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The Role of Microglia During the Sexual Differentiation of the Brain in Quail *(Coturnix Japonica)*

GIGA Neuroscience

Laboratory of Behavioral Neuroendocrinology

Julia Bous

Thesis presented to obtain the master's degree in biomedical sciences

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Summary

Brain sexual differentiation is a process that occurs during a period of sensitivity in development and is influenced by steroid hormones. Sex differences in brain and behavior appear due to the organizational and activational effects of steroid hormones. In rodents, estrogens are the primary driver of sexual differentiation: estrogens masculinize and defeminize the rodent brain and behavior. The microglia, the brain´s innate immune cell, seems to be involved in sexual differentiation. In rodents, more microglia are in the male preoptic area (POA), an important brain region in male sexual behavior, during the critical period of hormones. Estrogen treatment of females increased microglial counts to those of males. By inhibiting microglia, the masculinizing actions of estrogens on the rodent brain and behavior can be prevented. In the medial preoptic nucleus of female rats, more microglia actively engulf newborn cells. Thus, the role of microglia in brain sexual differentiation may be mediated through their phagocytic activity during the period of sensitivity to hormones. The Japanese quail is an interesting animal model to investigate since their sexual differentiation also depends on estrogens. In contrast to rodents, estrogens are responsible for the demasculinization of the brain and behavior in quail during the critical period of hormones. Opposed to what is observed in rodents, female quail exhibit more microglia in the medial preoptic nucleus during the critical period. This sex difference depends on estrogen availability. Interestingly, in quail, females remain sensitive to the demasculinizing effect of estrogens until two to four weeks of postnatal life. Microglia could, therefore, also influence brain development during the postnatal weeks.

The present research aimed to understand the role of microglia in brain sexual differentiation in the quail. The first experiment studied the number of phagocytic microglia on embryonic days 10 (E10) and 12 (E12). To visualize phagocytic microglia, a new method was tested in which eggs were injected with fluorescent liposomes. The brain tissue was then stained with a lectin specific to macrophages to visualize the total number of microglia in the POA. In a second experiment, the sex difference in the number of microglia on E12 and the first day of postnatal life (PN1) was investigated, as previous experiments reported a marked sex difference on E12, and we suspected that microglia might also play a role during the demasculinization process after hatching on PN1. To this end, quail embryos were treated with estradiol on E7 to confirm the results obtained by Delage & Cornil (2020) on E12 with a different microglial marker and to investigate a possible sex difference in the microglial count on the first day after birth. To visualize the total microglia, brain slices were stained with the same lectin as before.

No results concerning the phagocytic activity of microglia on E10 and E12 could be analyzed due to technical difficulties. Furthermore, there were no significant sex differences in the number of microglia, neither on E12 nor PN1. These differences were also not hormone dependent.

Résumé

La différenciation sexuelle du cerveau a lieu au cours d´une période de sensibilité aux hormones stéroïdiennes sexuelles. Au cours de cette période, des différences sexuelles s´établissent à la suite des effets organisationnels des hormones. Chez les rongeurs, de même que chez les oiseaux, les œstrogènes jouent un rôle clé dans ce processus, mais leur effet est opposé : chez les rongeurs, les œstrogènes sont responsables de la masculinisation et déféminisation du cerveau ainsi que du comportement, alors que chez les oiseaux, les œstrogènes démasculinisent. La microglie, une cellule immunitaire innée du cerveau, semble être impliquée dans ce processus. Chez les rongeurs, les mâles présentent plus de microglies dans l´aire préoptique, une région du cerveau impliquée dans le comportement sexuel mâle, lors de la période sensible aux hormones. Le traitement aux œstrogènes des femelles augmente le nombre de microglies qui atteignent des nombres similaires aux mâles. L´inhibition de la microglie prévient l'action masculinisante des œstrogènes sur le cerveau et le comportement. Chez le rat, dans le noyau préoptique median, les microglies peuvent phagocyter des cellules nouveau-nées et ceci est plus marqué chez la femelle. De ce fait, la microglie peut être impliquée dans la différenciation sexuelle par son activité phagocytique. Contrairement aux rongeurs, chez les cailles c´est la femelle qui présente plus de microglie dans le noyau préoptique médian lors de la période sensible et ceci est dépendant de la disponibilité en estrogènes. Ce qui fait que la caille japonaise offre un modèle pour étudier le rôle de la microglie lors la différenciation sexuelle du cerveau, puisque comme chez les rongeurs la différentiation sexuelle est médiée par les estrogènes. Curieusement, la démasculinisation du cerveau et du comportement de la caille femelle n´est pas limité à la période sensible aux hormones mais a également lieu les deux à quatre premières semaines de la vie postnatale.

La présente recherche visait à comprendre le rôle des cellules microgliales dans la différenciation sexuelle du cerveau chez la caille. Dans un premier temps, le nombre de microglie présentant une activité phagocytique à E10 et E12 a été étudié. Pour mettre en évidence l´activité phagocytique, des embryons de caille ont été traités avec des liposomes fluorescents. En plus, pour avoir une idée générale du nombre de microglies dans la POA, le tissu a également été traités avec une lectine qui marque la microglie totale. Dans un deuxième temps, les différences sexuelles du nombre de la microglie dans le POA à E12 et au premier jour postnatal (PN1) a été investiguée. Pour ce faire, les embryons de caille ont été traités avec de l´estradiol afin de confirmer les résultats de Delage & Cornil (2020) à E12 avec un autre marqueur de microglie et, d´investiguer le rôle de la microglie lors la démasculinisation de la femelle à PN1. Le tissu a également été traité avec une lectine afin de visualiser la microglie dans la POA.

Malheureusement, la première expérience n´a pas donné des résultats à cause des problèmes techniques rencontrés lors de la mise en évidence. Les résultats ont montré aussi qu´il n´y ait pas une différence significative dans le nombre de microglie et leur morphologie entre les mâles et les femelles, à E12, ni à PN1.

List of Abbreviations

Abbreviation	Definition
AMH	Anti-Müllerian hormone
AR	Androgen receptor
CA	Anterior commissure
CNS	Central nervous system
COX-2	Cyclooxygenase 2
CSF1	Colony stimulating factor
DHT	5α-dihydrotestosterone
DMRT1	Doublesex and mab-3-related transcription factor 1
E2	Estradiol
E6/7/8/9/10/11/12/18	Embryonic day six/seven/eight/nine/ten/eleven/twelve/eighteen
EB	Estradiol-benzoate
ER	Estrogen receptor
IGF-1	Insulin-like growth factor-1
IL-1β/IL-6/IL-34	Interleukin 1β/interleukin 6/interleukin 34
MPNc	Central part of the medial preoptic nucleus in rodents
PGE2	Prostaglandin E2
PN0/1/10	Postnatal day zero/one/ten
POA	Preoptic area
POM	Medial preoptic nucleus in quail
PR	Progesterone receptor
RCA-1	Ricinus communis agglutin 1
SDN-POA	Sexually dimorphic nucleus of the preoptic area
SRY	Sex-determining region of the Y chromosome
TDF	Testis determining factor
TNF-ɑ	Tumor necrosis factor alpha
TP	Testosterone propionate
VIII	Third ventricle

Table of Contents

Introduction

1. Context

In 1959, Phoenix and colleagues suggested that the sexual differentiation of the brain and behavior is influenced by exposure to steroid hormones during prenatal development. The idea was that exposure to steroid hormones leads to organizational changes during early development, and exposure later in life leads to activational changes (Phoenix et al., 1959). This is known as the "organizational or activational hypothesis" (VanRyzin et al., 2020). Sexual differentiation occurs secondarily to sex determination. During this process, the fate of the bipotential gonads to become either testes or ovaries is determined by the genetic sex. In fact, in humans and many mammals, the SRY gene on the Y chromosome is responsible for initiating testicular formation. The steroid production of the newly formed gonads directs and maintains sexual differentiation. Individuals then develop the characteristics associated with being male or female. In the rodent brain, estrogens are the main driver for the appearance of male traits, while females develop as a "default" without exposure to hormones. This phenomenon occurs within a specific period during development, the period of hormone sensitivity. Sex differences appear due to the organizational effects of steroid hormones, which are apparent in morphological traits, physiology, behavior, disease susceptibility, and much more. Physiological differences include that females are subject to hormone fluctuations during the menstrual cycle, pregnancy, and lactation, while men are exposed to relatively stable levels. Behavior can also be dimorphic. In rats, pup retrieval is more often observed in females. Juvenile rough-and-tumble play is more intense and more frequent in males across many species (McCarthy et al., 2017). Significantly, disorders can affect sexual development. For instance, girls with classic congenital adrenal hyperplasia are exposed to elevated testosterone levels during prenatal development as a consequence of a deficiency, usually in 21-hydroxylase, an enzyme in the pathway of steroid hormone synthesis (Hines, 2015; Witchel, 2017). These girls show more masculine play behavior. Comparably, XY individuals with complete androgen insensitivity syndrome have functioning testes but cannot respond to testosterone due to a mutation in the gene coding for the androgen receptor. These individuals show female-typical play behavior (Hines, 2015). There are sex differences in disease susceptibility; for example, most anorexia nervosa and bulimia patients (95%) are women (Zandian et al., 2007). Parkinson's disease is more prevalent among men (Strickland & Bertoni, 2004), whereas more women are diagnosed with multiple sclerosis (Schwendimann & Alekseeva, 2007). Concerning neurodevelopmental disorders, males are more susceptible to autism spectrum disorder (Christensen et al., 2016) and attention deficit hyperactivity disorder (Wang et al., 2017). The brain can also be seen as a "bipotential organ" by expressing either a male or female phenotype. Sex differences in neural structure, glial structure, and connectivity are observed. In canaries and zebra finches, the volume of several nuclei controlling singing is different between the two sexes, with higher volumes detected in males (Nottebohm & Arnold, 1976) which relates to the ability of males to sing. The region called SDN-POA,

for the sexually dimorphic nucleus of the POA, is eight times bigger in males (Gorski et al., 1978). These males show a higher neuronal density and size (Gorski et al., 1980).

The microglia, the brain's innate immune cell, is also reported to be sexually dimorphic. In the POA of neonate male rats, microglia are more abundant and have a more amoeboid shape because of the differential hormonal exposure (Lenz et al., 2013). One of the properties of microglia is their phagocytic activity. In the central part of the rats' medial preoptic nucleus, a region within the POA, microglia engulf newborn cells. By doing so, microglia can influence the preference for male odors and may participate as a regulator of sexual partner preference (Pickett et al., 2023). In rodents, another critical role of microglia is their contribution to neuroinflammation. Perinatal estrogens upregulate prostaglandin E2 (PGE2) production by microglia in the POA of rats, contributing to the establishment of a higher density of dendritic spine synapses in neurons of males (Amateau & McCarthy, 2004; Lenz et al., 2013). Birds are an interesting animal model to investigate because their sexual differentiation also depends on the organizational actions of estrogens, but their critical period to hormones occurs *in ovo* between embryonic days six (E6) and 12 (E12). Contrary to the observation in rodents, estrogens are responsible for the appearance of female traits in quail (Adkins, 1979; Balthazart et al., 1992). Female quail show more microglia in the medial preoptic nucleus (POM) than males (Delage & Cornil, 2020). Also, PGE2 does not seem to play a role in brain sexual differentiation in quail (Delage et al., 2021). Interestingly, the organizational actions of steroid hormones in quail are not restricted to prenatal development (Hutchison, 1978). It was proposed that females are pre-sensitized to the organizing action of estrogens during embryonic development and that complete female development requires exposure to estrogens during the first weeks after hatching (Balthazart & Schumacher, 1984; Schumacher & Balthazart, 1984).

Because a sex difference in the number of microglia can already be observed during prenatal development, and complete female development continues after hatching, we wondered whether a sex difference in the number of microglia in the POM could also be observed on the first day of hatching. Furthermore, inflammatory processes such as PGE2 production might not be implicated in brain sexual differentiation in quail, but new evidence suggests a role for microglia in phagocytosing newborn cells. Therefore, these assumptions raise the question if microglia in the quail POM are engaged in phagocytosis driving brain sexual differentiation during the period of sensitivity and if this phenomenon is sexually dimorphic.

2. Steroid Hormones

Steroid hormones are small hydrophobic and lipid-soluble cholesterol derivates produced and released by endocrine glands. Unbound steroids can cross the blood-brain barrier by unsaturable, transmembrane diffusion (Hampl et al., 2015). Steroid hormones have a characteristic chemical structure, including three six-carbon rings and one five-carbon ring (see Figure 1). In the nomenclature of steroid biochemistry, molecules are identified by the number of carbon atoms (from 1 to 21) in their chemical structure (Nelson & Kriegsfeld, 2017). There are five groups of steroid hormones: glucocorticoids, mineralocorticoids, estrogens, progestins, and androgens. The last three are known as sex hormones and are synthesized in both females and males but in different concentrations (Constanti et al., 2005). In mammals, the gonads primarily produce sex steroid hormones (Nelson & Kriegsfeld, 2017).

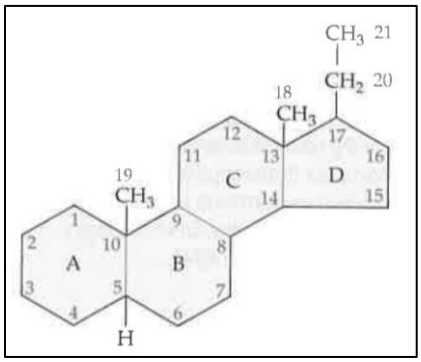

Figure 1: The chemical structure of steroids. All steroids are characterized by three six-carbon rings (A, B, C) and one conjugated five-carbon ring (D). In the nomenclature of steroid biochemistry, each carbon is referred to as a number between 1 and 21 (Picture from Nelson & Kriegsfeld, 2017).

As shown in Figure 2, the synthesis of sex-steroid hormones is a complex process catalyzed by multiple enzymes starting with cholesterol as the precursor. Cholesterol is first converted to pregnenolone, a progestogen, by a cytochrome P450 enzyme. Most of the enzymes in the steroid pathway are either cytochrome P450 enzymes or hydroxysteroid dehydrogenase enzymes (Miller & Auchus, 2011). Androgens are formed from progestogens by enzymatic cleavage of an ethyl group at carbon 17 (Nelson & Kriegsfeld, 2017). At a later stage, androgens are converted into estrogens due to aromatization by the cytochrome P450 enzyme aromatase (Cole et al., 2019). During this process, the carbon at position 19 is cleaved, leaving the estrogen with a phenolic ring, a known aromatic compound (Nelson & Kriegsfeld, 2017).

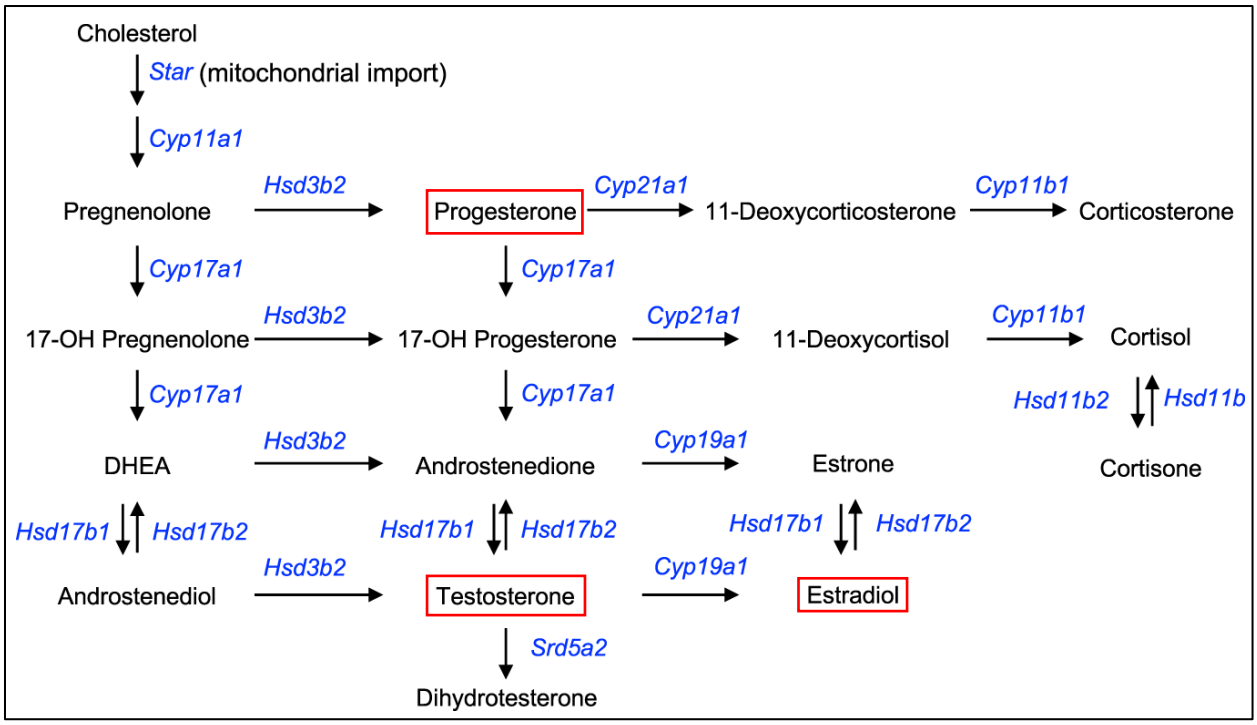

Figure 2: Overview of the steroid biosynthesis pathway. Cholesterol is converted into steroids by cytochrome P450 or hydroxysteroid dehydrogenase enzymes (respectively Cyp or Hsd, represented in blue). Sex hormones include testosterone, estradiol, and progesterone (red square) (Picture from Chakraborty et al., 2021).

2.1 Progestogens

Progestogens, such as pregnenolone, are the precursors to all other steroid hormones. Pregnenolone is a prohormone that can act as a hormone or be converted into another hormone. Progesterone, another prohormone, is the main progestogen secreted by the ovary (Nelson & Kriegsfeld, 2017) and is partly responsible for the changes in the uterus in preparation for pregnancy and lactation. During pregnancy, progesterone reduces contractions of the myometrium (Constanti et al., 2005).

2.2 Androgens

Testosterone is produced and secreted by the Leydig cells into circulation. The ovaries produce small amounts of testosterone (Constanti et al., 2005). The adrenal glands contribute to androgen synthesis by producing the precursor dehydroepiandrosterone and androstenedione (Dutt et al., 2022). Other androgens are, for example, 5α-dihydrotestosterone or androsterone (Constanti et al., 2005; Nelson & Kriegsfeld, 2017).

In the human fetus, testosterone is secreted by the developing testes during a critical period (8–24 weeks of gestation) (Nagamani et al., 1979; Reyes et al., 1974). Testosterone production leads to the differentiation of the male genitalia. During puberty, testosterone induces the growth of the male sex organs, contributes to skeletal growth, deepening of the voice, and growth of facial and body hair, as well as anabolic properties, e.g., increases the synthesis of proteins, leading to a higher muscle mass and strength. In adults, it maintains masculine traits such as libido and sexual potency

(Constanti et al., 2005). It is important to note that some of the effects of testosterone are mediated through its conversion via aromatization to estrogens (Boon et al., 2010).

2.3 Estrogens

The primary steroid secreted by the ovary is 17β-estradiol (E2). Other estrogens produced by the ovary are estrone and estriol. Estrogens are also secreted by the corpus luteum, the placenta, and in small amounts by the adrenal cortex (Constanti et al., 2005). Another estrogen is estradiol benzoate (EB), a synthetic form of E2. EB is a prodrug of E2, meaning that once it is metabolized into its active form, it has the same downstream effects within the body through binding to estrogen receptors (PubChem, n.d.).

During puberty, estrogens stimulate the growth of the uterus, breasts, and vagina. They also control fat deposition and distribution. Like testosterone in males, estrogens are the primary cause of skeletal growth in females. In the adult, estrogens play an important role during the menstrual cycle, pregnancy, and lactation and contribute to maintaining the sexual drive (Constanti et al., 2005). Estrogens affect water metabolism by favoring the retention of water. Moreover, estrogens influence calcium metabolism. More bone is formed in the presence of estrogens (Nelson & Kriegsfeld, 2017). Estrogens are also crucial in the central nervous system (CNS) by exhibiting neurotrophic properties and neuroprotection, as well as promoting synaptogenesis and differentiation starting during fetal development through adulthood (Boon et al., 2010).

2.4 The Mechanism of Action of Steroid Hormones

When they arrive in the target tissue, sex steroid hormones exert their function by mainly two modes of action: the genomic mode (or nuclear-initiated) and the non-genomic mode (membrane-initiated). The genomic mode can be described as slow compared to the non-genomic mode, whose effects on the tissue are rapid, from seconds to minutes (Figure 3) (Pillerová et al., 2021).

