < 0.05$, ** $p < 0.01$.

Figure 5. (A) Representative image of pyknotic cells (arrows) in the granule cell layer in degu pups (scale bar = 10 µm). Pyknotic cell density was affected by cross-fostering in the dorsal (B) but not ventral hippocampus (C). In the dorsal, full cross-fostered pups had the lowest density of pyknotic cells, followed by partial cross-fostered pups when compared to controls. No differences in pyknotic cell density were found across cross-fostering groups in the ventral region. Mean is shown as line. ** $p < 0.01$, *** $p < 0.001$.

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Figure 1

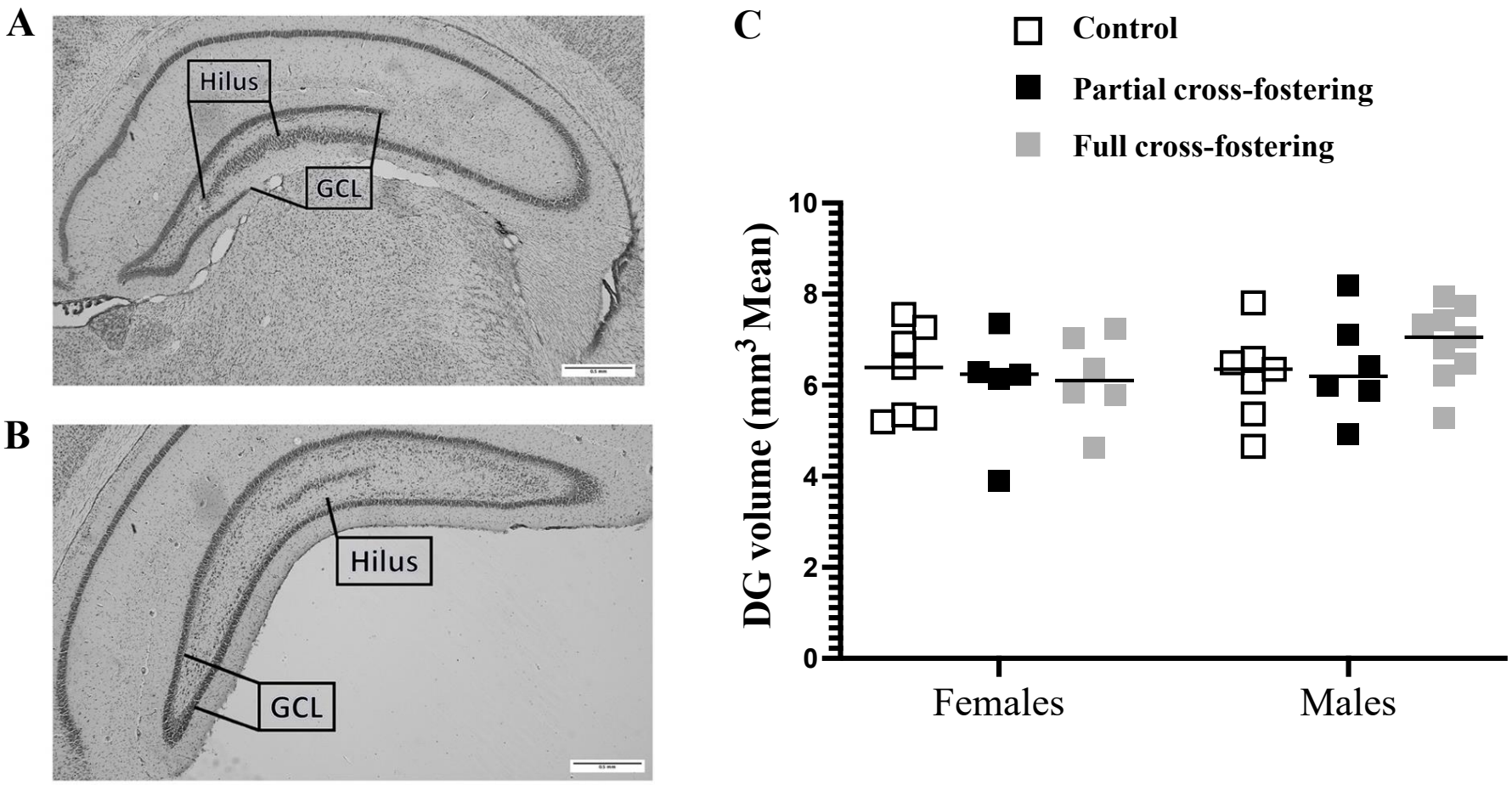

A
Hilus
GCL
B
Hilus
GCL
C
Control
Partial cross-fostering
Full cross-fostering
DG volume (mm3 Mean)
10
8
6
4
2
0
Females
Males

