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Risk evaluation of neurodevelopmental disorders in offspring conceived by Assisted Reproduction Technologies.

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"Risk evaluation of neurodevelopmental disorders in offspring conceived by Assisted Reproduction Technologies".

ABSTRACT

The use of Assisted Reproductive Technologies (ART) is increasing every year. More than 8 million ART babies have been born worldwide. The use of ART is frequently linked with advanced maternal age (AMA) which may ulteriorly affect the success of such obtained pregnancies. Both ART and AMA are associated with pregnancy and birth complications and higher risk of neurodevelopmental disorders (NDD) in the offspring. The origin of these disorders are not elucidated. Data linking the use of ART with development of NDD in children are discordant: some don't demonstrate any evident neurodevelopmental alterations; others strongly associate distinctive symptoms of Autism Spectrum Disorder (ASD) in offspring obtained by ART in both human and animal models. This dissertation hypothesises that embryo modifications caused by ART procedures are associated with an increased risk of neurodevelopmental afflictions. The general objective of this study was to verify the extent of ART induced neurodevelopmental alterations, their intergenerational transmissibility, and to determine the base of revealed neurodevelopmental traits in embryos and offspring obtained by ART using an animal model.

In the first part of the thesis, the effect of *in vitro* culture of embryos on offspring development as well as social and anxiety-like behaviour was evaluated. I proved that offspring generated by ART are characterised by decreased sociability. I also proved that pups obtained through ART are characterised by perturbed growth curves during preweaning period.

In the second part of my research, by extending the observation of ART effect to the second generation of offspring, I showed intergenerational transmission of decreased social motivation as a consequence of ART applied on fathers; and increased anxiety combined with reduced

social motivation consequent of ART applied on mothers. The level of penetrance of the observed changes differs quantitatively (offspring survival) and qualitatively (behavioural and growth perturbations) between F1 and F2. Furthermore, the level of penetrance of quantitative aberrancies was exacerbated in F2 offspring obtained following crossing ART males with ART females.

In the third part of the experiments, I investigated the possible causes of reduced sociability in ART offspring. By analysing antioxidant activity and lipid metabolism in the brains of F1 offspring at prenatal stage and adulthood, I showed that ART prenatal brain development is impaired due to increased oxidative stress, elevated lipid peroxidation and, reduced expression of genes associated with neurogenesis which might cause changes in brain functionality and connectivity. Furthermore, I found that these impairments persist until adulthood, are shown by increased oxidative stress in hypothalamus from ART offspring and altered proteome in hippocampus, highlighting altered expression of proteins involved in neurogenesis, synapsis and proteins associated with several neurological disorders.

In the fourth part of my dissertation, knowing that neurodevelopment is the first occurring during embryogenesis even before placentation, I evaluated if the lipid damage and compositional alterations in ART prenatal brains could be a consequence of alterations occurring prior to implantation, by analysing the lipid profile of ART oocytes and embryos. I showed that the lipid profile of ART embryos is altered presenting sign of peroxidation; relevantly, I found similar altered lipid profile also in AMA oocytes (mouse and human) and embryos, frequently used in ART procedures. I showed for the first time that lipid damage ART-induced in the embryos may have long-term health consequences, being the possible origin of NDD in the offspring.

This research shows that ART causes lipid modifications in embryos leading to altered prenatal and adult brain development, decreased sociability in adult offspring, and furthermore its effects are transgenerational. This study facilitates better understanding of the etiology of NDD and opens up new scientific horizons for subsequent lipidomic studies pinpointing mechanisms contributing to NDD.

Key words: Assisted Reproductive Technology, Neurodevelopmental disorders*, in vitro* culture, behaviour, mouse model.

"Ocena ryzyka zaburzeń neurorozwojowych u potomstwa poczętego za pomocą technologii wspomaganego rozrodu".