In the nuclear-initiated pathway, to exert their physiological effects, steroid hormones bind and activate specific intracellular proteins termed type one nuclear receptors, e.g., progesterone receptor (PR), androgen receptor (AR), and estrogen receptors (ER). It is important to note that estrogens can act through two isoforms of the estrogen receptor: ER-α and ER-β, encoded by the genes ESR1 and ESR2, respectively. Likewise, there exist two isoforms of the progesterone receptor (Saha et al., 2021). In the absence of a steroid ligand, nuclear receptors remain in an inactive complex associated with heat shock proteins. When the ligand diffuses across the cell membrane and binds to the nuclear receptor, the receptor translocates to the nucleus and homodimerizes. The homodimer then binds to specific palindromic sites called hormone response elements. These sites are located within the promotor regions of genes which eventually leads to the repression or activation of gene expression (Cole et al., 2019; Klinge, 2018; Saha et al., 2021). Although most receptors are localized in the

nucleus, around 5% are situated at the plasma membrane exerting a membrane-initiated pathway. This mode of action is responsible for extra-nuclear signaling (Saha et al., 2021). In this case, ER can activate intracellular cascades, including kinases such as MAP kinase or PI3 kinase. This pathway also includes mobilizing intracellular calcium and activating adenylate cyclase and cAMP production (Björnström & Sjöberg, 2005). The membrane-initiated pathway regulates different cellular responses, such as DNA synthesis, cell proliferation, migration, or survival (Pillerová et al., 2021).

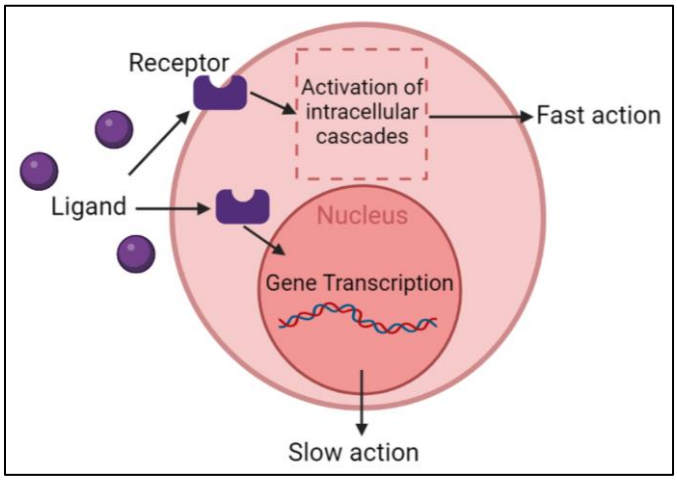

Figure 3: Simplified representation of the genomic and non-genomic signaling pathway. This picture was created with BioRender.

3. Classical Theory of Brain and Behavioral Sexual Differentiation

Sexual differentiation describes the process of becoming either male or female. This process starts at fertilization when an ovum (in mammals, usually bearing an X chromosome) is fertilized by a sperm (usually Y or X chromosome). This event is called sex determination. Homogametic mammals (XX) are females, while heterogametic mammals (XY) are males. Early in embryonic development, the primordial gonads that differentiate later into either testes or ovaries are bipotential (Nelson & Kriegsfeld, 2017). In mammals, whether the embryo will develop testes or ovaries is determined by the expression of the gene SRY (Sex-determining Region of the Y chromosome) (Nagamine, 1994; Nelson & Kriegsfeld, 2017). SRY encodes a protein called “testis determining factor” (TDF). TDF is a transcription factor that dictates the differentiation of the bipotential gonads into testes, thus, resulting in a phenotypically male fetus. TDF takes part in the development of Sertoli cells which then produce the anti-Müllerian hormone (AMH). AMH induces the regression of the Müllerian ducts and the differentiation of Leydig cells. Subsequently, Leydig cells start producing testosterone. Testosterone then facilitates the development of the Wolffian ducts and the final formation of internal and external genitalia. Females develop as “default” without the presence of TDF, AMH, or testosterone. In this context, the Wolffian ducts regress while the Müllerian ducts differentiate into structures, including oviducts, internal vagina, cervix, primordial oocytes, and vulva (Evans & Ganjam, 2017). As represented in Figure 4, because of the bipotential system, to develop as a male, defeminization (re-

pression of the female-typical phenotype, e.g., Müllerian duct regression) and masculinization (development of the male-typical phenotype, e.g., Wolffian duct development) is required. Likewise, females need to feminize (development of the female-typical phenotype) and demasculinize (repression of the male-typical phenotype) (Balthazart et al., 2009; Nelson & Kriegsfeld, 2017).

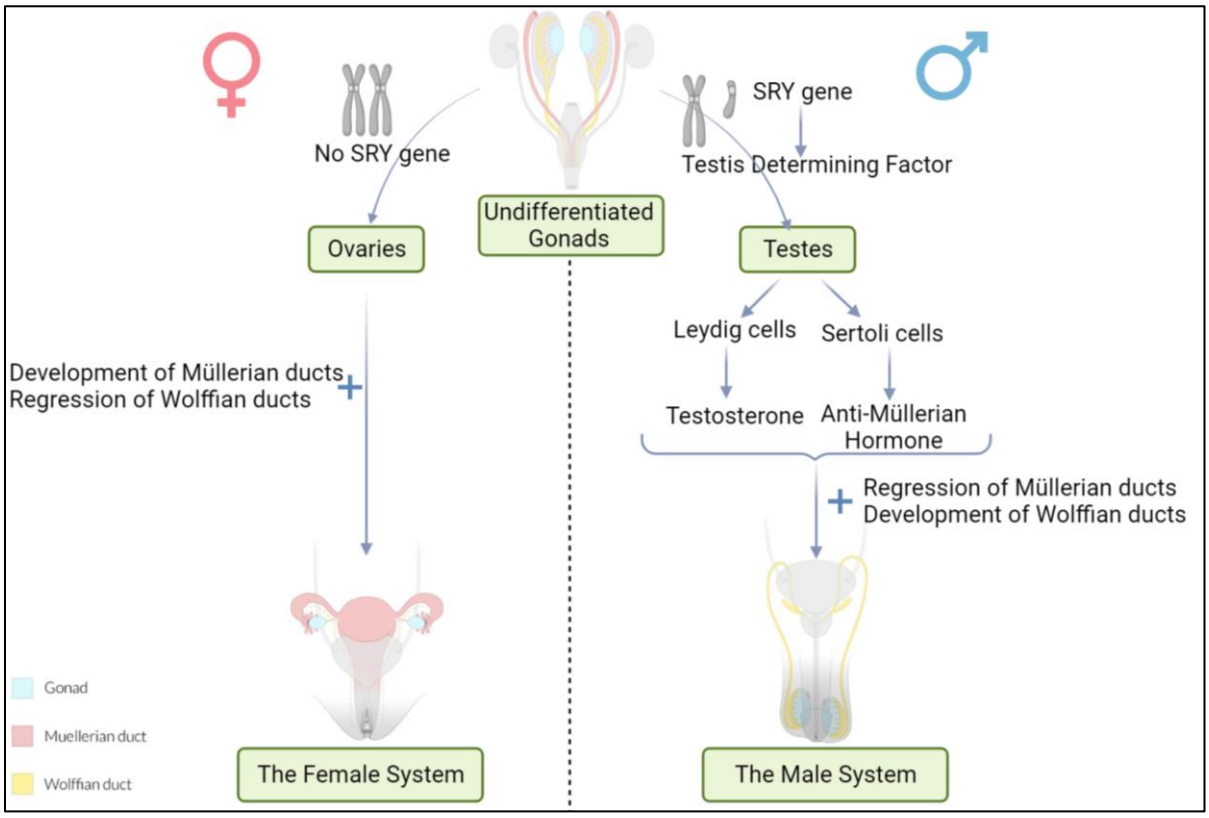

Figure 4: Sexual differentiation. The genetic (chromosomal) sex determines whether an individual develops as male or female. In the presence of the SRY gene, testes develop under the influence of the testis determining factor and produce testosterone leading to a male phenotype. However, when no SRY gene is present, ovaries develop, thus leading to a female phenotype (This picture was created with BioRender and amboss.com)

In 1959, while working on guinea pigs, Phoenix et al. were the first to state that sex steroids have organizational actions on tissues mediating sexual behavior during the prenatal period but activational actions later in life (Phoenix et al., 1959). The meaning behind his statement is that some sex differences in behavior result from an activational but reversible effect of sex steroids occurring in adulthood. Nevertheless, some sex differences that are organizational in nature are determined irreversibly by sex steroid action during prenatal development and do not disappear later in life when the hormone is no longer present (Balthazart et al., 2017). Later, his statement was confirmed in other animal models, such as rats, mice, hamsters, dogs, and ferrets (Baum, 1979). Moreover, the capacity to show sex-specific behavior in adulthood follows similar patterns as that of the genitals (Bakker & Baum, 2008). Female guinea pigs treated prenatally with testosterone propionate (TP) showed a suppressed capacity for female-typical behavior, like lordosis. This effect was permanent and thus demonstrated a defeminizing effect of androgens. The same amount of TP injected postna-

tally had no lasting effect in females. In contrast, males treated with TP showed no sign of impaired sexual behavior. In another attempt, EB treatment in castrated males induced male sexual behavior like mounting but not lordosis in females, also shown in Figure 5 (Phoenix et al., 1959). To summarize, in rodents, both males and females show male-like behavior when exposed to testosterone or estradiol during development. This observation created a paradox because all mammalian fetuses are exposed to maternal estrogens during development. How could female fetuses be protected from the effects of estrogens? Two observations acknowledged this question: the presence of alpha-fetoprotein in the fetal blood and the aromatization of testosterone into E2 (McCarthy et al., 2017). The former, a plasma glycoprotein mainly found in fetuses, can bind estrogens. Alpha-fetoprotein was shown to protect the female brain from exposure to maternal estrogens and protects the female brain from a male-typical organization (Bakker et al., 2006). Secondly, in rodents, testosterone is necessary for the sexual differentiation of male sexual behavior. The developing testis of the male embryo produces testosterone. It is then aromatized into 17β-estradiol by the cytochrome P450 aromatase in the brain leading to a masculinized and defeminized brain (Maekawa et al., 2014) (see Figure 5 for a summary).

Testosterone or its aromatized metabolites are crucial in development during so-called “critical periods” when sex steroids must be present to influence the development of sex differences in the brain and behavior (VanRyzin et al., 2020). In rats, the critical period for sexual differentiation of the brain begins prenatally around embryonic day 18.5 as testosterone synthesis in the testis peaks (Warren et al., 1973). Circulating testosterone levels fall within hours of birth, and the critical period closes shortly after (McCarthy et al., 2018). Females remain sensitive to exogenous testosterone until postnatal day 10 (Ichimura et al., 2015). In female rats, estrogens produced by the developing ovaries are not thought to influence embryonic development since they do not secrete any detectable levels of estrogens before postnatal day seven in rats (Lamprecht et al., 1976).

It should be noted that not all sex differences are due to gonadal hormones. The X chromosome and the Y chromosome contain many genes that drive sex-typical traits (Arnold, 2017), including immune-regulatory genes. Thus, the combination of both chromosomes and hormones influences the sexual differentiation of the brain (VanRyzin et al., 2020).

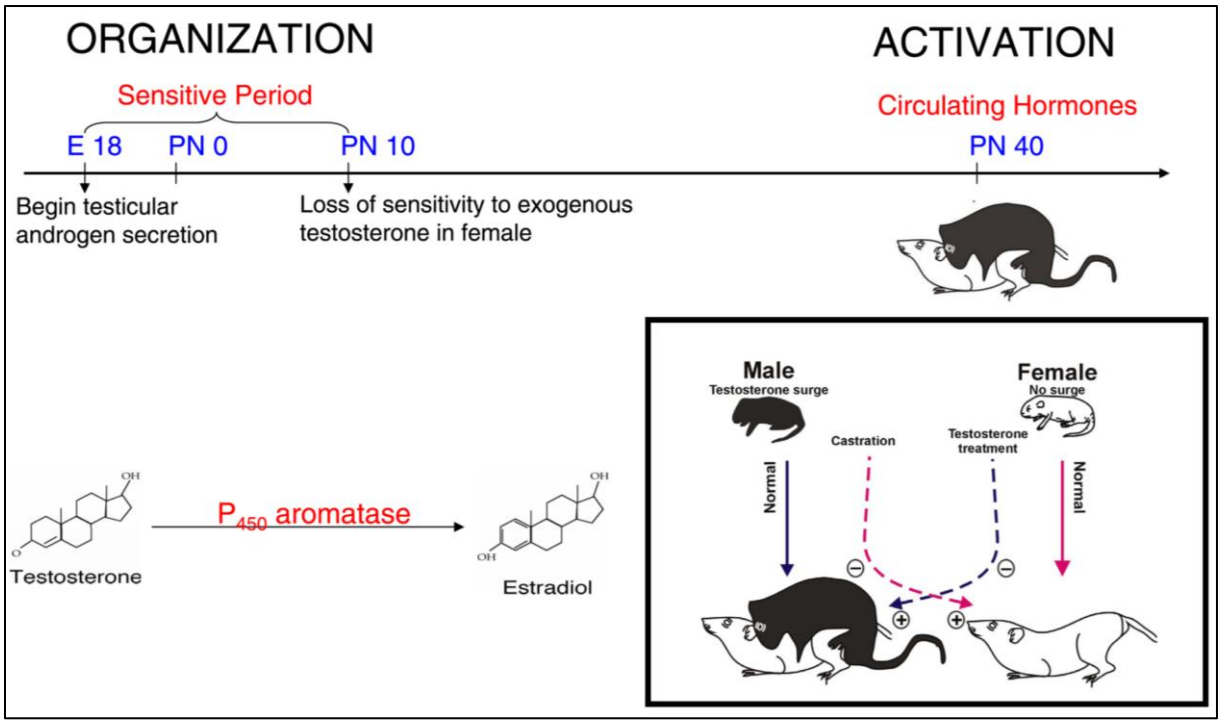

Figure 5: The organizational/activational and aromatization hypothesis. Testosterone is converted into estradiol by an aromatase. The organizational actions of estradiol take place during the sensitive period, between the 18th day of embryonic development and the tenth day of postnatal life. The activational actions of estradiol occur later in life. In the black square, the effect of hormones during sexual differentiation is represented. In males, a testosterone surge during the sensitive period leads to male-typical behavior in adulthood (represented as mounting). Females develop as "default" in the absence of hormones, leading to female-typical behavior (represented as lordosis). Castration of males neonatally leads to female-like behavior, while females treated with testosterone show male-like behavior. E18, embryonic day 18; PN0, postnatal day 0; PN10, postnatal day 10; PN40, postnatal day 40 (Picture adapted from (McCarthy, 2008).

4. Sexual Differentiation of the Quail Brain and Behavior

4.1 Sexual Differentiation During Embryonic and Early Postnatal Development

In birds, the sex chromosomes of birds are designated Z and W. Male birds are the homogametic sex with two Z chromosomes (ZZ), while female birds have one Z chromosome and one W chromosome (ZW). The DMRT1 gene on the Z chromosome is the avian counterpart to the SRY gene in mammals (Ioannidis et al., 2021). DMRT1 encodes the doublesex and mab-3-related transcription factor 1 (DMRT1) required for testis determination. Interestingly, in birds, as males are the homogametic sex, a higher dosage of DMRT1 compared to females may initiate testicular differentiation in male embryos (Smith et al., 2009).

Similar to rodents, estrogens play an important role during sexual differentiation in birds. Quails are interesting to investigate because their period of hormone sensitivity occurs *in ovo* without direct interaction between the embryo and its mother. The critical period of the brain to estrogens occurs from E6 to E12 in quail (Adkins, 1979; Balthazart et al., 1992). Contrary to rodents, male quail treated prenatally with either TP or EB show reduced male-typical features (see Figure 6 for further explanation). These features could not be restored by injecting TP later in life. The demasculinizing actions of testosterone in males can be inhibited by an aromatase inhibitor treatment (Ad-

kins-Regan et al., 1982). Female quail treated prenatally with either TP or EB are barely affected (Adkins, 1975; Panzica et al., 1987). When treated with an antiestrogen during embryonic development and with TP later in life, female quail showed increased male-typical behavior. Males are not affected by embryonic antiestrogen treatment (Adkins, 1976).

These observations are in line with sex-specific changes in gonadal development and circulating sex steroid concentrations. Sexual differentiation into testes or ovaries begins on E6 and is usually advanced between embryonic day 11 and 14 (Intarapat & Satayalai, 2014). In quail from E10 to hatching, E2 concentrations are higher in females than in males, similar to what is observed in the chick (Ottinger et al., 2001; Schumacher et al., 1988; Woods & Brazzill, 1981). Conversely, androgen concentrations are higher in males than in females during the same period. Hence, both the testes and ovaries are already implicated in steroid hormone production during development (Ottinger et al., 2001; Schumacher et al., 1988). Therefore, a mechanism protecting the male quail brain from testosterone exposure has been postulated in the form of high 5β-reductase activity in the embryonic male brain. 5β-reductase metabolizes testosterone into DHT instead of E2 and protects the male brain from being demasculinized (Balthazart & Ottinger, 1984).

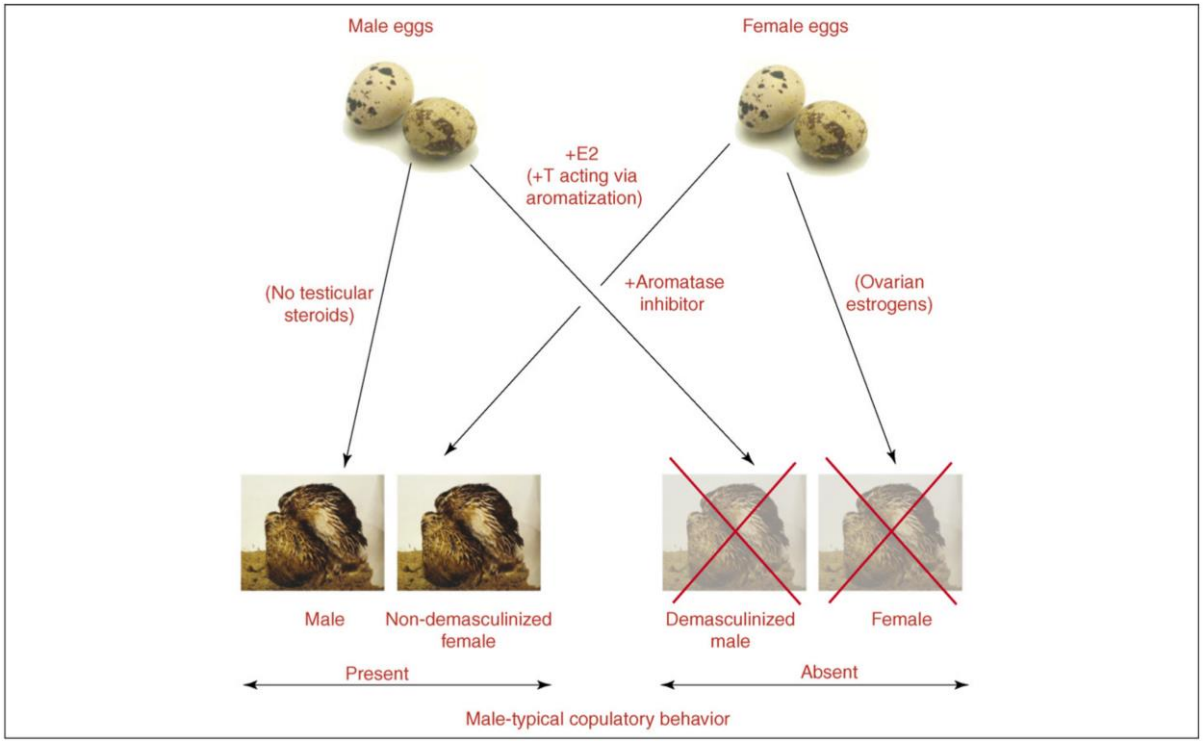

Figure 6: Schematic representation of the mechanisms controlling sexual differentiation of the brain and behavior in the Japanese quail. During embryonic development, the male quail develops as default, without exposure to hormones. In contrast, females are exposed to the organizational actions of estradiol, and therefore their brains and behavior are demasculinized. The male brain and behavior are masculinized. These events take place between embryonic days 6 and 12. However, if male embryos are injected with estradiol (E2) or testosterone (T, through aromatization into estradiol) between embryonic days 6 and 12, the male is demasculinized, and as adults, they will be unable to display male-typical copulatory behavior in response to testosterone. Similarly, if female embryos are injected with an anti-estrogen or an aromatase inhibitor, they will not be demasculinized and will show male-typical behavior in response to exogenous testosterone in adulthood. E2, estradiol; T, testosterone (Picture taken from Carere & Balthazart, 2007).