Figure 2

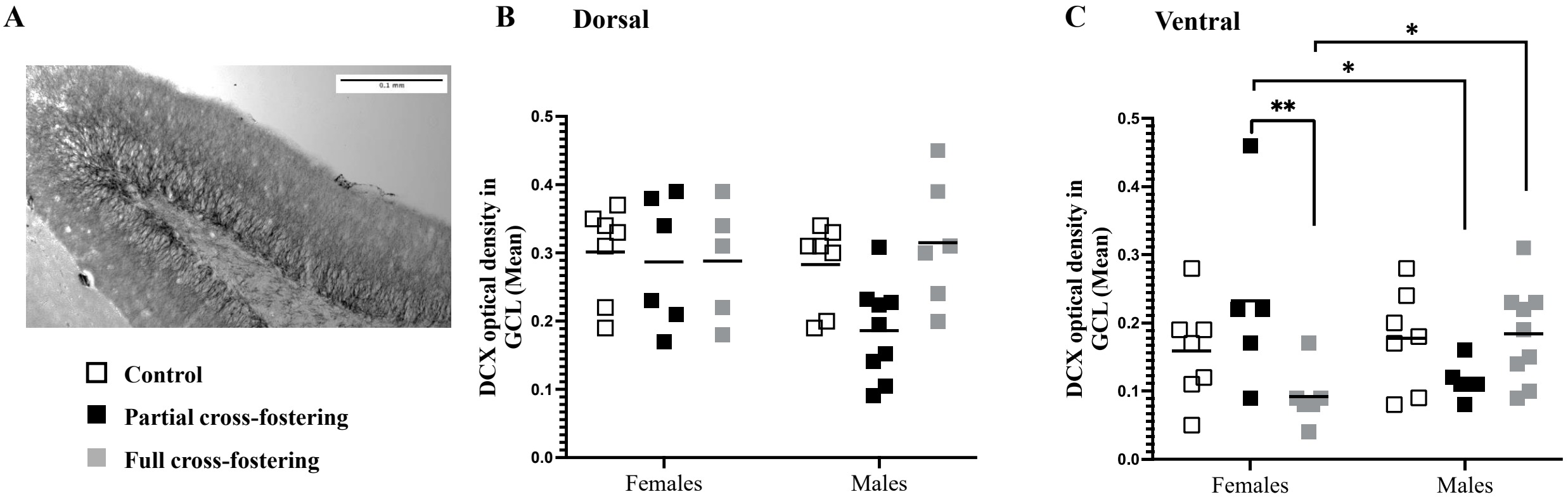

A
0.1 mm
Control
Partial cross-fostering
Full cross-fostering
B
Dorsal
DCX optical density in GCL (Mean)
0.5
0.4
0.3
0.2
0.1
0.0
Females
Males
C
Ventral
*
*
**
DCX optical density in GCL (Mean)
0.5
0.4
0.3
0.2
0.1
0.0
Females
Males