Wykorzystanie technologii wspomaganego rozrodu (ART) rośnie z każdym rokiem. Na całym świecie urodziło się ponad 8 milionów dzieci poczętych w wyniku ART. Stosowanie ART jest często związane z zaawansowanym wiekiem matki (AMA), co może mieć wpływ na powodzenie takich ciąż. Zarówno ART, jak i AMA wiążą się z powikłaniami ciążowymi i porodowymi oraz wyższym ryzykiem zaburzeń neurorozwojowych (NDD) u potomstwa. Pochodzenie tych zaburzeń nie zostało wyjaśnione. Dane łączące stosowanie ART z rozwojem NDD u dzieci są rozbieżne: część z nich nie wykazała żadnych wyraźnych zmian neurorozwojowych, inne wykazały, że ogólne zachowanie potomstwa uzyskanego za pomocą ART wykazuje charakterystyczne objawy zaburzeń ze spektrum autyzmu (ASD) w modelach ludzkich i zwierzęcych. W niniejszej pracy postawiono hipotezę, że modyfikacje zarodków spowodowane procedurami ART wiążą się ze zwiększonym ryzykiem zaburzeń neurorozwojowych. Ogólnym celem badań było zweryfikowanie zakresu zmian neurorozwojowych wywołanych ART, ich międzypokoleniowej przekazywalności oraz określenie podstawy ujawnionych cech neurorozwojowych u zarodków i potomstwa uzyskanego za pomocą ART na modelu zwierzęcym.

W pierwszej części pracy oceniono wpływ hodowli *in vitro* zarodków na rozwój potomstwa oraz ich zachowania społeczne i lękowe. Udowodniłam, że potomstwo uzyskane w wyniku ART charakteryzuje się obniżonymi interakcjami społecznymi. Udowodniłam również, że młode uzyskane za pomocą ART charakteryzują się zaburzonymi krzywymi wzrostu w okresie przedodsadzeniowym.

W drugiej części moich badań, rozszerzając obserwację efektu ART na drugie pokolenie potomstwa, wykazałam międzypokoleniową transmisję obniżonej motywacji społecznej,

będącą konsekwencją ART zastosowanego na ojcach. Podobnie, zaobserwowałam zwiększoną lękliwość połączoną z obniżoną motywacją społeczną będącą konsekwencją ART zastosowanego na matkach. Poziom penetracji obserwowanych zmian różnił się ilościowo (przeżywalność potomstwa) i jakościowo (zaburzenia zachowania i wzrostu) między F1 i F2. Co więcej, poziom penetracji aberracji ilościowych był nasilony u potomstwa F2 uzyskanego w wyniku krzyżowania samców ART z samicami ART.

W trzeciej części eksperymentów zbadałam możliwe przyczyny zmniejszonej socjalności u potomstwa ART. Analizując aktywność antyoksydacyjną i metabolizm lipidów w mózgach potomstwa F1 na etapie prenatalnym i w wieku dorosłym, wykazałam, że prenatalny rozwój mózgu ART jest upośledzony z powodu zwiększonego stresu oksydacyjnego, podwyższonej peroksydacji lipidów i zmniejszonej ekspresji genów związanych z neurogenezą, co może powodować zmiany w funkcjonalności mózgu i jego połączeniach. Co więcej, odkryłam, że upośledzenia te utrzymują się aż do wieku dorosłego, co objawia się zwiększonym stresem oksydacyjnym w podwzgórzu potomstwa ART i zmienionym proteomem w hipokampie, podkreślając zmienioną ekspresję białek zaangażowanych w neurogenezę, synapsy i białka związane z kilkoma zaburzeniami neurologicznymi.

W czwartej części mojej rozprawy doktorskiej, wiedząc, że neurorozwój jest pierwszym procesem zachodzącym podczas embriogenezy, nawet przed implantacją łożyska, oceniłam, czy uszkodzenia lipidów i zmiany składu w prenatalnych mózgach ART mogą być konsekwencją zmian zachodzących przed implantacją, analizując profil lipidowy oocytów i zarodków ART. Wykazałam, że profil lipidowy zarodków ART jest zmieniony, wykazując oznaki peroksydacji; co istotne, podobny zmieniony profil lipidowy stwierdziłam również w oocytach AMA (mysich i ludzkich) oraz zarodkach, często wykorzystywanych w procedurach ART. Po raz pierwszy wykazałam, że uszkodzenia lipidów indukowane ART w zarodkach

mogą mieć długoterminowe konsekwencje zdrowotne, będąc możliwym źródłem NDD u potomstwa.