4.2 Period of Hormone Sensitivity and Postnatal Female Demasculinization

The term "critical period" must be distinguished from "period of sensitivity". According to McCarthy et al. (2018), in rodents, "*the sexual differentiation of the brain is a unique critical period in that it is initiated by endogenous production of a critical signaling molecule in only one sex, testosterone in fetal males".* In male rodents, the critical period begins at embryonic day 18 (E18) with the initiation of testosterone production and ends around birth with the decline of testosterone. In contrast, females do not produce testosterone during embryonic development but are highly responsive to it and remain sensitive to it past the end of the critical period in males, designating the period of sensitivity that occurs from E18 until postnatal day 10 (PN10) (McCarthy et al., 2018). In quail, the critical period starts at E6 and ends around E12. During this time frame, endogenous E2 production leads to demasculinization in the female quail (Adkins, 1979; Balthazart et al., 1992). Similarly, treatment with an aromatase inhibitor before E12 blocked the behavioral demasculinization of females. Aromatase inhibitor injections after E12 showed that females are only partly demasculinized as some residual male sexual behavior is still observable in adulthood (Balthazart et al., 1992). In addition, blocking E2 production by antiestrogen treatment in females during the critical period showed that females can exhibit male behavior when treated with TP as adults (Adkins, 1976). Indeed, sexual differentiation is not fully completed at hatching. Females that were ovariectomized around hatching are still capable of weak male sexual behavior after TP treatment. This behavioral response to testosterone cannot be seen in females that were ovariectomized later than four weeks after hatching nor in neonatally ovariectomized females treated with estrogens during the first four weeks after hatching (Balthazart & Schumacher, 1984; Schumacher & Balthazart, 1984). Thus, these results suggest that sex steroids demasculinize male behavior in genetic females and that the organizational actions of steroid hormones are not completely restricted to the critical period in females (Hutchison, 1978). To conclude, neonatally ovariectomized females can show male sexual behavior in response to testosterone. This capacity is progressively lost during the first two to four weeks of postnatal life due to exposure to ovarian secretions. However, demasculinization in females is already partly achieved at hatching.

4.3 The Role of the Medial Preoptic Nucleus in Quail Behavior

The medial preoptic nucleus is a brain region within the POA. The POM is implicated in controlling male sexual behavior as EB or TP implants within this region activate sexual behavior in castrated males (Watson & Adkins-Regan, 1989). Lesions within this region impair male sexual behavior (Balthazart & Surlemont, 1990). The size of the POM is sexually dimorphic and tends to be 30-40% larger in male quail (Viglietti-Panzica et al., 1986). The POM volume is influenced by the availability of hormones in adulthood because adult quail treated with testosterone showed an enlarged POM volume compared to castrated males. When both sexes are exposed to testosterone in adulthood, the sex difference in the POM volume is eliminated (Panzica et al., 1987).

5. Microglia

"Glia" means glue in Greek, suggesting their "supportive" role. Glial cells represent more than 90% of the human brain (Greter & Merad, 2013) and comprise all non-neuronal cells in the CNS. They consist of two main populations: macroglia (astrocytes and oligodendrocytes) and microglia. The primary function of oligodendrocytes in the CNS is myelin production, which provides an insulating and conducting myelin sheath around axons. Astrocytes play an important role in providing trophic support to the surrounding cells (Borst et al., 2021). Compared to the other non-neuronal cells, microglia are smaller; their area of soma is generally under 50 µm2 (Savage et al., 2019). Microglia are representatives of the mononuclear phagocytic system (Boche et al., 2013) and are the innate immune cells of the CNS (Hanisch & Kettenmann, 2007). Nonetheless, the CNS is considered immune privileged, meaning that *"[…] foreign-tissue grafts placed in the immune-privileged site are tolerated and survive for prolonged, often indefinite, intervals, while placement of such grafts at conventional body sites leads to acute irreversible immune rejection*" (Thumann et al., 2013). However, the CNS can orchestrate inflammatory responses because it is inhabited by microglia, macrophages, and dendritic cells. Macrophages and dendritic cells occupy regions such as meninges, choroid plexus, and perivascular spaces. In contrast, microglia populate the brain parenchyma and comprise around 10–15% of all glial cells of the brain. They are often referred to as "tissue-resident macrophages" (Nayak et al., 2014). While macroglia and neurons derive from the neuroectoderm, microglia progenitors arise from the peripheral mesodermal tissue, the yolk sac (Chan et al., 2007). Macrophages also arise from the yolk sac during embryonic development, whereas in the adult, they derive from bone marrow monocytes (van Furth & Cohn, 1968).

5.1 The Morphology of Microglia

In birds and mammals, the first microglia detected in embryos are described as "amoeboid" or "immature" cells. They are highly proliferative, mobile, phagocytic cells with a rounded morphology (Streit & Xue, 2009). During development, these immature microglia are progressively replaced by ramified cells, also known as "adult," "mature," or "resting" microglia. Intermediate forms of these cell types, termed microglia with stout processes or with thick long processes, may represent transitional forms of differentiating microglia (Cuadros et al., 1994; Schwarz et al., 2012). Nonetheless, during embryonic development, amoeboid microglia and microglia with stout processes are more numerous than ramified microglia (Schwarz et al., 2012). It is important to note that microglia of the developing CNS and microglia cells that reside in the adult brain have functionally different roles. In a healthy adult brain, ramified microglia present a small cell body with a ramified morphology and express only a few molecules associated with macrophage function. These non-dormant cells are in resting mode, constantly scanning the CNS. Microglial cell bodies remain stationary, but their processes continuously scan the surrounding extracellular space and communicate with neurons, astro-

cytes, and blood vessels. This allows them to detect damage or infection and to respond by transforming into an activated phenotype called “activated microglia” and performing inflammatory functions (Nayak et al., 2014; Savage et al., 2019). Activated microglia have retracted processes and acquire a macrophage-like rounded morphology similar to amoeboid microglia during development. Activated microglia upregulate their migratory, proliferative, and phagocytic capacities to perform their macrophage-like defensive functions. These cells can produce neurotoxic and neurotrophic mediators, including nitric oxide (Sierra et al., 2014).

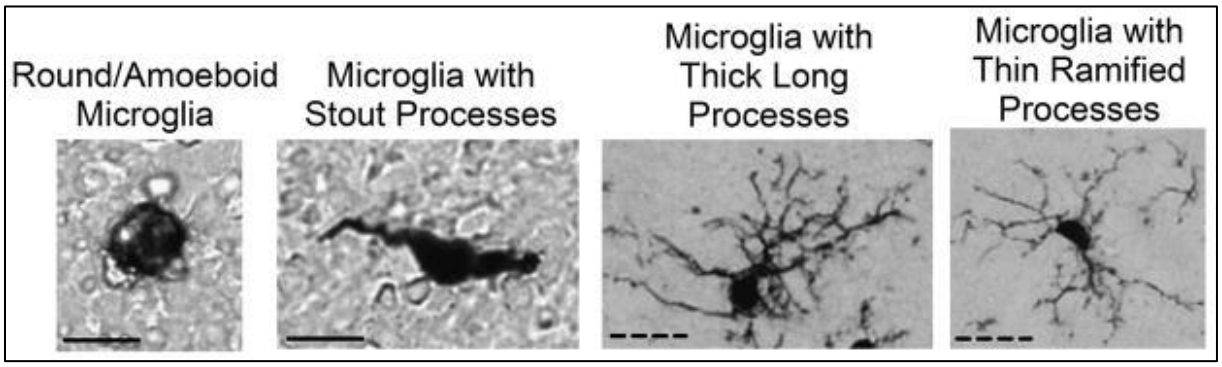

Figure 7: Classification of microglial morphology. The photos represent the four classifications of microglial morphology that also correspond to differences in microglial function. Round/amoeboid microglia, microglia with stout processes, and microglia with thick long processes are found during development. They represent immature microglia. In adults, the main morphology found corresponds to microglia with thin ramified processes. Scale bars: solid line represents 10 µm and dashed line 50 µm (Photo taken from Schwarz et al., 2012).

5.2 The Origins of Microglia

Many researchers wondered about the origin of microglial cells. Already in the early 20th century, the research team of Rio-Hortega hypothesized that mesenchymal pial cells (deriving from the mesodermal cells of the pia mater) invade the CNS to become amoeboid cells, which then transform into ramified cells. He pointed out that microglia invade the brain during late embryonic development when blood vessels begin to form (Cuadros et al., 1993). During the next years, other authors suspected that microglia, along with astrocytes and oligodendrocytes, arise from progenitor cells derived from the neuroepithelium (De Groot et al., 1992; Hao et al., 1991; Kitamura et al., 1984). Other authors assumed that microglia are derived from blood monocytes like other tissue-resident mononuclear phagocytes. Monocytes originate from bone marrow, and therefore it was thought that microglia are seeded within the nervous system around the time of birth and postnatally (Chan et al., 2007; Imamoto & Leblond, 1978).

Today, it is generally accepted that microglia derive from erythro-myeloid progenitor cells in the yolk sac during primitive hematopoiesis (Ginhoux et al., 2010; Kierdorf et al., 2013). It is thought that microglia are of myeloid (mesodermal) origin because they express the myeloid-specific transcription factor PU.1 (Kierdorf et al., 2013; Walton et al., 2000). Microglia that invade the CNS originate from the extraembryonic mesoderm of the yolk sac. Only a few are of intraembryonic origin.

The research team of Cuadros also observed that microglia invade the brain parenchyma through the pial mesenchyme, which agrees with the findings of Rio-Hortega (Cuadros et al., 1993). Other entry routes may be the cerebral ventricles and/or the parenchymal vascular network (Cuadros & Navascués, 1998). Through cell fate mapping, it was further demonstrated that microglia populating the adult brain derive from yolk sac primitive macrophages (Ginhoux et al., 2010). Despite the reported results, other data raise concerns about the exclusive yolk sac origin of microglia (Cuadros et al., 2022).

In mice, microglial invasion occurs at the end of the neurogenesis in the embryo. During fetal development, the progenitors originate from the first wave of hematopoietic cell production occurring in the extraembryonic yolk sac and then penetrate the brain. In mice, the first microglial progenitors are detected in the early neural folds around embryonic day 8 (E8) before the establishment of the blood-brain barrier (Alliot et al., 1999). Additionally, the vast majority (95%) of microglial cells are born during the first two postnatal weeks of the mouse (Alliot et al., 1999). In mammals, microglia mainly derive from precursors of YS origin, but in zebrafish and chicken, microglia that appear during development are replaced later by cells derived from intraembryonic origin (Ferrero et al., 2021; Garceau et al., 2015). In quail, microglia enter the developing brain between the ninth and 16th day of development (Cuadros et al., 1994, 1997).

As the brain matures, the microglia population expands through a combination of migration and proliferation (Swinnen et al., 2013). Once established in the CNS, microglia persist behind the blood-brain barrier and maintain themselves by self-renewal (Tay et al., 2016). In disease conditions, microglia can undergo rapid clonal proliferation (Füger et al., 2017). In addition, next to the microglia derived from myeloid cells of the yolk sac, another cell type can colonize the brain parenchyma in response to pathological situations. This population is composed of myeloid cells derived from bone marrow and are called bone marrow-derived microglia or microglia-like cells. Nonetheless, they are not only different in origin but also have slightly different characteristics (Cuadros et al., 2022)

5.3 The Role of Microglia in the Brain

In the absence of inflammatory stimuli or injury, microglia remain in a “surveying” state in which they scan the brain environment. The purpose of this “surveillance” state is to detect acute or chronic injuries, control the microenvironment and clear the brain parenchyma of accumulated metabolic products and deteriorated tissue (Nimmerjahn et al., 2005). To fight infections, microglia can recognize many infectious pathogens by expressing “pattern recognition receptors” (PRRs) that sense conserved microbial motifs, called pathogen-associated molecular patterns (PAMPs). Among these receptors are, for example, Toll-like receptor (TLR) and scavenger receptor families. In response to the stimulation of their receptors, microglia can produce proinflammatory mediators such as free oxygen

intermediates, nitric oxide, cytokines, and chemokines (Mariani & Kielian, 2009). Microglia are also central in the response to injury. Following brain or spinal cord injury, microglia retract their processes and transform into an amoeboid form. In this state, the microglia are highly motile and can infiltrate the injury site. Once at the injury site, proinflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α are released to recruit additional innate immune cells to the site of injury (Lenz & McCarthy, 2015). To limit the spread of damage, microglia then phagocytose dead or dying cells (Hanisch & Kettenmann, 2007). Microglia engage in anti-inflammatory signaling and growth factor secretion to limit chronic inflammation and promote repair (Lenz & McCarthy, 2015).

Microglia were known for a long time as “garbage collectors” (Lenz & McCarthy, 2015). However, besides their role in responding to injury or pathogens, they have a versatile role in the brain, including promoting survival, differentiation, and circuit formation of neuronal lineages, as well as eliminating synapses, sensing apoptotic cells, and brain sexual differentiation, to name a few.

The developmental functions of microglia are evolutionarily conserved, as they can also be found in species such as fruit flies or zebrafish (Sears et al., 2003; Casano et al., 2016). To execute their role in developing and maintaining neuronal circuits, microglia interact with many cells from the CNS, including neurons, astrocytes, and oligodendrocytes. These cells are responsible for cytokine production, such as colony-stimulating factor 1 (CSF1) and interleukin 34 (IL-34). CSF1 and IL-34 are crucial for microglial survival (Borst et al., 2021). Microglia also promote survival and differentiation of neuronal lineages as well as circuit formation by releasing trophic factors; for example, neurons in the V layer of the cortex require insulin-like growth factor-1 (IGF-1) that is released by the surrounding microglia (Ueno et al., 2013). Moreover, mice that lack resident microglia during embryogenesis have reduced precursor proliferation and astrogenesis, whereas cell survival and neurogenesis were unaffected, leading to the assumption that microglia may be an essential contributor to the regulation of proliferation and differentiation of embryonic cortical precursors. Besides, alterations in microglial number might perturb neural development by directly affecting neural precursors (Antony et al., 2011). Furthermore, microglial cells are highly activated during the first week after birth and express high levels of IGF1 and macrophage colony-stimulating factor, therefore suggesting a critical role in the genesis of or protection against axonal damage in the fetus (Hristova et al., 2010). Another important role of microglia is to engulf and eliminate synapses and other cells. When microglia numbers are low in the developing brain, synaptic pruning is delayed resulting in excess of dendritic spines and immature synapses (Paolicelli et al., 2011). Moreover, microglial processes make direct connections with presynaptic and postsynaptic elements. In the case of ischemia, the contact between the microglia and presynaptic boutons is prolonged, and this connection appears to be more intense (the microglial cell is wrapped more extensively around the bouton). Hence the assumption is that microglial cells may restore the function of synapses or may initiate their removal (Wake et al.,

2009). Microglia also eliminate neural precursors by phagocytosis, as shown in the cortex of rhesus monkeys with time-lapse microscopy (Cunningham et al., 2013). Microglia are sensors of apoptotic cells. The loss of neuronal cells during the development of the nervous system involves apoptosis followed by the removal of the cell corpses. Microglia contribute to the elimination of dead cells. In the cerebellum, apoptosis of Purkinje cells can be linked to superoxide ions produced by microglial respiratory bursts. These microglia showed a strong chemoattraction toward the death signal caspase-3 expressed by Purkinje cells (Marín-Teva et al., 2004). Also, microglial cells are actively implicated in cell death. The research team of Frade and Barde implied that in the chick, retina microglia express nerve growth factor as part of a cytotoxic mechanism leading to the elimination of newborn neurons, which are highly sensitive to survival signals (Frade & Barde, 1998). Rather than eliminating only dead cells, microglia also engage in primary phagocytosis, or the engulfment of a living cell and the induction of its death (Brown & Neher, 2012). This phenomenon was recently described by Pickett et al., explaining the sex differences occurring in the SDN-POA[1], which correlates with the sex difference for sexual partner preference (Pickett et al., 2023). Therefore, microglial cells can be identified as key drivers or targets of brain sexual differentiation. Microglia themselves are also sexually dimorphic. The role of microglia during sexual differentiation will be further discussed in the next chapter.

5.4 Microglia and their Role in Brain Sexual Differentiation

As previously discussed, estrogens play a crucial role in the sexual differentiation of the brain and behavior. Notably, microglia also contribute to the process of brain sexual differentiation through mediating some of the effects of estrogens.

In the rats, males have more microglia in early postnatal development, whereas females have more microglia later in the dentate gyrus of the hippocampus, parietal cortex, the amygdala (Schwarz et al., 2012), as well as in the POA (Lenz et al., 2013). In the POA, in addition to the sex difference in the microglial number, a sex difference in the morphology of microglia can be observed, with females exhibiting fewer amoeboid microglia during early postnatal development. The sex differences observed in microglia can be influenced by the availability of hormones, particularly estrogen (E2). When females were treated with E2, their microglia exhibited similarities to those found in males. Furthermore, the inhibition of microglia prevented the masculinizing effects of E2. This observation suggests that E2 has a masculinizing effect on microglia and that microglia play a central role in mediating the effects of E2 on sexual differentiation (Lenz et al., 2013).

[1] SDN-POA: The sexually dimorphic nucleus (SDN) of the preoptic area. The medial preoptic nucleus (MPN) resides within the POA and is subdivided into lateral, medial, and central parts. The SDN overlaps in part with the central part of the MPN and is sometimes referred to as the MPNc (Pickett et al., 2023).

In the context of the role of microglia in brain sexual differentiation, neuroinflammatory processes such as PGE2 production may contribute to brain sexual differentiation in rodents via microglia. PGE2 action is thought to be mediated by E2 which upregulates cyclooxygenase 2 (COX-2) expression (Amateau & McCarthy, 2004). COX-2 synthesizes the prostaglandin PGE2 from arachidonic acid derived from the cell membrane (Park et al., 2006). In the developing POA, COX-2 is upregulated by estradiol which eventually leads to an increased synthesis of PGE2. Similarly to E2, PGE2 exposure during development leads to male sexual behavior in adults (Amateau & McCarthy, 2004). Microglia were found to be required for the E2-induced synthesis of PGE2. PGE2 treatment increases the number of microglia in females, while a COX inhibitor diminishes the microglial count. The presence of microglia is also found to play a role in masculinizing spine-like protrusions on neurites in POA-derived neuron cultures, as in the absence of microglia, a lower density of such protrusions was seen. Inhibiting microglia in neonatal pups prevented the long-term masculinizing actions of E2 through PGE2 on behavior observed in adulthood (Lenz et al., 2013).

Furthermore, the phagocytic activity of microglia has been found to significantly impact sexual differentiation. In the developing amygdala of the male rat, which is important for juvenile social play, microglia were found to be more phagocytic than in females. The higher phagocytic activity in males leads to a reduced density of astrocytes in the male amygdala. Therefore, phagocytic microglia contribute to masculinization and to the development of sex differences in the brain (VanRyzin et al., 2019). In contrast, in the SDN-POA, females show more phagocytic microglia, and these microglia engulf viable neurons (Pickett et al., 2023; VanRyzin et al., 2019). The SDN-POA tends to be smaller in females, but its volume (and the number of neurons) in females could be increased to levels seen in males when microglial phagocytosis was blocked (Pickett et al., 2023). These results suggest that the characteristics associated with being male or female within the brain may result due to a difference in the phagocytic activity of microglia. This difference in their phagocytic activity could stem from differential hormone exposure during development. Additionally, when the number of neurons in the SDN-POA was increased, females lost their preference for male odors in adulthood. This further proves that the SDN-POA may function as a regulator of sexual partner preference (Pickett et al., 2023), as already stated by other authors (Byne et al., 2001; Roselli et al., 2004). However, further research is needed to fully understand how microglia are implicated in this process.

In summary, research on rodents has revealed that microglia play a vital role in the process of sexual differentiation by sex hormones. They are involved in both producing and responding to the E2-induced PGE2 synthesis. Furthermore, microglia in the female rodent brain were also shown to be more phagocytic, which could explain some sex differences. Therefore, microglia may contribute to brain sexual differentiation through a variety of mechanisms.

In quail, sexual differentiation also depends on estrogens, but as opposed to rodents, they are responsible for the demasculinization in female quail. In contrast to rodents, in the quail POM microglia are more abundant in the female brain during E9 and E12, and males are the ones with fewer amoeboid microglia. Similar to rodents, the sex difference in microglia is driven by estrogens. E2 treatment in males increases the number of microglia, whereas blocking estrogen synthesis diminishes microglial count (Delage & Cornil, 2020). Unlike in rodents, PGE2 treatment during embryonic development does not seem to affect the sexual behavior of both male and female quail. Furthermore, COX inhibition does not affect the behavior of females but impairs male sexual behavior and eliminates the sex difference in the microglial count at E12 (Delage et al., 2021). This leads to the assumption that even though PGE2 treatment does not seem to affect sexual behavior, prostanoids might still be implicated in brain sexual differentiation.

To summarize, in quail, E2 is responsible for brain demasculinization and dimorphisms in microglial cells. However, contrary to what is observed in rodents, PGE2 is not the prostanoid involved in sexual differentiation. Also, the role of phagocytic microglia in the quail brain remains unknown.

Objectives, Hypotheses, and Strategy

1. Objectives and Strategies

As part of this master thesis, we wanted to further explore the role of microglia during the sexual differentiation of the brain in quail. We defined two goals. Our first goal was to study the number of microglia implicated in phagocytosis during embryonic development in the POM. The second goal was to examine the number of microglia during brain development in the POM.