Figure 3

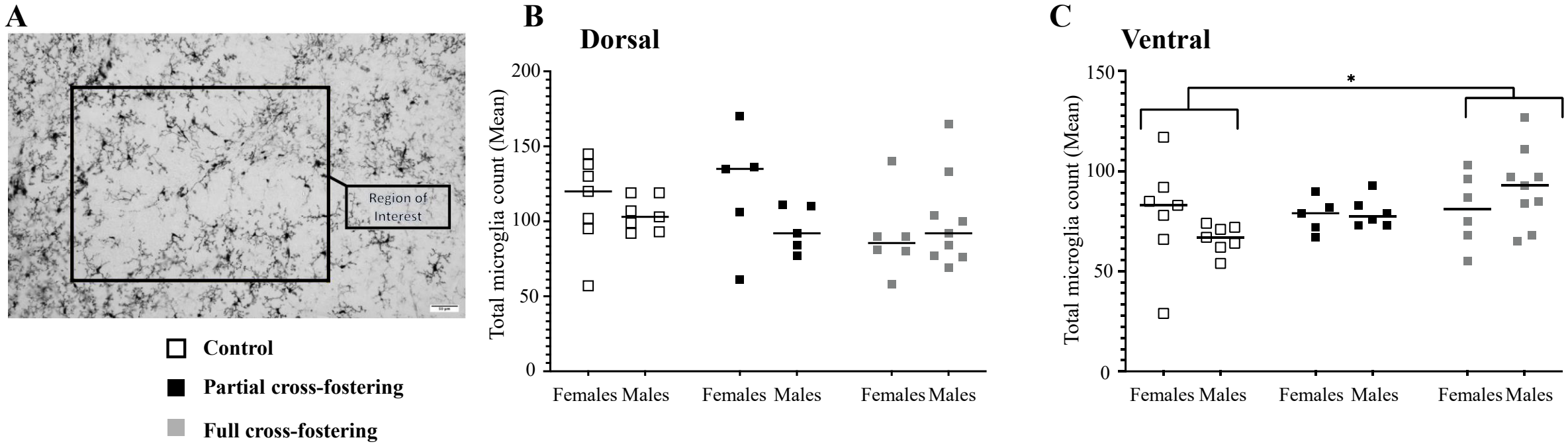
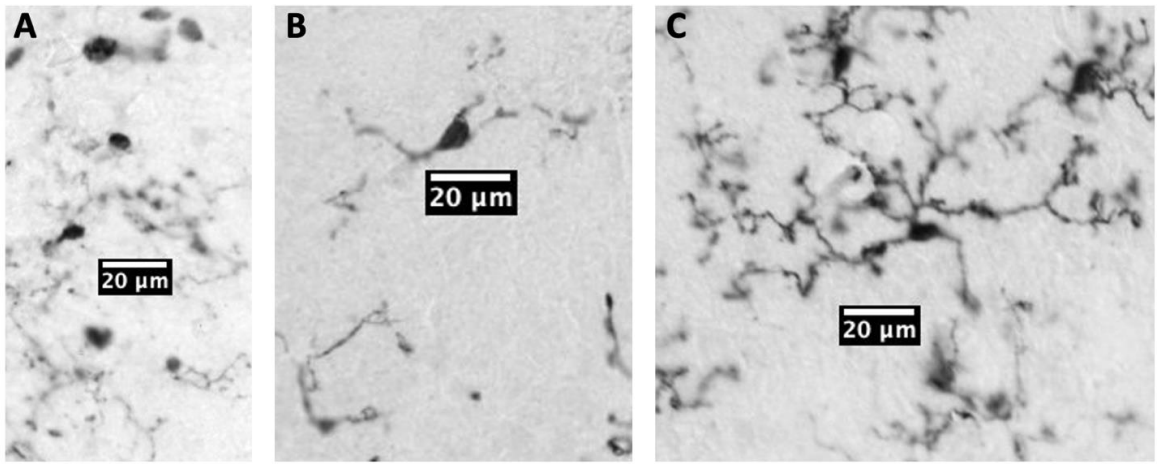

A
Region of Interest
Control
Partial cross-fostering
Full cross-fostering
B
Dorsal
Total microglia count (Mean)
200
150
100
50
0
Females Males
Females Males
Females Males
C
Ventral
Total microglia count (Mean)
150
100
50
0
*
Females Males
Females Males
Females Males