Badania te wykazały, że ART jest związana z modyfikacją lipidów w zarodkach, prowadząc do zmienionego rozwoju mózgu w okresie prenatalnym i dorosłym, zmniejszonej socjalności u dorosłego potomstwa, a ponadto jego skutki są transgeneracyjne. Badania te pozwalają na lepsze zrozumienie etiologii NDD i otwierają nowe horyzonty naukowe dla kolejnych badań lipidomicznych wskazujących mechanizmy przyczyniające się do NDD.

Słowa kluczowe: Technologia Wspomaganego Rozrodu, zaburzenia neurorozwojowe, hodowla *in vitro*, behawior, model mysi

ABBREVIATIONS

4-HNE= 4-hydroxy-2-nonenal

ADHD= attention deficit hyperactivity disorder

AI=Artificial insemination

AMA: Advanced Maternal Age

ART: Assisted Reproductive Technology

ASD: Autism Spectrum Disorder

CARs= Coherent Anti-Stoke RAMAN spectroscopy

Cntnap2= Contactin-associated protein-like 2

Cox2= Cyclooxygenase-2

CP= Cerebral Palsy

DHA= Decosahexaenoic acid

ET: Embryo Transfer

Fgf2= Fibroblast growth factor 2

GABRA1= Gamma-amminobutyric acid receptor subunit alpha-1

GPx= Glutathione Peroxide

GSH= reduced Glutathion

GSSG=oxidised Glutathion

hCG= Human chorionic gonadotropin

HMOX1= Heme Oxigenase 1

HT= Hypothalamus

ICSI= intracytoplasmic sperm injection

ID= Intellectual disability

I_{NP} = Index-Novel Preference

I_{SP} = Index-Social Preference

IVC: *in vitro* culture

IVF= *in vitro* fertilization

LDs= Lipid Droplets

MapK3= Mitogen-activated protein kinase 3

MDA= Malondialdehyde

MO= Medulla Oblongata

N= Novel stimuli

NDD: Neurodevelopmental Disorders

NM: Natural Mating

NMDA=N-methyl-D-aspartate

NN= Non-Novel stimuli

NPC= Neopallial cortex

NS= Non-Social stimuli

NSCs= neural stem cells

OS= Oxidative Stress

PGE2= Prostaglandin E_2

PGT= Preimplantation Genetic Testing

PMSG= Pregnant mare's serum gonadotropin

PND: Post Natal Days

PPAR-α = Peroxisome proliferator-activated receptor alpha

ROS= Reactive Oxygene Species

S: Social stimuli

SOD= Superoxide Dismutase

T_N= Time Novel

T_{NN} = Time Non-Novel

T_{NS} = Time Non-Social

T_S = Time Social

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Figure 20: Altered morphology indicating inflammation in IVC prenatal brain.

Figure 21: LDs profile is altered in IVC embryos.

Figure 22: Altered lipid profile and lipid peroxidation in AMA oocytes and embryos (mouse and human model).

1. INTRODUCTION

1.1 Assisted Reproductive Technology (ART)

Assisted reproductive technologies (ART) refer to any treatment related to fertility in which oocytes and/or embryos undergo manipulation, as defined by the American Centers for Disease Control (CDC). ART refers to a range of medical procedures that are used to help individuals and couples achieve pregnancy. These technologies have been developed for use in both human and animal reproduction and have had significant impacts on reproductive medicine and biology.

Since 1978 when the first ART treatment was performed in humans (Steptoe and Edwards 1978), several ART procedures have achieved widespread use including ovarian stimulation, oocyte retrieval, *in vitro* maturation, fertilization via *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI), *in vitro* embryo culture, embryo transfer, Preimplantation Genetic Testing (PGT), and cryopreservation of gametes and embryos (Niederberger et al. 2018) (Fig. 1). ART methodologies have become increasingly optimised over the past few decades, with millions of babies born worldwide using these technologies showing to be effective in helping couples overcome infertility (Andersen et al. 2008).