1.1 First Objective

We wondered whether there exists a sex difference in the number of microglia implicated in phagocytosis during embryonic development in the POM of the quail. Microglia may play an important role during brain sexual differentiation as they are the brain's resident immune cells. They are essential in sculpting neural circuits, they are highly phagocytic and capable of pruning synapses (Cunningham et al., 2013; Paolicelli et al., 2011; VanRyzin et al., 2019). In rats, the sex difference in the SDN-POA volume resides in a differential phagocytic activity of microglia between males and females (Pickett et al., 2023). In the rat´s developing amygdala, microglia are more phagocytic and engulf more viable newborn cells in males. Microglia phagocytosis, therefore, contributes to masculinization and to the development of sex differences in the brain (VanRyzin et al., 2019). The POM was chosen as a region of interest, as the existence of a sex difference in the number of microglia between E9 and E12 was already identified within this region. Female quail exhibit more microglia than males, and therefore microglia are sexually differentiated in the developing POM under the action of estrogens (Delage & Cornil, 2020). The POM is known for its role in male sexual behavior (Panzica et al., 1996) and was shown to be sexually dimorphic in quail (Viglietti-Panzica et al., 1986). We decided to investigate specifically E10 and E12 for several reasons. Both development days fall within the critical period of the brain to estrogens that occurs from E6 and E12 and mediates sexual differentiation (Adkins, 1979; Balthazart et al., 1992). Additionally, microglial cells only start invading the brain around E9 until E16 (Cuadros et al., 1994, 1997). To summarize, we wanted to examine whether microglia are implicated in phagocytosis during brain sexual differentiation on E10 and E12 in the POM and to do this, we tested whether the number of phagocytic microglia is different between the sexes.

To facilitate the visualization of phagocytic microglia, we tested a novel method. To this end (Figure 9), quail eggs were injected on either E9 or E11 with fluorescent DiI-liposomes, similar to what Tay et al. did (Tay et al., 2020). We compared the effect of 30µl or 100µl of liposomes in the yolk or the albumen to determine which injecting method was the most robust. The brain dissection was done 24h after injection to allow the injected product to propagate throughout the egg. Besides, Tay et al. (2020) reported that the effect of their injections was already visible 24h following injection (Tay et al., 2020). To quantify microglia, a biotinylated lectin termed *Ricinus communis agglutinin-1* (RCA-1) was used (Cuadros et al., 2006). The POM was then analyzed for its microglial count and if

microglia had incorporated the fluorescent liposomes under a microscope. The incorporation of the liposomes by microglia could hint to a phagocytic activity.

1.2 Second Objective

Our initial intention was to reproduce the findings reported by Delage & Cornil (2020) by utilizing another microglial marker, anti-CSF1R, which is believed to be highly specific to microglia (Garceau et al., 2010; Garcia-Morales et al., 2014). Unfortunately, we were unable to achieve any results with this marker. Consequently, we decided to change our approach and utilize a different marker, specifically a lectin known as RCA-1. Therefore, by using the lectin, we wondered whether there still exists a sex difference in the number of microglia in the POM of quail during brain sexual differentiation and whether this sex difference depends on hormone exposure, as shown by Delage & Cornil (2020). We investigated the number of microglia in the POM on E12 to verify with a different immunostaining method the results of Delage & Cornil (2020), who previously reported more microglia in females between E9 and E12. This sex difference depends on estrogen bioavailability (Delage & Cornil, 2020). Furthermore, in male rodents, the critical period of hormone sensitivity begins at E18 with the initiation of testosterone production and ends around birth with the decline of testosterone. Female rodents do not produce testosterone but are sensitive to its action, even past the critical period in males, until PN10 (McCarthy et al., 2018). Therefore, we suspected there might be a similar phenomenon in the female quail. The female quail is not fully demasculinized after E12, and complete demasculinization is thought to be achieved during the first two to four weeks after hatching (Balthazart & Schumacher, 1984; Schumacher & Balthazart, 1984). Thus, we wondered whether there exists a sex difference in the number of microglia in the POM on PN1, as it was reported that females remain sensitive to the demasculinizing effect of estrogen during the first weeks of postnatal life (Balthazart & Schumacher, 1984; Schumacher & Balthazart, 1984).

To this end (Figure 10), quail eggs were injected with estradiol benzoate (EB) or its vehicle on embryonic day seven (E7). E7 was chosen as injection day as the day E7 falls in the critical period (Adkins, 1979; Balthazart et al., 1992). To confirm the results of Delage & Cornil (2020) by using a different staining method than they did, quail brains were collected on E12 or PN1 and treated with the biotinylated lectin RCA-1 (Cuadros et al., 2006). The POM was then analyzed for its microglial count under the microscope.

2. Hypotheses

The first goal was to determine whether there exists a sex difference in the number of microglia implicated in phagocytosis in the POM during embryonic development on E10 and E12 in quail. We expected to see a higher number of phagocytic microglia in the POM of females given that more micro-

glia are in the female POM (Delage & Cornil, 2020). Hence, we suspect more microglia to be involved in engulfing newborn cells in the female POM.

The second goal was to determine if there is a sex difference in the number of microglial cells in the POM during brain development. We investigated 2 developmental stages: E12 and PN1. For E12, we expected to observe similar results as already reported (Delage & Cornil, 2020), meaning that microglia are more abundant in the female POM than in males and that this sex difference is eliminated by treatment with estrogens. As females remain sensitive to the demasculinizing effects of estrogens after hatching and complete demasculinization of females is acquired during the first two to four weeks of postnatal life, the first day of hatching was also examined in the number of microglia in the POM. In the case of a sex difference in the microglial count, we wondered also if this was mediated by differential hormone exposure. We expected to see more microglia in the female POM on PN1 and that this sex difference would be eliminated by estrogen treatment.

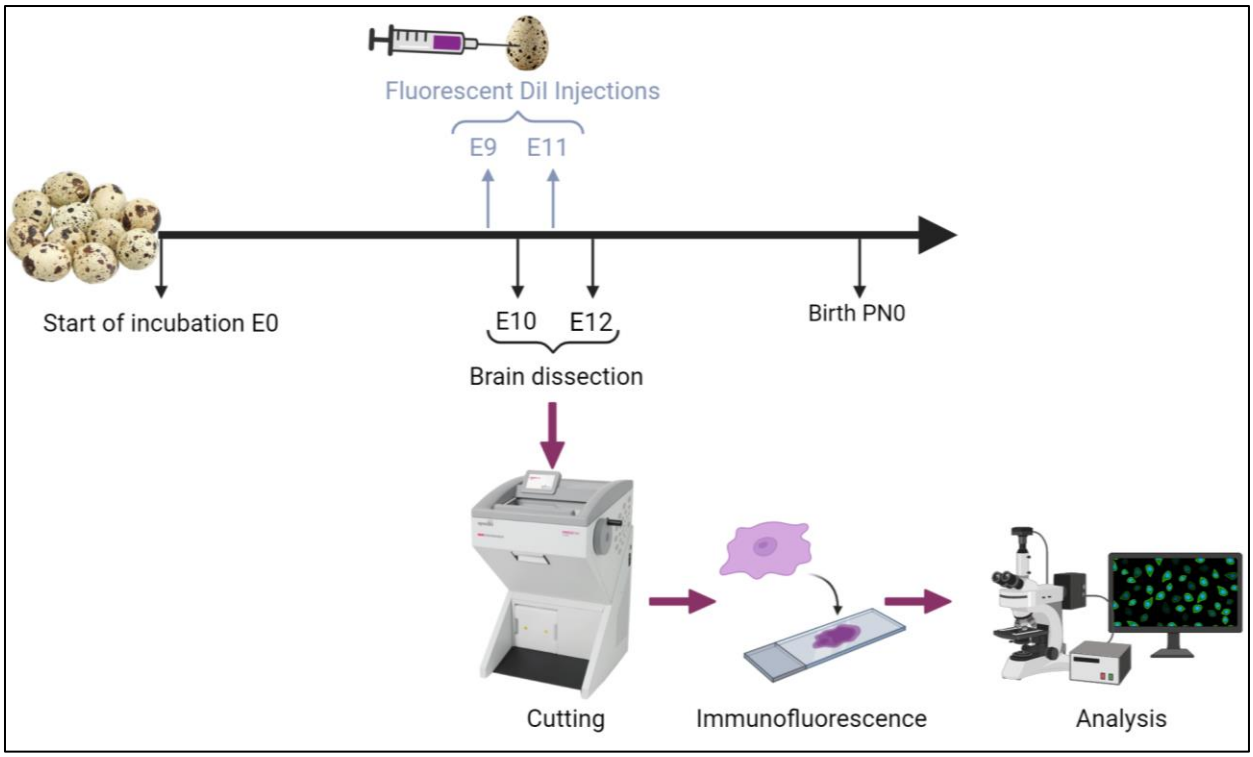

Figure 8: Experimental strategy of the first project. Eggs were incubated, marking E0. After nine (E9) or eleven days (E11), the eggs were injected with fluorescent DiI liposomes and then collected on E10 or E12, respectively. The brains were cut on a cryostat and stained with immunofluorescence to be visualized and analyzed under a microscope. E0/9/10/11/12, embryonic day 0/9/10/11/12; PN0, postnatal day 0 (The picture was created with BioRender).

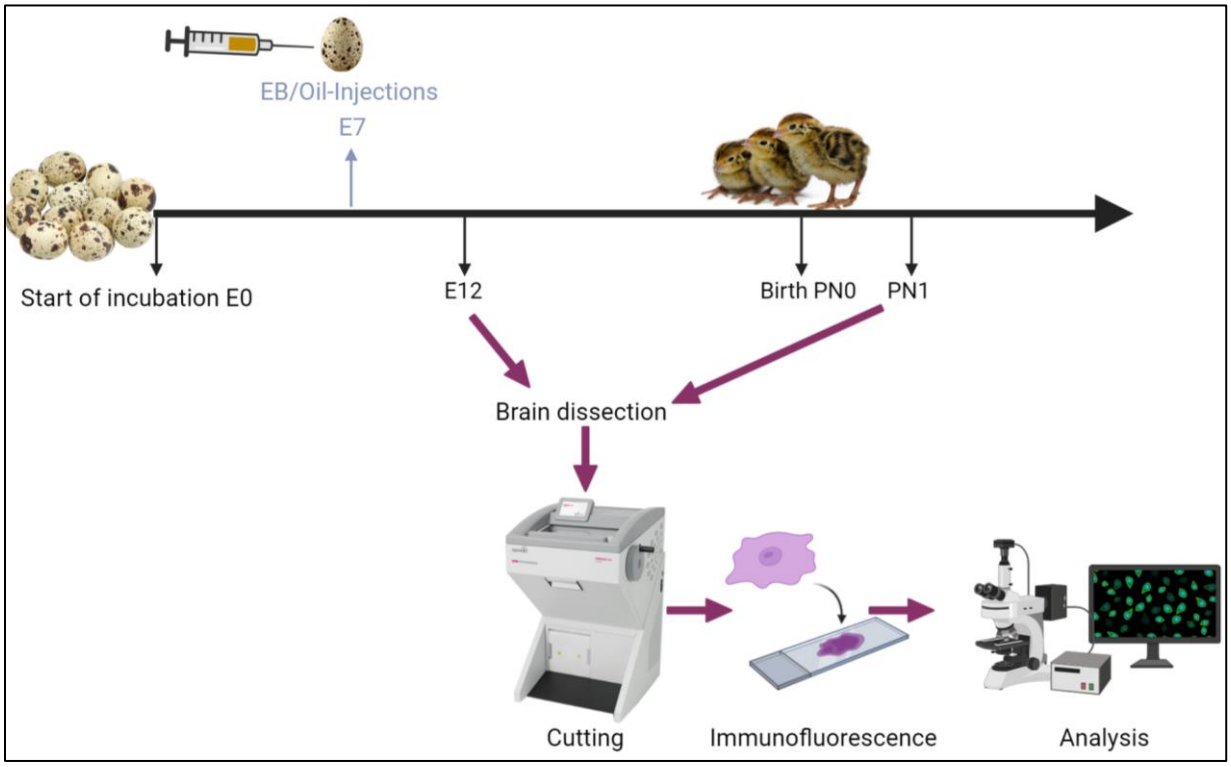

Figure 9: Experimental strategy of the second project. Eggs were incubated, marking E0. After seven days (E7), the eggs were injected with EB or its vehicle. After collection on E12 or PN1, the brains were cut on a cryostat and stained with immunofluorescence to be visualized and analyzed under a microscope. E0/7/12, embryonic day 0/7/12; EB, estradiol benzoate; PN0/1, postnatal day 0/1 (The picture was created with BioRender).

Material and Methods

1. Subjects

A total of 174 embryonic or early postnatal Japanese quail *(Coturnix japonica)* served as subjects in this study. They were obtained from fertilized eggs produced in our breeding colony at the GIGA Neurosciences (Animalerie Centrale de l'Université de Liège, LA1610002). Details about the number of animals used in each experiment and experimental groups are provided in specific experiment sections and each figure, respectively. All experimental procedures were in agreement with the Belgian laws for the use of animals in scientific research and approved by the Ethics Committee at the University of Liège (Protocol #2522). All measures were obtained from an experimenter who was blind to treatments.

2.1 First Experiment: Sex Difference in the Number of Phagocytic Microglia in the Medial Preoptic Nucleus During Development

On E9 or E11 (counting the day the eggs were put in the incubator as embryonic day zero (E0)), the eggs were candled to verify the viability of the embryo. The fertilized eggs were assigned to one of the prenatal treatment groups. Fluorescent DiI-liposomes were injected in eggs at E9 or E11 through a hole by using a 0.45x25mm needle or a 0.45x13mm needle to inject either in the yolk or the albumen, respectively. For each developmental age and injected compartment, one group was injected with 30µl and another 100µl (Tay et al., 2020), resulting in a total of 8 experimental groups. A total of 123 eggs were injected. After injection, the eggs remained in the incubator for another day. Male and female embryos of these eight groups were collected at embryonic day 10 (n=41), and embryonic day 12 (n=34) and their brains were processed and analyzed for microglia in the POM.

2.2 Second Experiment: Effect of Estradiol Benzoate on Microglial Numbers in the Medial Preoptic Nucleus

Before injection, the eggs were candled to verify the viability of the embryo. Then, fertilized eggs (n=130) were injected at E7 with 50µl EB or its vehicle, sesame oil. Male and female embryos were collected at E12 (n=49) and PN1 (counting the day the chick hatched as postnatal day 0 (PN0)) (n=50). The brains of the four treatment groups were then processed and analyzed for microglia in the POM.

2. General Procedures

2.1 Egg Incubation and Hatching

Eggs were collected daily and stored for up to 15 days at 4°C until incubation. The collected eggs were set in an incubator (Fiem; Model: MG200/300) at 37.8° C and 47% relative humidity. The eggs were turned every six hours throughout incubation. When incubation was carried out beyond day 15, eggs were placed in hatching baskets on day 15, and the relative percentage of humidity was raised to 60% to ensure proper hatching (E17–18).

2.2 Preparation of Solutions, Sterilization, and Egg Injections

For the first experiment, eggs were injected with 0,1% fluorescent DiI-Liposomes (Liposoma Research Liposomes, Amsterdam, Netherlands; No. D6-4E1122). The manufacturer recommends injecting 1000ul per 100gr of animal. As quail embryos weigh around 2gr at E10 and 3.7gr at E12, 30ul and 100ul fluorescent DiI-liposomes were injected (Tay et al., 2020). According to the manufacturer, the average size of the liposomes is 1.7 µm with a maximum of 3 µm. These liposomes are artificially prepared lipid vesicles encapsulating a PBS solution labeled with the fluorochrome DiI. The excitation and emission maxima of fluorescence DiI liposomes are at 549 nm and 656 nm, respectively (ThermoFisher Scientific, n.d.),

For the second experiment, the eggs were injected with 50 µl sesame oil (mpbio; REF: 8008-74-0) containing estradiol benzoate (beta-estradiol-3-benzoate, 25 µg; REF: E8515 1gr.SIGMA) or only with sesame oil as a control. The solutions were sterilized by boiling for 15 min at 110°C. The control solution underwent the same procedures. Before each injection, needles were sterilized in a flame. The sterile solutions were then injected through a hole made in the shell of the apex of the egg, opposite to the air chamber (Delage & Cornil, 2020). The hole was then sealed with melted paraffin. These steps were done in an aseptic environment.

2.3 Sexing of Quail Embryos

Quail gonads are located ventrally to the mesonephron. They can be observed under binoculars following laparotomy. The sex can already be determined as early as E7 based on anatomical observations. As indicated in Figures 12 and 13, males display two bilateral testes resembling two small grains of rice, while females possess asymmetrical gonads with only the left gonad that develops into a functional ovary, while the right one degenerates (Intarapat & Satayalai, 2014).

2.4 Brain Collection and Fixation

At the end of the experimental procedure, embryos and newborns were euthanized by rapid decapitation, and their brains were dissected from the skull and fixed by immersion in 4% paraformaldehyde for 24 hours. After three washes in PBS for 30 minutes, they were transferred to 30% sucrose at 4°C for 48 hours. The brains were then embedded into 7.5% gelatin and 15% sucrose in PBS at 4°C (Delage & Cornil, 2020). The brains were frozen in isopentane, cooled with dry ice, and stored at −80°C until needed.

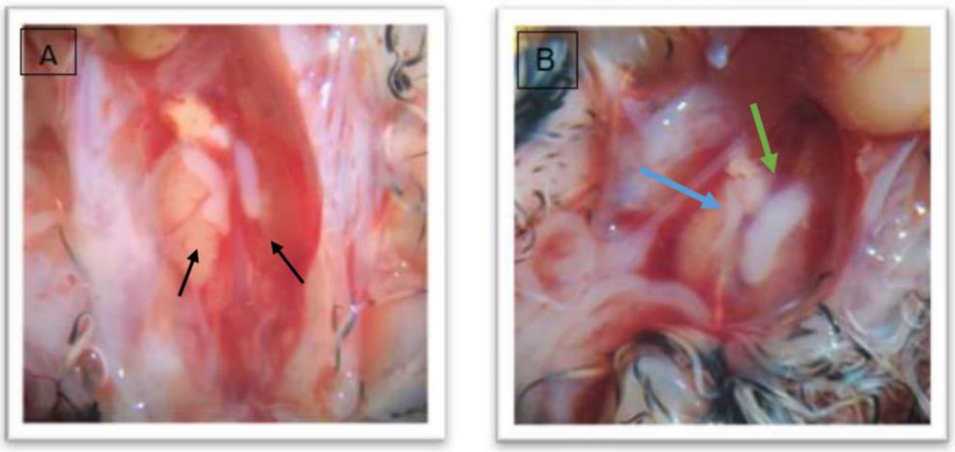

Figure 10: Picture of male and female gonads taken at E12 under binoculars. A. Male gonads. Males display two bilateral testes resembling two grains of rice (black arrows). B. Female gonad. The left gonad (green arrow) is more developed than the right one. The right gonad (blue arrow) is regressed but still visible (photos from the master thesis of Elisa Dardenne).

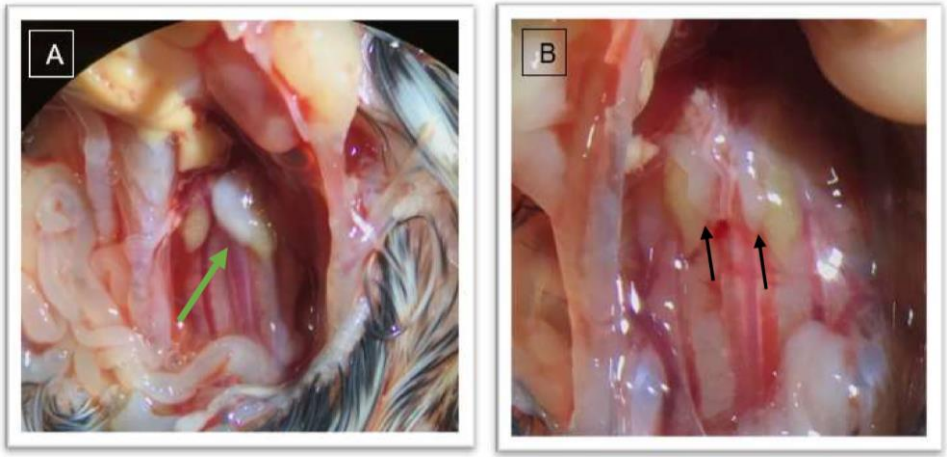

Figure 11: Picture of male and female gonads taken at PN1 under binoculars. A. The left gonad of a female quail (green arrow) is well developed with a thick cortex. B. Two bilateral gonads of a male quail with a thinner cortex compared to the female gonad (photos taken from the master thesis of Elisa Dardenne).