Figure 4


A
20 µm
B
20 µm
C
20 µm

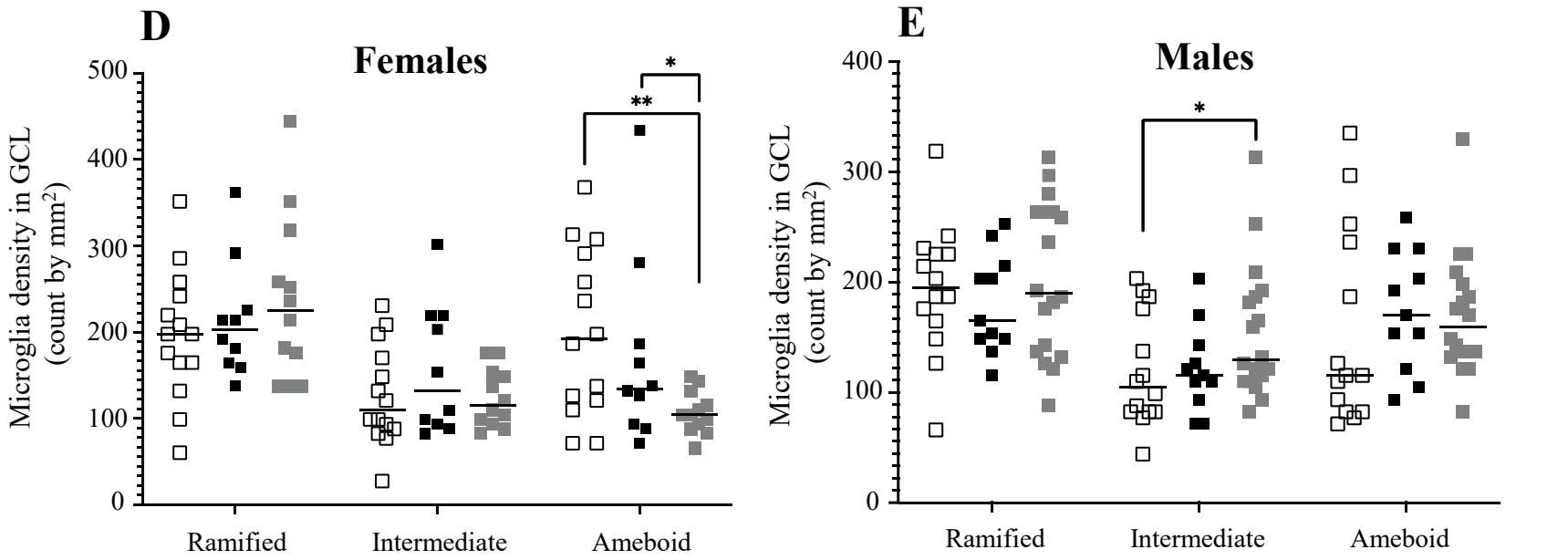
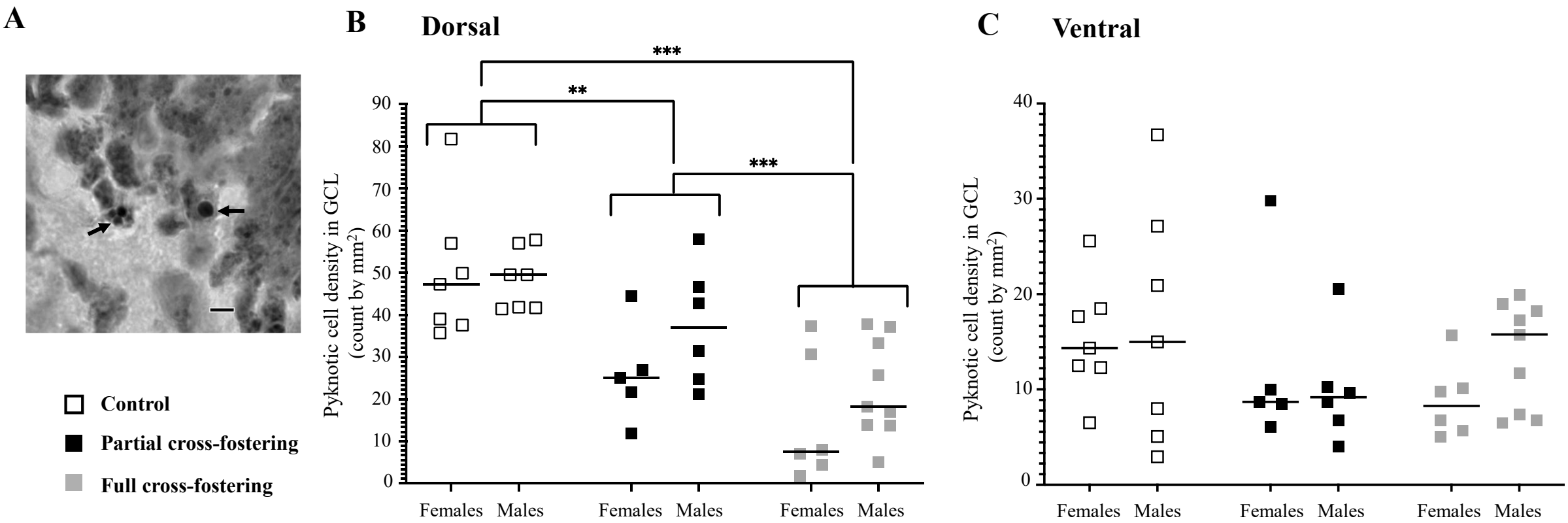

Control
Partial cross-fostering
Full cross-fostering
D
Females
Microglia density in GCL (count by mm²)
500
400
300
200
100
0
*
**
Ramified
Intermediate
Ameboid
E
Males
Microglia density in GCL (count by mm²)
400
300
200
100
0
*
Ramified
Intermediate
Ameboid

Figure 5


A
B
Dorsal
C
Ventral
Control
Partial cross-fostering
Full cross-fostering
Pyknotic cell density in GCL (count by mm²)
***
**
***
Females
Males
Females
Males
Females
Males
Pyknotic cell density in GCL (count by mm²)
Females
Males
Females
Males
Females
Males