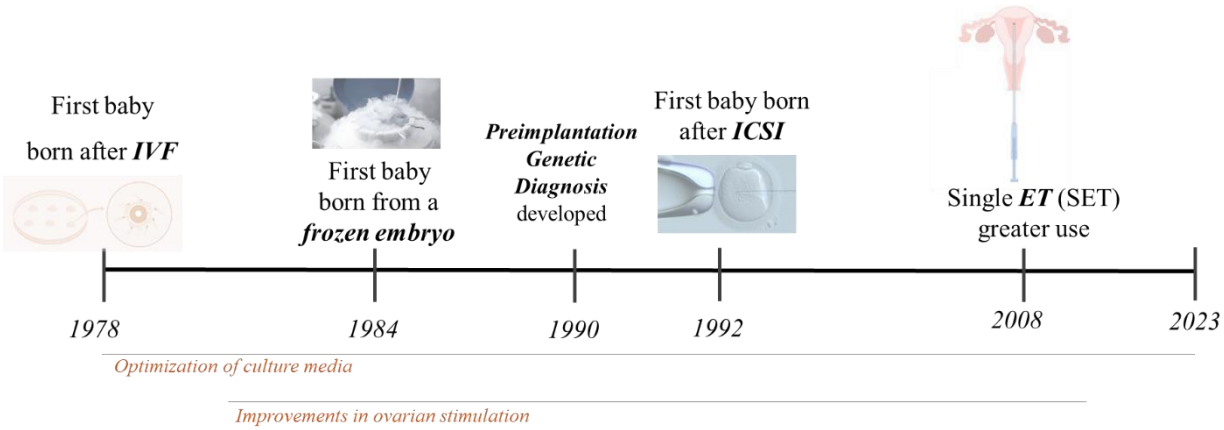


Figure 1. Schematic representation of ART history.

In addition, ART has also been used in animals for many years, particularly in livestock breeding programs. Techniques such as Artificial Insemination (AI), embryo transfer, and *in vitro* fertilization have been used to improve the genetics of livestock and increase productivity (Hansen 2014).

1.1.1 ART implications on offspring health

Assisted Reproductive Technologies (ART) have revolutionised the treatment of infertility, enabling millions of couples worldwide to conceive children. However, there are growing concerns about the potential health implications of ART on offspring. While the success rates of ART have improved over the years, several studies have suggested that ART children may be at an increased risk of certain health problems, including low birth weight, congenital malformations, and imprinting disorders (Barberet et al. 2022; Berntsen et al. 2019; Ericson and Källén 2001; Olson et al. 2005).

ART children are more likely to be born with low birthweight than naturally-conceived children which is associated with several health problems, such as respiratory distress syndrome, neurological deficits, and increased risk of death. The higher incidence of multiple births in ART pregnancies may contribute to the increased risk of low birth weight, although the underlying mechanisms are not yet fully understood (Maheshwari et al. 2012). Moreover, there is evidence to suggest that ART children have an increased risk of congenital malformations, such as heart defects and neural tube defects, compared to naturally-conceived children (Hansen et al. 2012). Some researchers have suggested that the use of ART may affect the development of the embryo, leading to an increased risk of malformations as well as imprinting disorders. Indeed, the normal process of genomic imprinting during early embryonic development is thought to be disrupted by ART procedures, leading to a higher incidence of rare epigenetic disorders such as Beckwith-Wiedemann syndrome and Angelman

syndrome (Chang et al. 2005; Chen et al. 2015). Several other factors, such as the type of ART used, and the underlying fertility status and age of the patient(s), may also affect the health outcomes of ART children (Basso and Baird 2003; Messerlian, MacLagan, and Basso 2013).

On the other hand, other studies investigating the risk of health problems in ART children have reported that ART is generally safe and there is no higher risk of birth defects in offspring (Wise et al. 2015), imprinting disorders (Sutcliffe et al. 2023), as well as no higher risk of developmental delays (Lyall et al. 2013).

Despite ART procedures being widely applied for several decades and much research carried out, data about the potential health implications of ART on offspring remain controversial and not yet conclusive because the majority of studies are mainly based on epidemiological data and there are several limitations in their interpretation. Firstly, the field of ART is relatively new, and the number of individuals conceived through these techniques is still relatively small compared to the general population. This limited sample size is a challenge to detecting rare or long-term health outcomes accurately. Additionally, ART procedures often involve multiple confounding factors, such as various infertility causes, specific treatment protocols, and individual patient characteristics, thereby complicating the recognition of isolated ART effects. Furthermore, ethical concerns and privacy issues associated with collecting data on children conceived through ART can impede comprehensive follow-up studies. Lastly, the diversity of ART techniques utilised, and the rapid advancement of technologies further complicate the interpretation of epidemiological data. Therefore, while epidemiological studies provide valuable but limited insights, experimental studies on homogenous animal populations absent of underlying fertility issues could help in shining a light on potential health implications of ART itself.