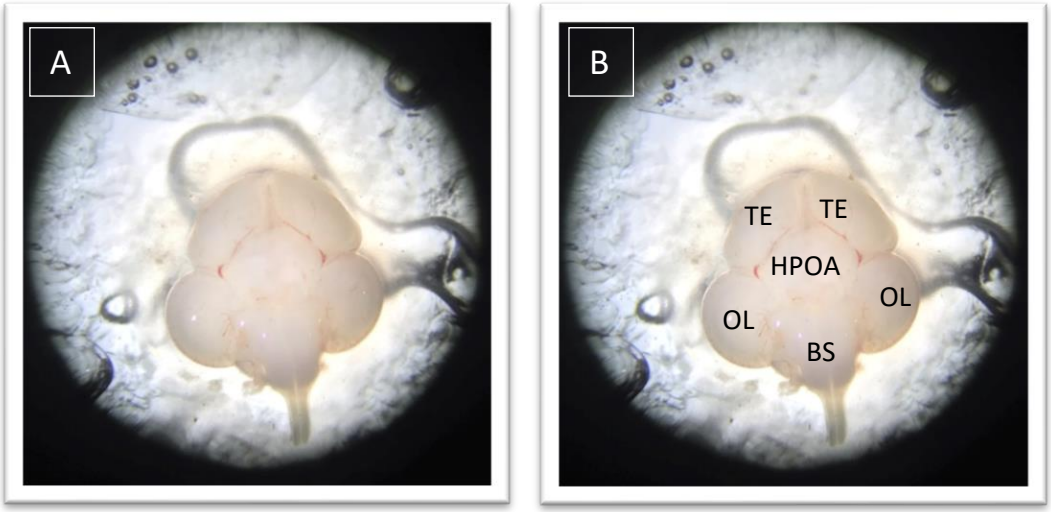

Figure 12: Pictures of a quail brain taken at E8 under binoculars. A. Ventral view of a quail brain. B. Several important brain regions are represented: the telencephalon (TE) is rostral, and the optic lobes (OL) are lateral. Caudal of the brain is the brainstem (BS), and the region consisting of the hypothalamus and the preoptic area (HPOA) is in the center (photos taken from the master thesis of Elisa Dardenne)

2.5 Histology and Immunostaining

Brains were cut with a cryostat (Epredia; CryoStar NX70) into four series of 20 µm thick coronal sections. The plane of the section matches the stereotaxic atlas of the chicken brain (Kuenzel & Masson, 1988). Sections were collected directly on Superfrost Plus Adhesion Microscope slides (Epredia; REF: J1800AMNZ) and kept at −80°C. Brains were then immunostained using standard procedures. The slides were thawed before immunostaining. Then, the slides were rinsed in Tris-buffered saline (TBS 0.05 M). To reduce the unspecific binding of the lectin, the slides were saturated for 60 min in 5% normal goat serum (NGS, Vector Laboratories; REF: S-1000; LOT: ZJ101 and ZJ0412) and in TBST (TBS containing 0.1% Triton X-100). Subsequently, the slides were incubated overnight at RT with the biotinylated lectin Ricinus Communis Agglutinin 1 (1/250; RCA I; Vector Laboratories; REF: B-1085; LOT: ZJ0929) and 5% NGS in TBST. The next day, slides were incubated for two hours with streptavidin conjugated to Alexa 488 (1/100; Invitrogen Thermofisher; REF: S11223; LOT: 2480092 and 2555707) in TBST. Then, the slides were stained with DAPI (1ul per 5ml in TBS; Sigma; REF: D9542-10MG; LOT: #0000116964). Slides were left to dry overnight and coverslipped with Aqua-Poly/Mount (Polysciences Inc.; REF: 18606-20) the next day.

Our initial intention was to use a novel marker for microglia, the antibody anti-Chicken CSF1R. It recognizes the chicken homolog of human CSF1R. Its ligand CSF-1 is required for normal differentiation, proliferation, and survival of macrophage-lineage cells. The expression of CSF1R on the cell surface appears to be one of the earliest events of macrophage-lineage cells. It was also reported that its expression is restricted to the cells deriving from the macrophage-lineage, throughout embryonic development and in adults (Garceau et al., 2010; Garcia-Morales et al., 2014). The staining protocol was applied as follows. The slides were rinsed in Tris-buffered saline (TBS 0.05 M). To reduce the unspecific binding of the antibody, the slides were saturated for 60 min in 5% normal goat serum (NGS, Vector Laboratories; REF: S-1000; LOT: ZJ101 and ZJ0412) in TBST (TBS containing 0.1% Triton X-100). Subsequently, the slides were incubated for two nights at 4°C with different concentrations of the primary antibody Mouse anti-CSF1R (1/250-1000-5000; Biorad; REF: MCA5956GA; LOT: 151276) and 2% NGS in TBST. Afterwards, the slides were incubated for two hours with two different concentrations of the secondary antibody Goat Anti-Mouse Alexa 488 (1/200-500; Invitrogen; REF: A11001; LOT: 2015565) in TBST. Then, the slides were stained with DAPI (1ul per 5ml in TBS; Sigma; REF: D9542-10MG; LOT: #0000116964). Slides were left to dry overnight and coverslipped with Aqua-Poly/Mount (Polysciences Inc.; REF: 18606-20) the next day.

2.6 Image Analysis

The POM is immediately lateral to the third ventricle (VIII) and extends throughout the preoptic region. The POM extends dorsally to the anterior commissure (CA) but disappears caudally shortly after the appearance of the anterior commissure (Viglietti-Panzica et al., 1986). As DAPI staining did not reveal the region of interest as we anticipated, the section containing the POM at its most caudal level, where the anterior commissure reaches its largest extension, was selected for image analysis.

Pictures were taken with an Echo Revolve microscope. The quantification field (H x L, 370,8x443,3 µm) was acquired when aligned with the lateral edge of the third ventricle and the ventral edge of the anterior commissure (see Figure 13). It was then moved 443,3 µm (the length of the field) ventrally along the third ventricle, and a second picture was taken. This process was repeated in the other hemisphere. Using the cell counter plugin of Image J (ImageJ 1.53t; Java 1.8.0 322, 64bit), microglial cells were manually counted and classified based on the shape of the cell bodies and their processes. They were considered either amoeboid (round with no processes), transitioning (elongated cell bodies with stout or long and thick processes), or ramified (long and thin processes) as described in quail (Figure 14a-c) (Delage & Cornil, 2020). During development, only immature microglia are observed. Immature microglia have an amoeboid or transitioning shape. Later in life and adulthood, mature microglia with long and thin processes can be seen. Mature microglia are also called surveying or ramified microglia (Figure 14d). No mature microglia were observed in the present study. Cells whose cell bodies were not entirely within the boundaries of the regions of interest were excluded. To compare the immature microglial cell numbers across treatment groups, the cell numbers of amoeboid and transitioning microglia were summed into one group, the total microglia.

For the first experiment, concerning immunofluorescence analysis of the DiI staining, pictures were also taken with the inverted confocal microscope Leica SP5 with 7 lasers at the wavelength 561 nm for excitation. Gas lasers are the light source that emit at various wavelengths. The specific wavelengths available for excitation were 405 nm, 458 nm, 476 nm, 488 nm, 494 nm, 561 nm, and 633 nm. This type of microscope is also equipped with AOBS (Acousto-Optical Beam Splitter) capabilities. The AOBS facilitates efficient separation of the excitation and emission light. The AOBS enables precise selection of the emission wavelength, ensuring accurate detection of fluorescence signals. This feature allows for the elimination of unwanted excitation light and significantly enhances the detection sensitivity and image quality (GIGA Cell Imaging Platform Liège University - Confocal Microscope Leica SP5).

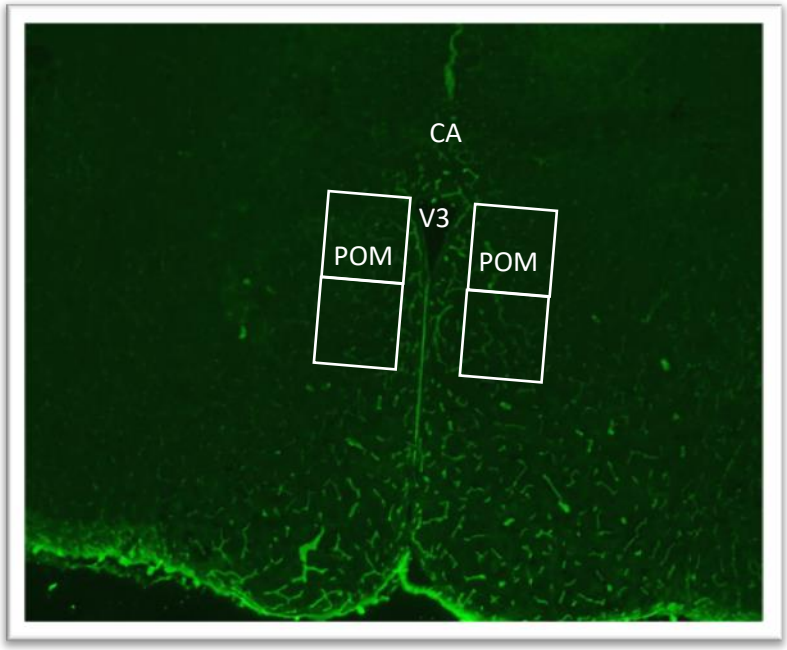

Figure 13: Representative photomicrograph on postnatal day 1. The white squares represent approximately the boundaries of the acquired image. CA, anterior commissure; POM, medial preoptic nucleus; V3, third ventricle.

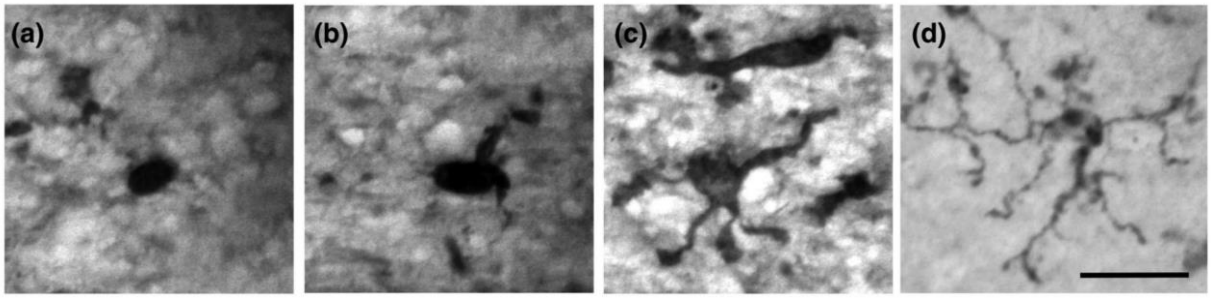

Figure 14: Different microglial morphotypes during development (A-C) and adulthood (D) in Japanese quail. Immature microglia comprise amoeboid microglia (a) and transitioning microglia with stout processes (b) or long and thick processes (c). Adult microglia are thinly ramified (d). Scale bars, 25 µm (Picture taken from Delage & Cornil, 2020).

2.7 Statistical Analysis

Data were analyzed using GraphPad Prism 8 (Version 8.0.2) by two-way ANOVA with sex and embryonic treatments as independent factors. Normality and heterogeneity of variance were verified by Shapiro–Wilk and Spearman tests, respectively. Effects were considered significant when $p<0.05$. Data are reported as means ± standard error of the mean (SEM). Due to technical problems (torn or damaged sections) and time management, accurate counts could not always be performed for each subject and nucleus.

Results

1. Sex Difference in Phagocytic Microglia in the POM During Brain Development

The first experiment was conducted to identify a possible sex difference in the number of phagocytic microglia in the POM on two developmental days, E10 and E12. The POM, a key region in establishing male sexual behavior (Panzica et al., 1996), was chosen as a region of interest because a sex difference in the microglial number was reported previously (Delage & Cornil, 2020). To this end, we injected 30µl or 100 µl of fluorescent DiI liposomes in quail eggs. As we wanted to test which injecting method was the most reliable, we injected into either the yolk or the albumen. The diameter of fluorescent DiI liposomes can be up to 3 µm and can thus be easily taken up by macrophages through phagocytosis (Naito et al., 1996; van Rooijen et al., 1996). No signal could be observed with low exposure time of the tissue to the light. Some "fluorescent bodies" could, however, be identified when the exposure was increased up to 520 ms (see green arrows in Figure 15-17). These fluorescent bodies were primarily found in the meninges and at the border of the third ventricle. Some could even be found within the brain parenchyma, but the staining was weak. However, since extending light exposure increases the signal/noise ratio, the observed "fluorescent bodies" are probably non-specific staining. Many blood vessels could also be observed within the brain parenchyma, as represented in Figure 15-17 (see white arrows). Concerning the different treatment groups, no differences in terms of staining quality could be observed, neither on E10 nor on E12. Injecting in the yolk or the albumen with different volumes of liposomes also did not make a difference. Upon transitioning to the confocal microscope Leica SP5 to carefully select the excitation wavelength corresponding to DiI, we conducted another examination of the DiI liposomes in the red spectrum. No signal was seen under normal excitation. Only faint and sparse signals could be detected upon higher excitation and/or exposure time of the selected wavelength, reinforcing the conclusion that the staining we could observe following longer exposure likely corresponded to non-specific staining.

Furthermore, microglia were identified by using the biotinylated lectin RCA-1 with Alexa Fluor 488 streptavidin. RCA-1, also known as *Ricinus communis agglutinin 1*, is a lectin that binds to β-δ-galactose residues and stains microglia and macrophages. This lectin has previously been described to label microglial cells in quail, rats, and the developing human brain (Andjelkovic et al., 1998; Boya et al., 1991; Delage & Cornil, 2020). The immunofluorescence analysis revealed the presence of a signal not only in the green spectrum but also in the red spectrum for the biotinylated RCA-1, as shown in Figure 18. This dual signal posed challenges in isolating and analyzing the DiI staining independently. In contrast to the DiI liposome staining, the RCA-1 staining required a significantly shorter exposure time, averaging 5 ms, and was not detected when using the confocal (or only when increasing the exposure time significantly). However, we were able to detect a signal in the red spectrum for RCA-1 staining only when the exposure time was drastically prolonged. Moreover, blood vessels

were also labeled by this lectin, as stated by other authors (Seitz et al., 1988). To conclude, as we probably only observed non-specific staining for the DiI liposome staining and the staining with the DiI liposomes did not provide any reliable signal, microglia could not be counted, their phagocytic activity could not be assessed, and therefore, statistical analysis could not be done.

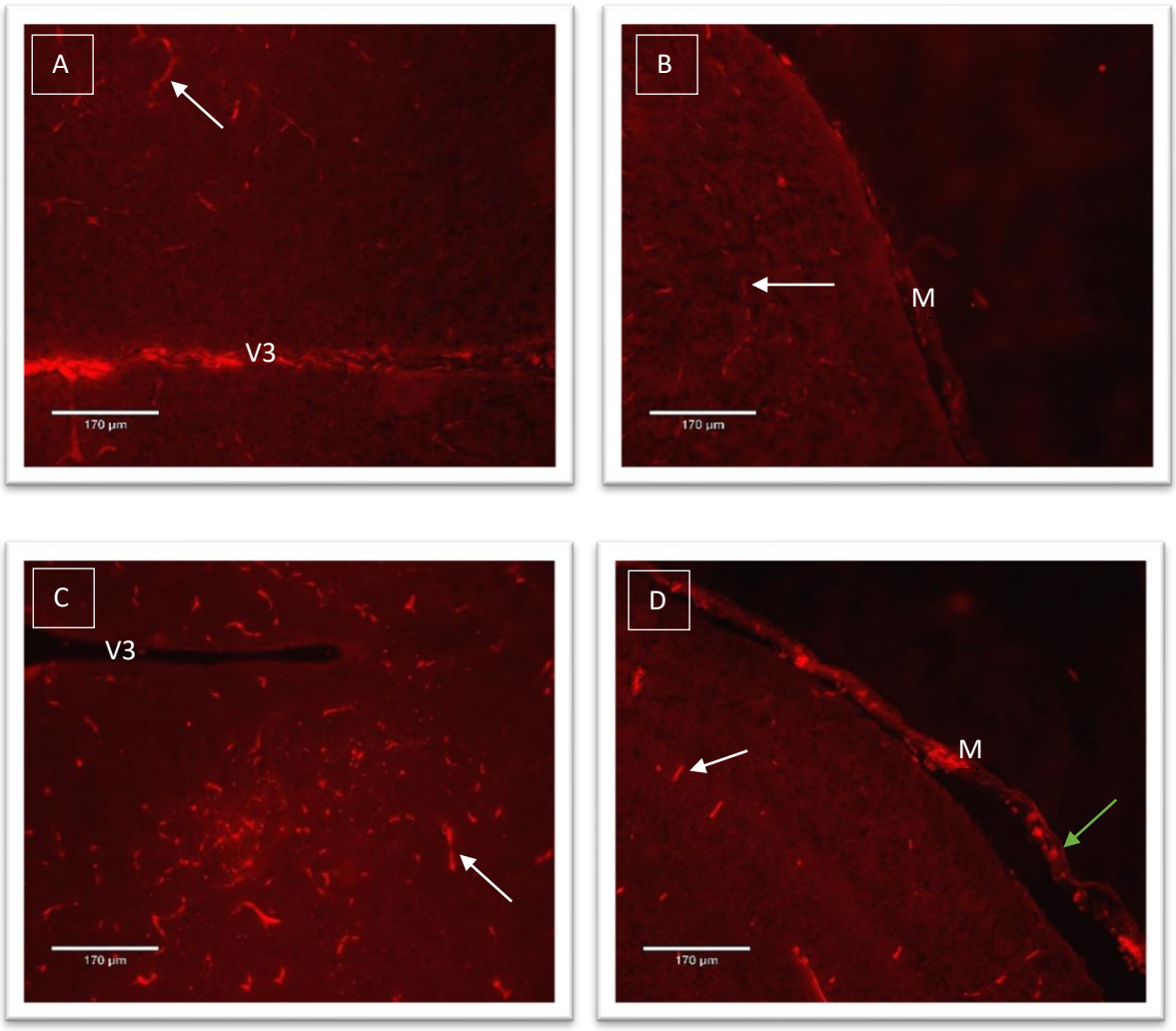

Figure 15: Representative photos taken with 10x magnification from brain regions with the microscope Echo Revolve. A. Brain parenchyma along the third ventricle in a female quail (E10) injected with 30µl of fluorescent DiI liposomes in the albumen. B. Brain parenchyma near the meninges in a male quail (E12) injected with 100µl of fluorescent DiI liposomes in the albumen. C. Brain parenchyma along the third ventricle in a female quail injected with 30µl of fluorescent DiI liposomes in the yolk. D. Brain parenchyma of the optical lobe in a male quail (E12) injected with 30µl of fluorescent DiI liposomes in the yolk. V3, third ventricle; M, Meninges; Scale bars: 170µm; white arrows: pointing to blood vessels; green arrows: pointing to cells.

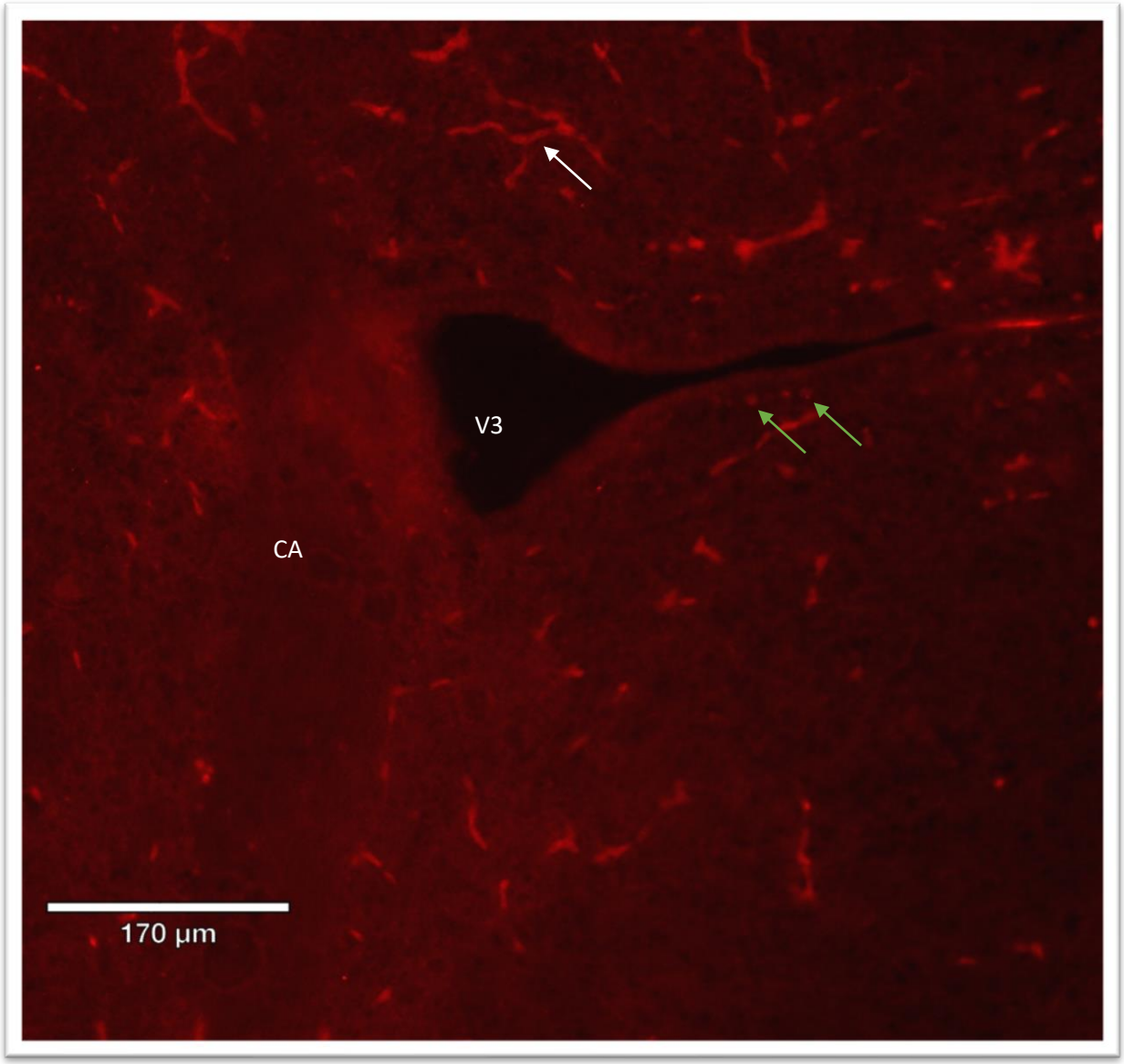

Figure 16: Representative photo taken with 10x magnification from the brain parenchyma along the third ventricle in a female quail (E10) injected with 30µl of fluorescent DiI liposomes in the yolk brain regions with the microscope Echo Revolve. V3, third ventricle; CA, anterior commissure; Scale bars: 170µm; white arrows: pointing to blood vessels; green arrows: pointing to cells.

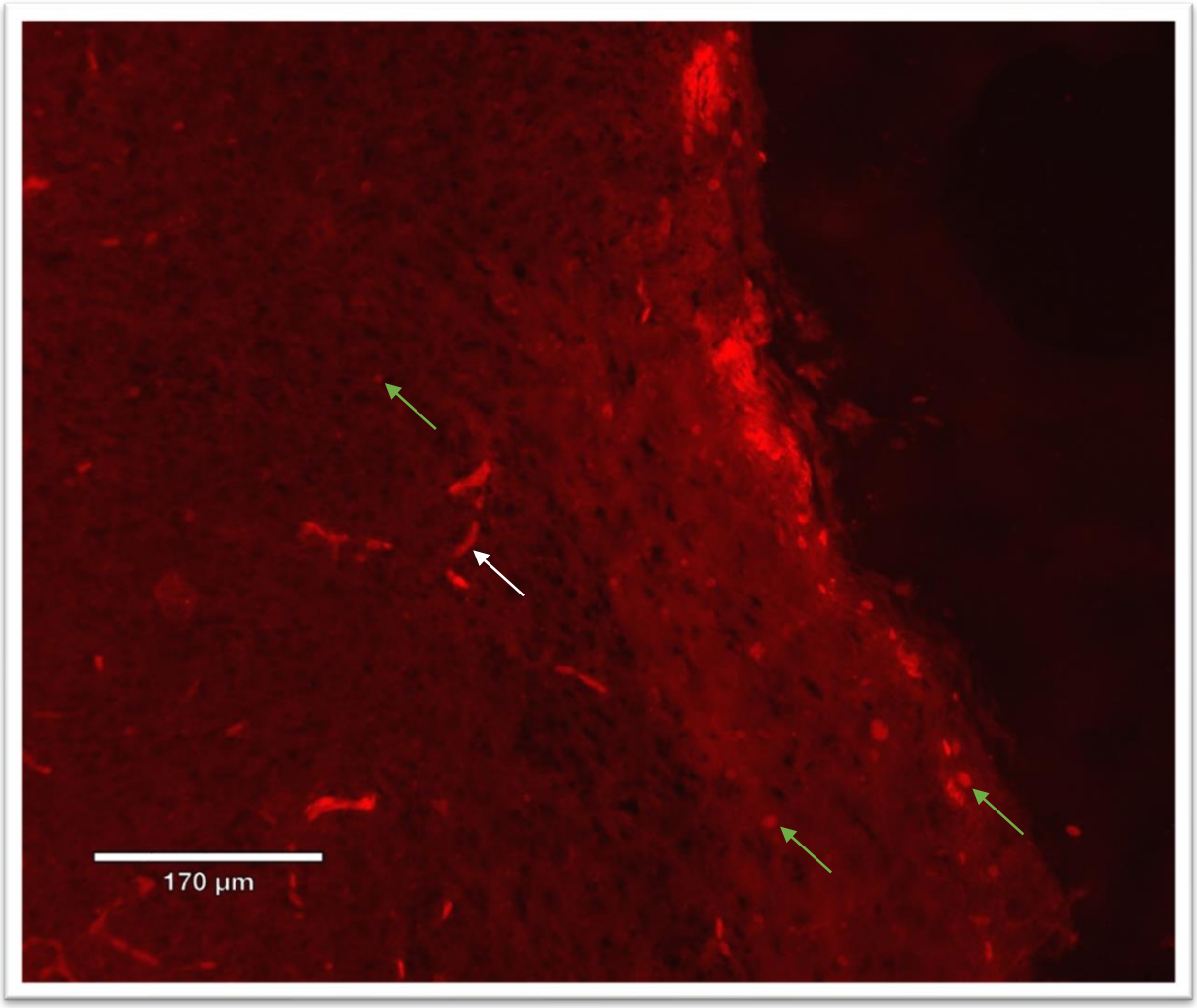

Figure 17: Representative photo taken with 10x magnification from the brain parenchyma along the meninges in a female quail (E10) injected with 30µl of fluorescent DiI liposomes in the albumen with the microscope Echo Revolve. M, meninges; Scale bars: 170µm; white arrows: pointing to blood vessels; green arrows: pointing to cells.

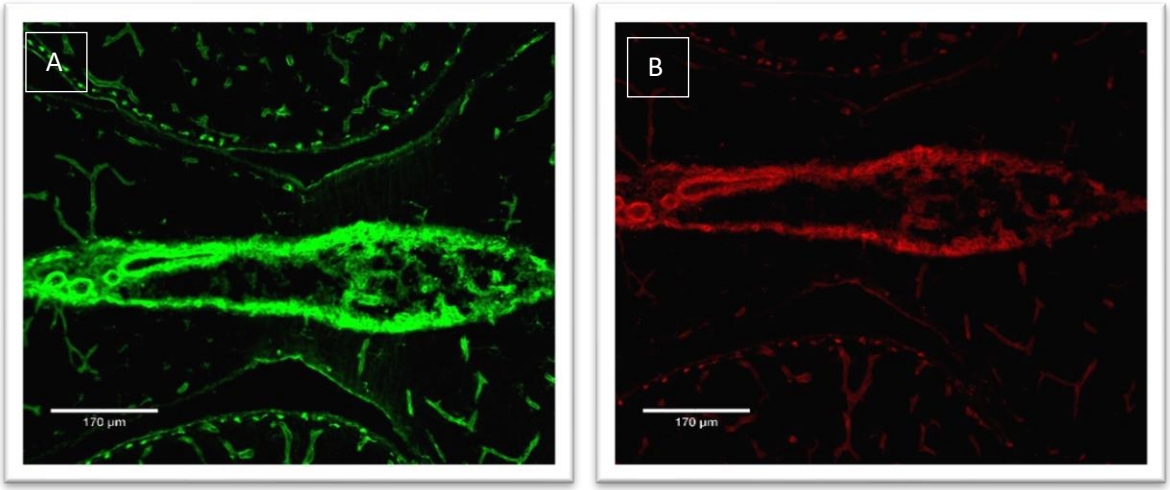

Figure 18: Representative photo taken with 10x magnification from the brain parenchyma along the lateral ventricles in a female quail (E12) injected with 100µl of fluorescent DiI liposomes in the yolk with the microscope Echo Revolve. The two picture show that RCA-1 staining can be detected in both the green and red spectrum. A. Immunofluorescence analysis in the green spectrum B. Immunofluorescence analysis in the red spectrum. VL, lateral ventricle; Scale bars: 170µm; white arrows: pointing to blood vessels; green arrows: pointing to cells.

2. The Effect of Estradiol Benzoate on Microglial Numbers in the Medial Preoptic Nucleus

The sensitive period of the quail brain to estrogens occurs from E6 to E12 in the quail (Adkins, 1979; Balthazart et al., 1992). Previous experiments conducted by Delage & Cornil (2020) showed the existence of a sex difference in the number of microglia between E9 and E12, with female quail having more microglia than males. This sex difference depends on estrogen bioavailability (Delage & Cornil, 2020). We wanted to replicate the results of Delage & Cornil (2020) on E12 using a different immunostaining method, namely the lectin RCA-1. Furthermore, the female quail is not fully demasculinized after E12, and complete demasculinization is thought to be achieved during the first two to four weeks after hatching (Balthazart & Schumacher, 1984; Schumacher & Balthazart, 1984). Hence the decision to investigate the number of microglia on PN1. For both development days, eggs were treated at E7 with exogenous estradiol (EB), a treatment known to sex reverse the expression of male sexual behavior (Balthazart et al., 1992; Cornil et al., 2011). Afterward, the brains were collected, and microglia were identified by the biotinylated lectin RCA-1 coupled to a streptavidin AlexaFluor 488.

2.1 No Sex Difference in the Microglial Count and Morphology on E12

Many blood vessels within the parenchyma were stained. As illustrated in Table 1, immunostained microglia were either amoeboid, poorly, or not ramified (see also Figures 20 and 21), representing immature microglia. Most of the immature microglia were microglia with long and thick processes. No surveying microglia, characteristic of adult microglia, was found in any animal (see Figure 14d for comparison). This confirms that only immature microglia are present during embryogenesis. Other authors reported that the proportion of ramified to amoeboid microglia and the complexity of their ramifications increase in newborn quail (Cuadros et al., 1994, 1997). Transitioning microglia represented the most abundant category, which was also observed by Delage & Cornil (2020). To compare the number of microglia with the data of Delage & Cornil (2020), we pooled all microglia with ramifications (transitioning microglia with stout processes or long and thick processes) into one group called transitioning microglia. We also calculated for each group the number of microglia per mm^2. On average, we observed 88 total microglia in the POM per mm^2 on E12.

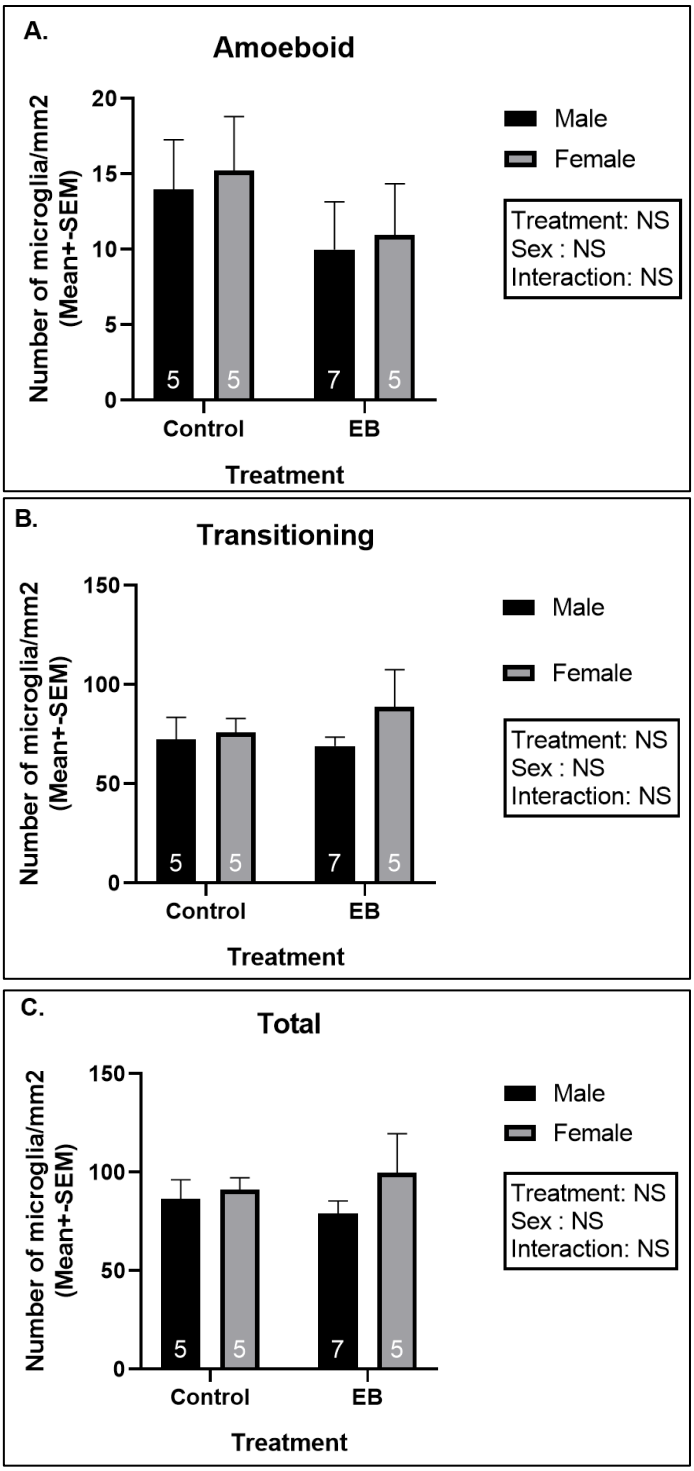

Figure 19: Number of microglia of each subtype per mm2 in the medial preoptic nucleus of males and females on embryonic day E12. The statistical boxes provide the results of separate two-way ANOVAs with sex and treatment as independent factors. EB, estradiol-benzoate treatment; N.S., non-significant. The number in each bar indicates the number of animals per group.

Table 1: Analysis of the densities (microglia/mm^2) of different types of microglia in the medial preoptic nucleus and the effect of treatment at E12.

	Sex	CTRL	EB	Statistics
Amoeboid	M	14 ± 3	10 ± 3	Treatment: $F_{1,18}$= 1.465; p=0.2417
	F	15 ± 4	11 ± 3	Sex: $F_{1,18}$= 1.015; p=0.7537
				Interaction: $F_{1,18}$=0.001641; p=0.9699
Transitioning microglia with stout processes	M	11 ± 3	6 ± 2	Treatment: $F_{1, 18}$= 0.01477; p=0.9046
	F	8 ± 2	14 ± 6	Sex: $F_{1, 18}$= 0.6786; p=0.4208
				Interaction: $F_{1, 18}$ = 3.130; p=0.0938
Transitioning microglia with long and thick processes	M	61 ± 11	63 ± 3	Treatment: $F_{1, 18}$= 0.1795; p=0.6768
	F	68 ± 7	75 ± 18	Sex: $F_{1, 18}$= 0.7765; p=0.3898
				Interaction: $F_{1, 18}$= 0.05234; p=0.8216

Note: The table shows the means ± SEM of the measures. The last column represents the statistical results of the two-way ANOVA with sex and treatment as independent factors. CTRL, control group; EB, estradiol-benzoate treatment.

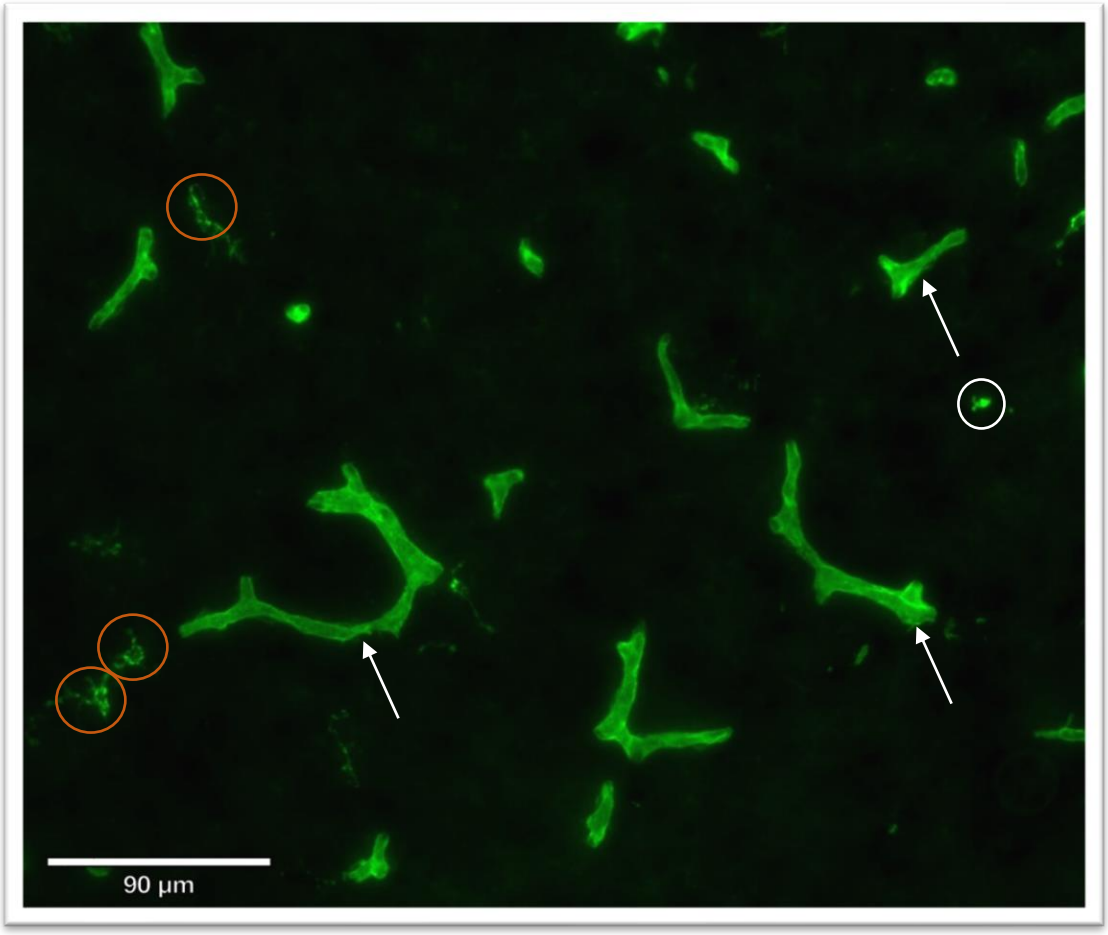

Figure 20: Representative photo taken with 20x magnification from the medial preoptic nucleus at E12 with the microscope Echo Revolve. The tissue was stained with biotinylated RCA-1. White circle: transitioning microglia with stout processes; Orange circle: transitioning microglia with long and thick processes; White arrow: blood vessels; scale bar: 90µm.

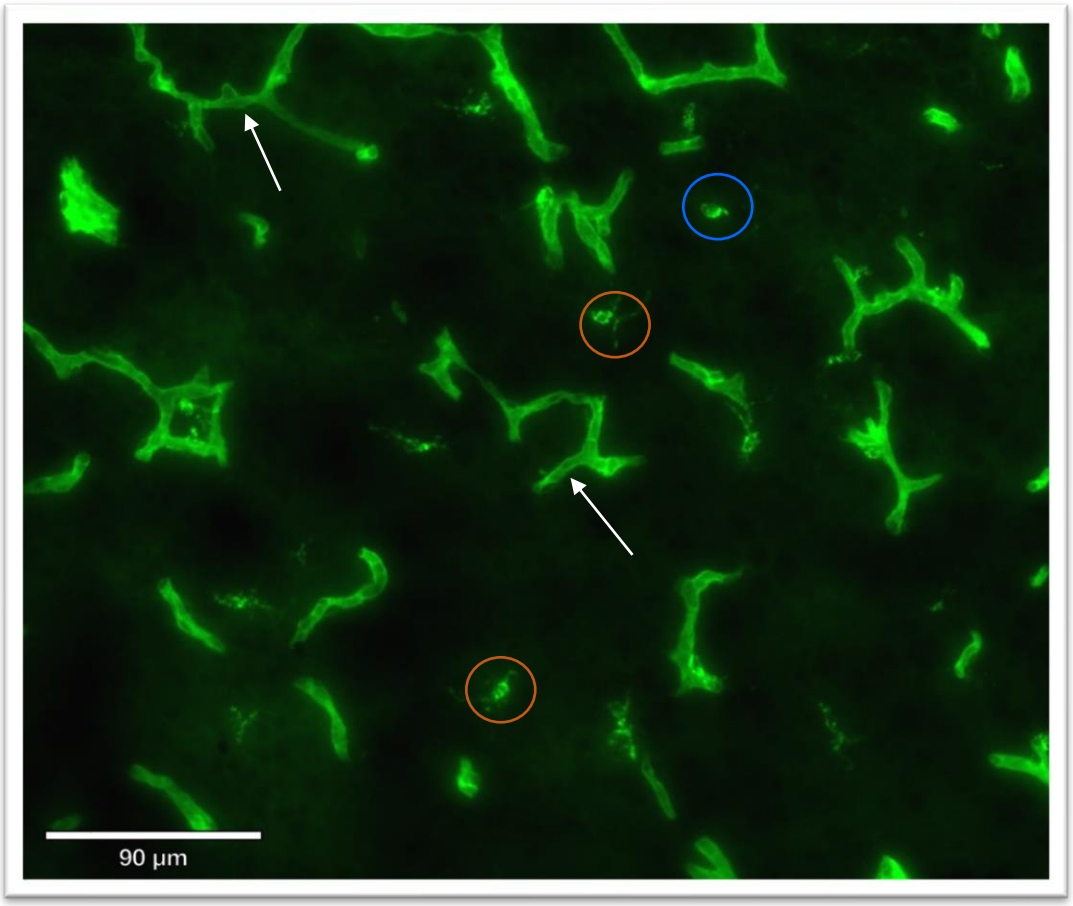

Figure 21: Representative photo taken with 20x magnification from the medial preoptic nucleus at E12 with the microscope Echo Revolve. The tissue was stained with biotinylated RCA-1. Blue circle: amoeboid microglia; Orange circle: transitioning microglia with long and thick processes; White arrow: blood vessels; scale bar: 90µm.