1.1.2 Risk of neurodevelopmental disorders in ART-conceived offspring

There is a considerable concern surrounding mental health and neurodevelopmental outcomes in ART-conceived children (Hart and Norman 2013). Neurodevelopmental disorders are a group of conditions that affect the development of the nervous system and brain function. These disorders include attention deficit hyperactivity disorder (ADHD), cerebral palsy (CP), intellectual disability (ID), and autism spectrum disorder (ASD). These disorders can significantly impact cognitive, social, and emotional abilities, posing unique challenges for individuals affected.

Studies have suggested that children conceived through ART may be at a higher risk of developing these disorders compared to naturally-conceived children (Liu et al. 2017; Rönö et al. 2022; Strömberg et al. 2002). Similarly, studies in mouse models indicate that ART offspring show more frequent anxiety and depression-like behaviours in comparison to naturally-conceived offspring (Qin et al. 2021a)

The underlying mechanisms for the increased risk of neurodevelopmental disorders in ART-conceived children are not yet fully understood. However, several factors have been proposed, including epigenetic changes, gene mutations, and environmental factors. Epigenetic changes refer to changes in gene expression without alterations to the underlying DNA sequence. These changes may be caused by the *in vitro* culture environment, ovarian stimulation, or the use of cryopreserved gametes and embryos (Mani et al. 2020). Gene mutations may also occur during the *in vitro* fertilisation process, leading to changes in the genetic makeup of the embryo (Barberet et al. 2022; Cannarella et al. 2022). Environmental factors such as exposure to drugs and stress during ART may also contribute to the increased risk of neurodevelopmental disorders (Mulder et al. 2002) .

However, some studies show contrary results and their authors deny the increased risk of neurodevelopmental disorders (such as communication disorder or ASD) in ART-conceived children and moreover in ART-conceived children from advanced aged mothers (Ackerman et al. 2014; Kamowski-Shakibai, Kollia, and Magaldi 2017).

It seems evident that it is yet to be conclusively determined if the use of ART increases the risk of neurodevelopmental disorders in the offspring, and, if so, what are the underlying origins of these disorders.

1.2 Brain development

In the realm of neurodevelopmental disorders (NDD), the brain takes centre stage, as these conditions arise from atypical brain development or functioning. Understanding the role of the brain and its involvement in NDDs is crucial for unravelling the mysteries surrounding their origins, improving diagnosis and treatment strategies, and ultimately enhancing the lives of those affected.

Brain development is a complex process that starts in the embryonic stage and continues throughout childhood and adolescence. Specifically, brain development begins at the very early embryonic developmental stage called neurula-stage, characterised by the formation of the neural tube, which gives rise to the central nervous system. The initial stages of brain development include neural induction, neural proliferation, and neural migration, during which time the neural tube is formed, and neurons begin to develop. In mouse, the neural tube formation occurs by day 7 of gestation when even the placenta is not functioning yet (Copp 2005). This is followed by the formation of synapses – which allow neurons to communicate

with each other – and the growth of axons and dendrites which form the complex neural networks that are critical for brain function. In the prenatal period, neurogenesis and migration continue and the brain rapidly grows in size and complexity. During the first few years of life, the brain undergoes a period of rapid synaptogenesis and pruning, during which time connections between neurons are strengthened or eliminated based on experience and environmental factors. Throughout childhood and adolescence, the brain continues to develop and mature, with changes in grey and white matter density, myelination, and functional connectivity. The process of brain development is highly sensitive to environmental influences, such as nutrition, stress, and early life experiences, and disruptions to normal brain development can have significant consequences for cognitive and emotional development (Houston, Herting, and Sowell 2014; Johnson 2001; Kandel 2014).