No clear sex difference concerning the number of microglia was observed at E12. EB treatment also did not affect the microglial count (Figure 19). Accordingly, separate two-way ANOVAs with sex and treatment as independent factors did not unveil any sex ($F_{1,18}$ = 1.015; p = 0.7537) or treatment effect ($F_{1,18}$= 1.465; p = 0.2417) as well as no interaction between the two factors for amoeboid microglia ($F_{1,18}$= 0.001641; p = 0.9699). There was no sex ($F_{1,18}$=1.174; p=0.2929) or treatment ($F_{1,18}$=0,1928; p=0.6658) effect for transitioning microglia and no sex ($F_{1,18}$= 1.299; p= 0.2693) or treatment ($F_{1,18}$= 0.002946; p= 0.9573) effect for total microglia. Moreover, no interaction between the two factors for transitioning microglia ($F_{1,18}$= 0.5553; p= 0.04658) nor total microglia ($F_{1,18}$= 0.4979; p= 0.4895) could be observed.

These results indicate an absence of a sex difference in the microglial count. Providing EB to embryos did not result in a sex reversal of the number of microglia in the POM. Therefore, our results contradict previous reports from Delage & Cornil (2020).

2.2 No Sex Difference in the Microglial Count and Morphology on PN1

Many blood vessels within the parenchyma were stained too, as already observed on E12. Only a few immunostained microglia were poorly or not ramified at all. As shown in Table 2, microglia with long and thick processes were primarily observed as expected, as amoeboid cells are progressively replaced by ramified cells with age (Cuadros et al., 1994, 1997; Schwarz et al., 2012). Microglial ramifications and processes seem to be increased and thinner, and the cell body is smaller compared to E12 (Figure 24). It appears that microglia are more numerous at this age as compared to E12, similar to what is observed by the research team of Cuadros (Cuadros et al., 1994).

The quality of the tissue was compromised, particularly in the central region of the brain, as evident in Figure 22, indicated by a white square, and Figure 23. It appears that the tissue was damaged, potentially due to an issue with the fixation process, leading to the subsequent destruction of the brain tissue during freezing. Because of that, quantification of microglia was done on only a fraction of the collected brain tissues. Additionally, counting microglia proved to be more challenging compared to E12 due to insufficient brightness of the staining, which was probably a consequence of the fixation issue. Microglia could also not be easily counted compared to E12, as the staining was not bright enough. The staining intensity progressively decreased from the periphery to the center of the tissue.

To compare the number of microglia with the data of Delage & Cornil (2020), we pooled all microglia with ramifications (transitioning microglia with stout processes or long and thick processes) into one group called transitioning microglia. We also calculated for each group the number of microglia per mm^2. On average, we observed 162 total microglia in the POM per mm^2.

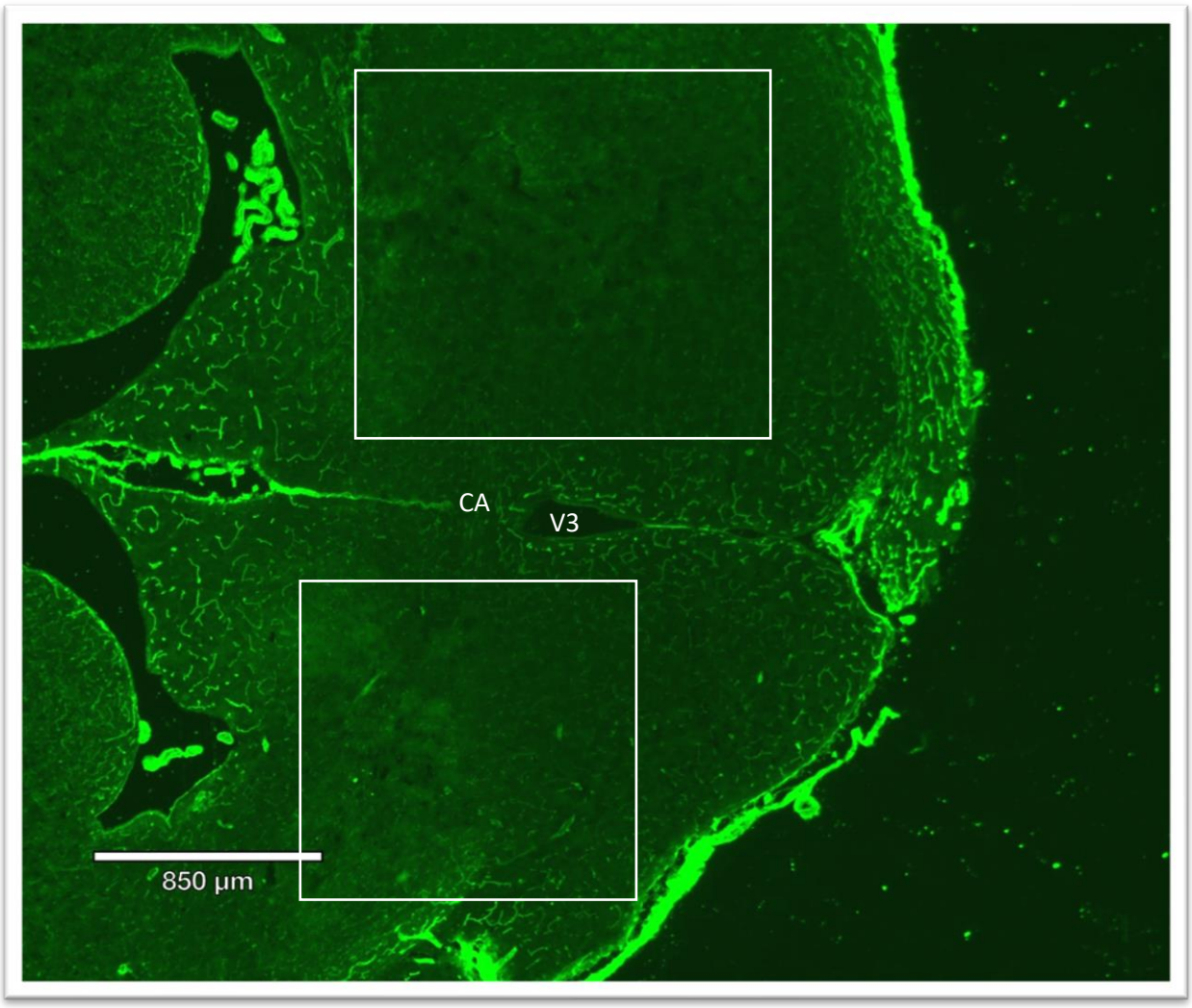

Figure 22: Representative photo taken with 2x magnification from the brain at PN1 with the microscope Echo Revolve. The tissue was stained with biotinylated RCA-1. White square: destroyed brain parenchyma; CA, anterior commissure; V3, third ventricle; scale bar: 90µm.

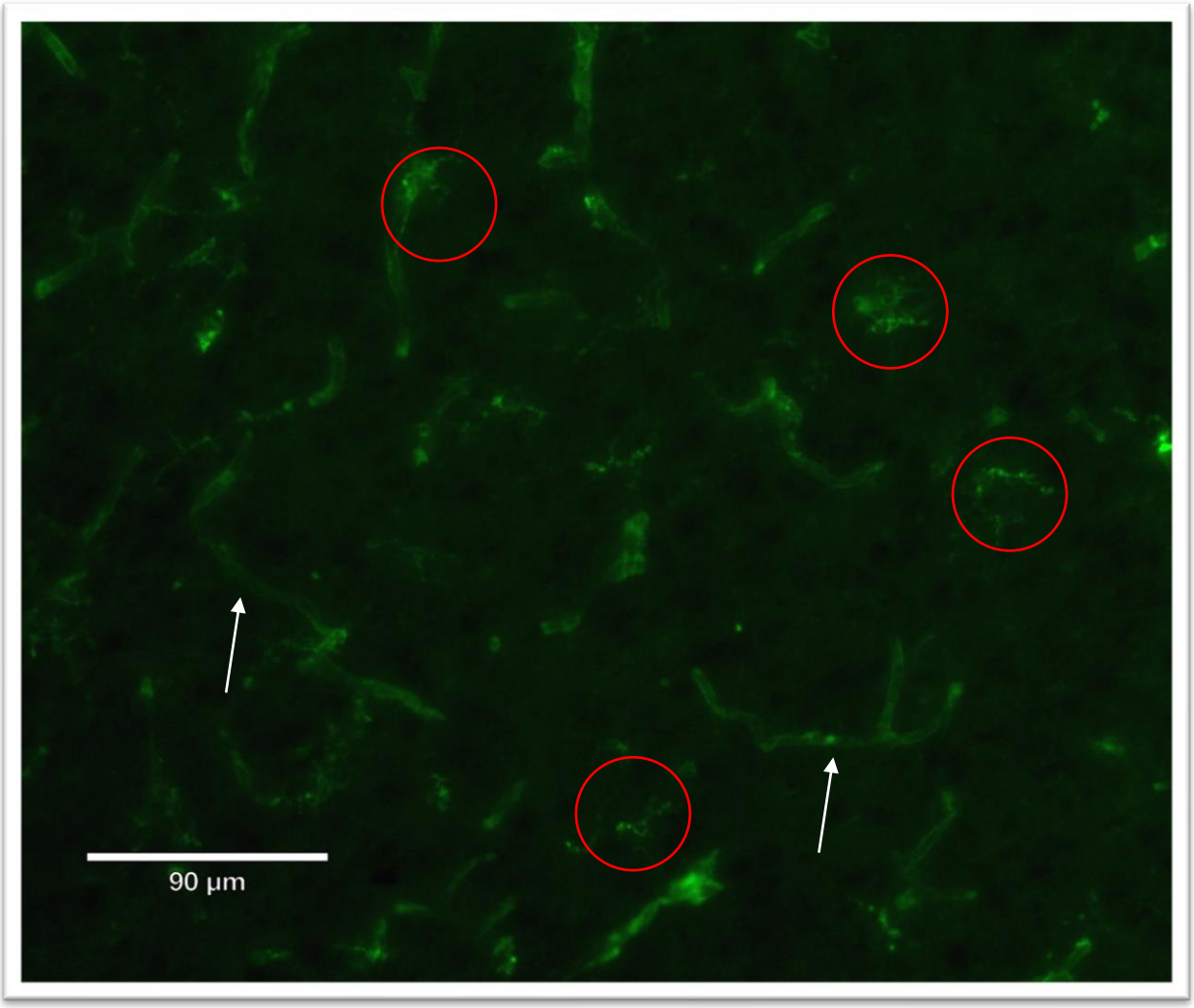

Figure 23: Representative photo taken with 20x magnification in the medial preoptic nucleus at PN1 with the microscope Echo Revolve. The tissue was stained with biotinylated RCA-1. The tissue seems to be destroyed, and the staining is not bright. White arrow: blood vessel; red circle: potential microglia scale bar: 90µm.

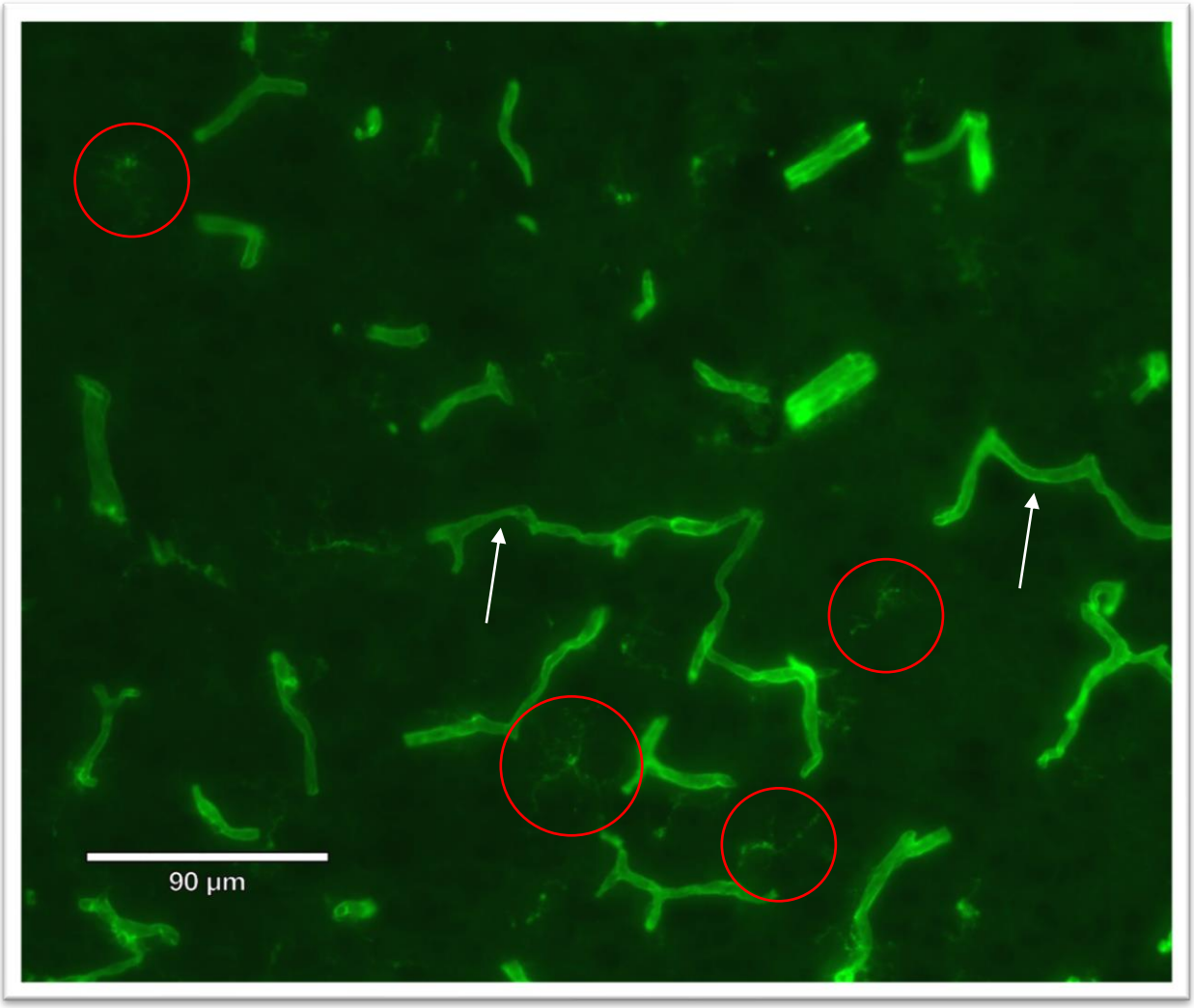

Figure 24: Representative photo taken with 20x magnification in the medial preoptic nucleus at PN1 with the microscope Echo Revolve. The tissue was stained with biotinylated RCA-1. Microglia with thinner ramifications and a smaller cell body can be observed. White arrow: blood vessel; red circle: potential microglia scale bar: 90µm.

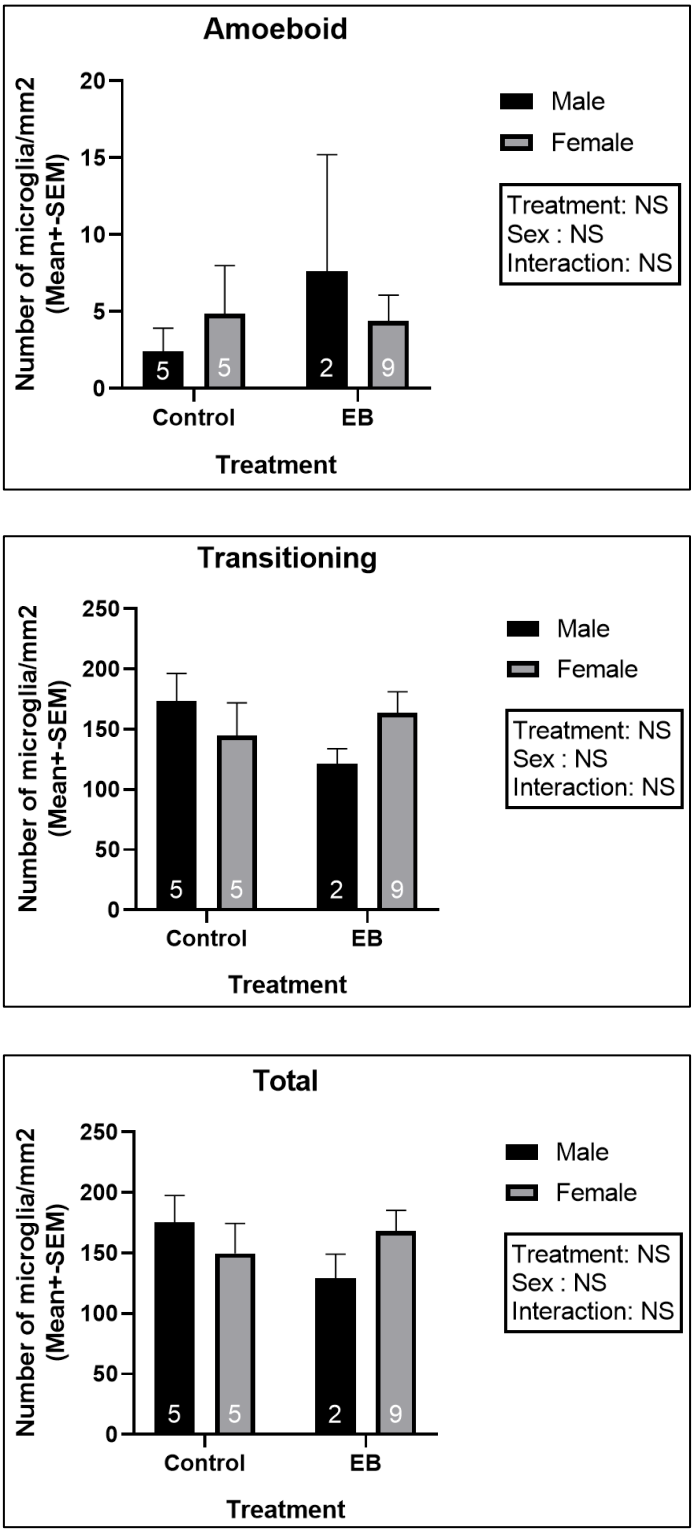

Figure 25: Number of microglia of each subtype per mm² in the medial preoptic nucleus of males and females on postnatal day 1. The statistical boxes provide the results of separate two-way ANOVAs with sex and treatment as independent factors. The number in each bar indicates the number of animals per group. EB, estradiol-benzoate treatment; N.S., non-significant.

Table 2: Analysis of the densities (microglia/mm2) of different types of microglia in the medial preoptic nucleus and the effect of treatment on PN1.

	Sex	CTRL	EB	Statistics
Amoeboid	M	2 ± 1	8 ± 8	Treatment: $F_{1,17}$= 0.6605; p=0.4276
	F	5 ± 3	4 ± 2	Sex: $F_{1,17}$= 0.01808; p=0.8946
				Interaction: $F_{1, 17}$= 0.9533; p=0.3426
Transitioning microglia with stout processes	M	5 ± 4	5 ± 2	Treatment: $F_{1, 17}$= 0.02007; p=0.8889
	F	4 ± 1	5 ± 3	Sex: $F_{1, 17}$= 0.002887; p=0.9577
				Interaction: $F_{1, 17}$ = 2.386e-005; p=0.9962
Transitioning microglia with long and thick processes	M	168 ± 26	117 ± 14	Treatment: $F_{1, 17}$= 0.3605; p=0.5562
	F	141 ± 28	159 ± 17	Sex: $F_{1, 17}$= 0.06676; p=0.7992
				Interaction: $F_{1, 17}$= 1.650; p=0.2162

Note: The table shows the means ± SEM of the measures. The last column represents the statistical results of the two-way ANOVA with sex and treatment as independent factors. CTRL, control group; EB, estradiol-benzoate treatment.

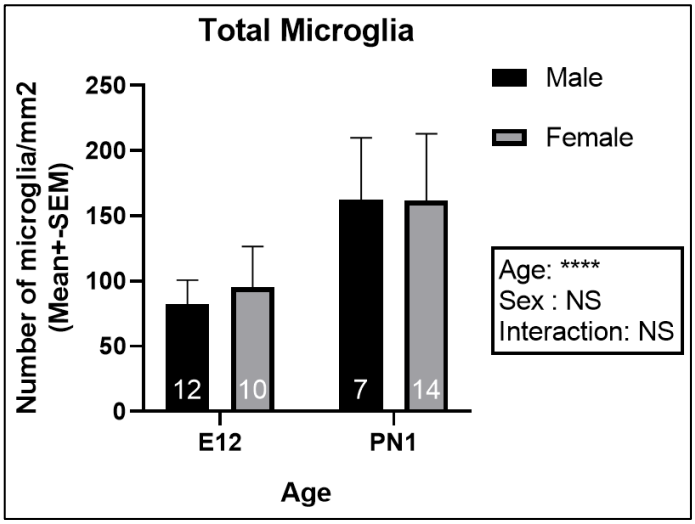

*Figure 26: Number of total microglia per mm² in the medial preoptic nucleus of males and females on embryonic day 12 (E12) and postnatal day 1 (PN1). The statistical boxes provide the results of separate two-way ANOVAs with sex and treatment as independent factors. The number in each bar indicates the number of animals per group. E12, embryonic day 12; PN1, postnatal day 1; ****p < 0.0001; N.S., non-significant.*

No clear sex difference concerning the number of microglia was observed at PN1. EB treatment did also not affect the microglial count (see Figure 28). Accordingly, separate two-way ANOVAs with sex and treatment as independent factors did not unveil any sex ($F_{1,17}$ = 0.01808; p = 0.8946) or treatment effect ($F_{1,17}$= 0.6605; p = 0.4276) as well as no interaction between the two factors for amoeboid microglia ($F_{1,17}$= 0.9533; p = 0.3426). There was no sex ($F_{1,17}$=0.06693; p=0.7990) or treatment ($F_{1,17}$=0.3811; p=0.5452) effect for transitioning microglia and no sex ($F_{1,17}$= 0.06865; p= 0.7965) or treatment ($F_{1,17}$= 0.2885; p= 0.5981) effect for total microglia. Moreover, no interaction between the two factors for transitioning microglia ($F_{1,17}$= 1.801; p= 0.1972) nor total microglia ($F_{1,17}$= 1.609; p= 0.2217) could be observed.

These results indicate an absence of a sex difference in the number of microglia. Providing EB to embryos did not result in a sex reversal of the number of microglia in the POM. Therefore, our results imply that microglia are probably not sexually differentiated on PN1 in the POM of Japanese quail.

2.3 Increase of the Total Microglial Count with Age

We wanted to compare the number of total microglia between E12 and PN1. As we could not observe any treatment effect on both E12 and PN1, we pooled all treatment groups per development day into one group. As represented in Figure 26, there are significantly more total microglia per mm^2 on PN1 than on E12. Accordingly, separate two-way ANOVA with sex and age as independent factors revealed an effect of age ($F_{1,39}$ = 34.69; p<0.0001) but not an effect of sex ($F_{1,39}$=0.2686; p=0.6072) as well as no interaction ($F_{1,39}$=0.3139; p=0.5785) between the two factors. These results indicate an increase in the number of microglia between E12 and PN1.

Discussion

The focus of this master´s thesis was the role of microglia during the brain sexual differentiation in quail. Investigating the role of microglia during brain sexual differentiation in quail is beneficial. Sexual differentiation is a complex process, thus, studying the role of microglia during this process might further enhance our understanding of the underlying processes. Also, as briefly mentioned during this master´s thesis, microglia play a role in neuroinflammatory processes such as PGE2 production. Bringing light into microglial function could unravel the potential effects of neuroinflammatory conditions on sexual differentiation. Exploring the role of microglial phagocytosis during brain sexual differentiation may provide clues about the prevailing sex difference in the susceptibility to many diseases, such as autism spectrum disorder and attention deficit hyperactivity disorder. The Japanese quail is an interesting animal model to investigate, as their sexual differentiation also depends on estrogens, but as opposed to rodents, in the quail, estrogens demasculinize the females (McCarthy et al., 2017). While the involvement of microglia in brain sexual differentiation has been studied to some extent in rodents, there is limited knowledge regarding what is happening in the quail brain. Hence, exploring microglial dynamics in the context of brain sexual differentiation in quail can provide valuable insights into this unexplored area.

For the first objective, we wondered whether there exists a sex difference in the number of phagocytic microglia in the POM of the developing brain on E10 and E12 in quail as increased microglial phagocytosis might drive to sex differences in the quail brain. To visualize the phagocytic microglia in the POM, we wanted to test a new method by injecting fluorescent DiI liposomes directly into either the yolk or the albumen of the quail egg 24h before collecting the brains. No phagocytic microglia were detected under the microscope.

The second objective was to replicate the findings of Delage & Cornil (2020) using a different but more specific microglial marker, namely the antibody anti-CSF1R. The rationale behind this approach was that potential variations in microglial detection and quantification techniques are observed depending on which marker or method is used (Bridlance & Thion, 2023; Delage & Cornil, 2020). Our aim was then to determine if there exists a sex difference in the number of microglial cells in the POM on E12 and whether the sex difference relies on hormonal exposure during the sensitive period to estrogens. We also wondered whether there exists a sex difference in the microglial count on PN1 in quail as it was reported that females remain sensitive to the demasculinizing effect of estrogen during the first weeks after hatching, a process that may be driven by microglia (Balthazart & Schumacher, 1984; Schumacher & Balthazart, 1984). If a sex difference is observed, we also wanted to know whether it relies on hormonal exposure during the sensitive period to estrogens. Unfortunately, we were unable to obtain any results with the novel microglial marker antibody anti-CSF1R, leading us to proceed with our research using the lectin RCA-1. Concerning the results of E12, no clear sex difference could be observed in the number of microglia in the POM, and manipulation of

estrogen availability at E7 also did not affect microglial numbers and morphology in males. These findings disagree with the results of Delage & Cornil (2020), where immature microglia were more abundant in the POM of females (Delage & Cornil, 2020). In addition to analyzing the microglial count on E12, we also extended our analysis to PN1. Unfortunately, during the quantification of microglia on PN1, we encountered compromised brain tissue, likely resulting from issues during the fixation process. This compromised tissue impeded the microglial analysis. Despite these difficulties, our analysis did not reveal any significant sex differences in microglial numbers but revealed an increase in the number of microglia counted between E12 and PN1, as expected based on previous work in quail and rodents. Furthermore, we found no evidence to suggest that estrogen availability had an impact on microglial counts during the sensitive period.

The following section focuses on the challenges and limitations encountered during the analysis of DiI liposomes, including potential reasons for the absence of signal and alternative methods to study phagocytic microglia in the quail POM. The discussion also addresses the absence of the EB treatment on the microglial numbers, as well as the increase of the number of microglia during development and alternative methods for further research.

1. No Immunofluorescence Analysis for DiI Liposome Injections

Our aim was to develop a novel detection method to address the current lack of techniques for studying the number of phagocytic microglia in the quail POM. In order to track microglia that might be implicated in phagocytosis, we performed injections of fluorescent DiI-liposomes into the quail egg. Analysis of the brain tissue under the microscope showed that some “fluorescent bodies” along the ventricles and in the meninges could be observed, but only when the intensity and the opening time of the microscope camera were increased drastically. Extending the opening time of the camera can lead to increased non-specific staining and hence the assumption that the observed staining does not correspond to a microglial cell that had engulfed the DiI liposomes. Several arguments may explain these results:

1) An insufficient volume of DiI liposomes was injected. We injected 30ul and 100ul of fluorescent DiI liposomes as recommended by the manufacturer. However, as we injected into the egg and not directly into the embryo, it is possible that the liposomes were diluted in the content of the egg. This dilution could have led to a lower concentration of liposomes reaching the quail POM, potentially affecting the visibility and detection of phagocytic microglia.

2) The existence of the blood-brain barrier should be considered. Our method involved injecting liposomes into the apex of the egg using a needle. However, it is plausible that the liposomes were unable to penetrate through the developing cranium and blood-brain barrier. In mice, the blood-brain barrier starts developing between E15 and E19 (Haddad-Tóvolli et al., 2017). Very little is

known about the blood-brain barrier in quail and other avians. In the quail, it is speculated that the blood-brain barrier may form around E11, as indicated by various blood vessel markers used to assess the integrity of the blood-brain barrier (Ikeda et al., 2008; Stewart & Wiley, 1981). The liposomes may have difficulties crossing the blood-brain barrier. One potential approach is administering the liposomes directly into the brain (Han et al., 2019). However, as the quail develops *in ovo*, reaching the brain is quite difficult. Intracerebral injections come with risks, as they can induce injury and release of inflammatory cytokines. Additionally, the process of reaching the embryo within the egg, performing the injection, and subsequently sealing the egg while ensuring the proper development of the embryo until hatching poses a significant challenge. Another method would be injecting the liposomes directly intravenously into the chorioallantoic membrane (CAM)[2] after having transferred the content of the egg into a little container allowing the further development of the embryo (Dunn & Boone, 1976; H. Tay et al., 2020). Further investigation is required to evaluate the feasibility of this approach.

3) No further verification of the phagocytic capability of microglia in quail was conducted. Our objective was to assess the number of microglia involved in phagocytosis by injecting DiI liposomes. However, we did not verify whether microglia in quails at E10 and E12 are indeed phagocytic. It is possible that the absence of a signal was due to the lack of microglial cells engaged in phagocytosis. To address this issue, an alternative method to analyze the phagocytic activity of microglia could be employed. Unfortunately, Delage had attempted to address this problem previously but was unable to identify a marker that could be used to evaluate the phagocytic activity of microglia in quails (as stated in Delage's dissertation).

4) Another explanation for the lack of a signal could be attributed to the timing of the DiI liposome injections. Microglia start invading the quail brain around E9 (Cuadros et al., 1994, 1997). If the injection was performed too early in development when microglia are in the early stages of invading the brain, it could result in an absence of a detectable signal. An alternative approach would be injecting during post-natal development, as this would allow us to inject the liposomes directly into the ventricles of the brain. Injecting directly into the brain could enhance their delivery and uptake by microglia, increasing the chances of obtaining a visible signal.

We encountered another technical obstacle while analyzing the brain tissue. The excitation and emission maxima of fluorescence DiI liposomes are at 549nm and 656nm, respectively (ThermoFisher Scientific, n.d.), meaning it covers the orange spectrum. Regarding the AlexaFluor 488 streptavidin, its fluorescence excitation and emission maxima are at 495nm and 519nm, respectively,

[2] Chorioallantoic membrane: a highly vascular yet not innervated membrane resulting from the fusion of two extraembryonic membranes, the chorion, and the allantois. It is the avian homolog of the mammalian placenta, whose primary function is exchanging gases and nutrients (Yuan et al., 2014).

and therefore, it covers the green spectrum (Invitrogen Thermofisher; REF: S11223). Surprisingly, immunofluorescence analysis on the microscope Echo Revolve of RCA-1-stained brain tissue of embryos showed a positive signal within the green and when the exposure time was extended, also in the red spectrum. The observed phenomenon can be attributed to the emission signal from streptavidin Alexa Fluor 488 leaking into the red channel. This occurs when there is a spectral overlap between the emission spectra of the fluorophore used due to the limitations of the optical filters used in the microscope. For this reason, we conducted a second examination of the tissue on the confocal microscope Leica SP5. The signal for Alexa Fluor 488 in the red spectrum was absent, which can be attributed to the improved spectral separation and elimination by confocal imaging. Moreover, no signal for the DiI liposomes was seen, reinforcing the conclusion that our experiment was unsuccessful.

1.1 Alternative Methods and Future Investigations

A possible alternative method to the liposome injections would be, for example, using a general marker for microglia in quail such as QH1 or RCA-1, and to assess the number of phagocytic microglia, a marker for apoptotic cells could be used, as reported by Sierra-Martin et al., (2023) in the quail retina (Sierra-Martín et al., 2023). Also, it would be interesting to test further whether injecting the DiI liposomes in the CAM or directly in the brain would allow us to analyze the number of phagocytic microglia. If this technique works, it will allow the multi-parametric analysis of those cells with FACS.

2. Effect of Estradiol Benzoate on Microglial Numbers in the Medial Preoptic Nucleus

2.1 Absence of EB Treatment Effect in Quail

Our goal was to replicate the results from Delage & Cornil (2020) but with a different microglial marker. We observed no sex difference in the microglial count, and this sex difference could not be modulated by E2 treatment. Our observation contrasts with the findings of Delage & Cornil (2020), who observed that the administration of exogenous E2 resulted in a sex reversal of the microglial count (Delage & Cornil, 2020). Also, we observed overall less microglia than Delage & Cornil (2020). We counted around 88 total microglia per mm^2, and they reported more total microglia, around 160 total microglia per mm^2, on E12 (Delage & Cornil, 2020). These discrepancies between our results and those of Delage & Cornil (2020) could be explained by:

1) Difficulties arose in identifying the region of interest as the POM was not visualized by DAPI staining. This limitation made it challenging to accurately distinguish the POM from the surrounding tissue. To overcome this issue, we aligned the quantification field of the microscope along the third ventricles and the anterior commissure. As a result, there is a possibility that during the quantifica-

tion process, microglia from the neighboring tissue were inadvertently counted instead of those specifically belonging to the POM.

2) Delage & Cornil (2020) used a different quail breeder and, consequently, different animals with potentially distinct genetic backgrounds. This variation in the genetic makeup of the experimental subjects would introduce an important factor that can influence the results and interpretation of the study.

3) A different approach was taken in our study by employing a staining protocol using the biotinylated lectin RCA-1 coupled to a fluorescent streptavidin to visualize microglia, whereas Delage & Cornil (2020) used an antibody anti-QH1 to do immunohistochemistry. RCA-1 is a lectin that binds to β-δ-galactose residues and stains microglia and macrophages. This lectin has previously been described to label microglial cells in quail, rats, and the developing human brain (Andjelkovic et al., 1998; Boya et al., 1991; Delage & Cornil, 2020). QH1 is a monoclonal antibody that binds to quail endothelial and hemopoietic cells, but the recognized antigen sequence is unknown (Pardanaud et al., 1987). Co-labeling of RCA-1 and QH1 revealed some discrepancies between the two staining methods (Delage & Cornil, 2020), suggesting that both methods do not stain uniformly microglia. This variation between the staining methods could explain why we counted less microglia than Delage & Cornil (2020). The observed inconsistencies in microglial count between RCA-1 staining and QH1 staining, coupled with the unknown target of QH1 binding, suggest the potential staining of distinct subpopulations within the microglial population. These discrepancies raise the possibility that the two staining methods may have labeled different subsets of microglia, each representing specific subpopulations with unique characteristics or functions.

For PN1, while analyzing the brain tissue under the microscope, we observed that the tissue was destroyed. Microglia could not be easily counted compared to E12, as the staining was losing its brightness from the periphery to the center of the brain. We believed inadequate freezing was the culprit. The brains at PN1 are bigger in size but were bathed in sucrose and PAF4% as long as the brains at E12. It is possible that we should have extended the fixation process or reduced the brain size by cutting off brain regions that are not of interest. Because of that, quantification of microglia was done only on a fraction of the collected brains that were still intact. It is worth noting that even though we only used intact brain tissues, the staining was not uniform, and it might be possible that not all microglia were counted. Similar to the findings of Delage & Cornil (2020), we could not observe any sex difference in the number of microglia or their morphology on PN1. Administering E2 at E7 did not sex reverse the microglial count in male quail. One limitation of this research is that we did not manipulate estrogen availability directly after hatching but on E7. Therefore, we only assessed if E2 injection on E7 has a lasting effect on brain development, meaning whether it influences the brain also post-hatching. Furthermore, it is important to acknowledge that our findings might be influ-

enced by potential biases, particularly regarding the detection and quantification of microglia. Different techniques and methodologies for microglial detection can provide varying results, leading to differences in the reported numbers. Microglial detection and quantification methods can differ based on factors such as staining protocols, image acquisition, thresholding criteria (e.g., the morphological characteristics used to classify microglia in amoeboid microglia, microglia with stout processes, and microglia with long and thick processes), and quantification techniques. These variations can impact the accuracy and consistency of microglial counts across studies.

2.2 Increase of Microglial Count During Development

The results from the second experiment showed a significant increase in microglia between E12 and PN1. At E12, we counted, on average, 88 total microglia per mm^2, while on PN1, 162 total microglia per mm^2 were spotted. These findings agree with the observations of several other authors. In her master´s thesis, Elisa Dardenne observed a higher number of microglia indirectly by looking at their gene signature (Dardenne, 2022). For example, the expression of RUNX-1, a marker for microglia (Ginhoux et al., 2010), increases between E8 and PN1 (Dardenne, 2022). Cuadros et al. (1997) also observed growing numbers of microglial cells during development, starting at around E10, overlapping with the period of microglial invasion (Cuadros et al., 1997). In contrast, Delage & Cornil (2020) reported no change in the microglial count between E12 and PN1 (Delage & Cornil, 2020).

We could not identify any sex difference in the microglial count between males and females neither on E12 nor on PN1, regardless of their treatment. Interestingly, this coincides with the findings of Dardenne (2022), who observed no significant differences in the gene signature of microglia between males and females (Dardenne, 2022).

2.3 Microglial Morphology and Distribution within the Brain

Our data further confirmed the observations of other authors that embryonic microglia are immature, as no microglia with long and thin processes were detected at E12 (Cuadros et al., 1997; Delage & Cornil, 2020; Schwarz et al., 2012). With aging, we observed less amoeboid microglia and more microglia of the transitioning type. Furthermore, it also seemed that microglia had thinner processes and a smaller cell body at PN1 when compared to E12. However, our data did not reveal any sex difference in the morphology of microglia, regardless of their treatment, neither on E12 nor on PN1. Interestingly, Delage & Cornil (2020) showed that females had higher numbers of transitioning microglia and, to some extent, amoeboid microglia. This sex difference was detected at E12 but not at PN1 (Delage & Cornil, 2020).

We noticed many cells along the ventricles and meninges. Several authors suggest that microglia may use the third ventricle to enter the brain (Cuadros & Navascués, 1998; Delage & Cornil,

2020). Then, microglia migrate to the brain parenchyma, coinciding with our observations as we could observe many microglia throughout the brain (Cuadros & Navascués, 1998).

2.4 Alternative Methods and Future Investigations

During embryonic development, quail can be sexed by anatomical observations: males display two bilateral testes resembling two small grains of rice, while females possess asymmetrical gonads with only the left gonad that develops into a functional ovary, while the right one degenerates (Intarapat & Satayalai, 2014). However, because of the presence of the regressing right gonad, sexing of embryos can be challenging. Therefore, doing an RT-PCR to determine the sex additionally to the anatomical observations would be more beneficial.

To improve the staining outcome for the brains collected on PN1, the fixation process can be extended, or brain reduced in size by cutting off brain regions that are not of interest. Also, another fixative could be used, such as acrolein which is efficaciously fix adult brains (Taziaux et al., 2007). We also reported issues with the DAPI staining. As Nissl staining is known to visualize the POM (Delage & Cornil, 2020), another possible option would be testing if fluorescent Nissl can also be applied in the quail and if it visualizes the POM.

Conclusion and Perspective

To conclude, this master´s thesis gave an insight into the role of microglia during brain sexual differentiation. Also, we tried to introduce a new method to identify phagocytic microglia in the quail brain by using fluorescent DiI liposomes. For the latter, we injected different volumes of the liposomes in either the yolk or the albumen one day before collecting the quail brains, either on E10 or on E12. Unfortunately, the number of phagocytic microglia could not be analyzed after the liposome injections regardless of the treatment group, and therefore we cannot confirm the presence of phagocytic microglia. The absence of a detectable signal may be attributed to multiple factors. Firstly, it is possible that the injected volumes of DiI liposomes were too low to produce a measurable signal. Additionally, the possible presence of the blood-brain barrier could have limited the uptake of the liposomes by microglia, further hindering the detection of phagocytic microglia. In addition to the technical problems encountered, the absence of phagocytic microglia in our study may be due to their developmental stage at the time of injection and observation, suggesting they were not yet in a phagocytic state. Therefore, it would be interesting to conduct further investigations into the role of phagocytic microglia in quails, specifically by administering the DiI liposomes during postnatal development. Additionally, injecting the liposomes directly into the brain ventricles after hatching could enhance their delivery and uptake by microglia, enabling the staining process.

We could also not confirm the previous results of our lab, stating that females have more microglia in the medial preoptic nucleus at E12. In contrast to their findings, our results did not show any sex difference in the microglial count on E12. Also, administering exogenous E2 on E7 did not influence the microglial count on E12. It is noteworthy that we did not replicate the results of Delage & Cornil (2020) in the same conditions, as we used a different staining method and a different quantification field of the POM. We also investigated the microglial count on the first day after hatching. Similar to the findings reported by our lab, our results did not show a sex difference in the microglial number on PN1, and this could not be influenced by exogenous E2 treatment either. Quantification of microglia on PN1 might be impaired, as the brain tissues collected on PN1 were of low quality due to encountered issues during fixation and freezing. In contrast to what was observed by Delage & Cornil (2020), our results showed that there is a significant increase in the microglial count between E12 and PN1, which is in line with other recent data of microglial markers assessed by qPCR at different developmental ages (Dardenne, 2022). We also observed that embryonic microglia are immature, with an amoeboid morphology and stout processes. In light of these findings, a future objective would be to replicate the experiment investigating the role of microglia on PN1, but with a slightly modified fixation process to address the challenges encountered.

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