The most critical steps in brain development that, if disrupted, can lead to neurodevelopmental disorders are neural tube formation, neural proliferation and migration, synaptogenesis and pruning, and myelination. Neural tube formation occurs in the first few weeks of gestation and gives rise to the brain and spinal cord; failure of the neural tube to close properly can result in neural tube defects such as spina bifida, which can lead to a range of neurological and developmental problems (Detrait et al. 2005; Harris and Juriloff 2007). Another critical step is neural proliferation and migration: during this stage, the brain generates the appropriate number and types of neurons and glial cells which migrate to their appropriate locations in the brain. Disruptions in this process can lead to conditions such as lissencephaly – in which the brain has a smooth surface due to abnormal migration of neurons – or microcephaly, where the brain is smaller than normal (Mochida 2009). Also, synaptogenesis and pruning has a pivotal role in brain formation: this stage involves the formation of synapses and the strengthening or elimination of connections based on experience and environmental factors. Disruptions in this process can lead to conditions such as autism spectrum disorder (ASD), which is characterised

by difficulties in social interaction and communication, and attention deficit hyperactivity disorder (ADHD), which is characterised by hyperactivity, impulsivity, and inattention (Martínez-Morga et al. 2018; Watanabe et al. 2021; Yenkoyan et al. 2017). The final important step is myelination, the process by which axons are coated with a fatty substance called myelin, which speeds up neural transmission. Disruptions in this process can lead to conditions such as multiple sclerosis, which is characterised by demyelination of the nervous system, or leukodystrophies, which are a group of inherited disorders that affect myelin formation (Fancy et al. 2010).

1.2.1 Role of lipids in brain development

Within all the components playing crucial roles in brain development and contributing to its structure and function, lipids are undeniably one of the most important. They are essential building blocks for the formation and maintenance of neuronal membranes, providing structural integrity and facilitating cellular communication (Borroni, Vallés, and Barrantes 2016; Bush, Lee, and Nagele 1992; Poitelon, Kopec, and Belin 2020)(Borroni, Vallés, and Barrantes 2016; Bush, Lee, and Nagele 1992; Poitelon, Kopec, and Belin 2020). As brain development starts at the very early stage of post-implantation embryonic development called the neurula-stage, it is important to mention that the first source of lipids contributing to brain development comes from the embryo itself: lipid supply through maternal nursing via placental blood stream starts later (Copp 2005). Indeed, lipid droplets present in mammalian oocytes are utilised during the post implantation period (Arena et al. 2021) and have been found to not be essential for embryo pre-implantation development(Nagashima et al. 1995).

The brain is one of the most lipid-rich organ in the body, with lipids accounting for approximately 50% of its dry weight (Montesinos, Guardia-Laguarta, and Area-Gomez 2020).

Unsurprisingly, lipid metabolism disorders can cause severe neurological symptoms and developmental abnormalities (Bazinet and Layé 2014), highlighting the importance of lipids for a correct brain development.

Lipids are involved in various aspects of brain development, including the formation and growth of neuronal cells and the myelination of axons. In particular, lipids are critical for the development and function of glial cells, which are the primary support cells of the brain (Barber and Raben 2019). Glial cells play a crucial role in maintaining the structural integrity of the brain, regulating neurotransmitter signalling, and providing metabolic support to neurons (Puchkov and Haucke 2013). One important group of lipids involved in brain development is the phospholipids. Phospholipids are a class of lipids that are composed of a glycerol backbone, two fatty acid chains, and a phosphate group. They are a major component of the cell membrane, where they form a bilayer that separates the interior of the cell from the external environment. Phospholipids are critical for the formation and maintenance of neuronal and glial cell membranes and are involved in the regulation of membrane fluidity and stability (Goracci et al. 1973; Lehninger 1968). Another important group of lipids involved in brain development is the sphingolipids. Sphingolipids are a class of lipids that are composed of a sphingosine backbone, a fatty acid chain, and a polar head group. They are involved in various cellular processes, including signal transduction, cell adhesion, and apoptosis. Sphingolipids are particularly important for the formation and maintenance of myelin, which is a fatty sheath that surrounds axons and enables rapid and efficient signal transmission between neurons (Hussain et al. 2019; Kalinichenko et al. 2022).

Moreover, lipids play a crucial role in synaptogenesis – the formation of synapses – and pruning – the elimination of unnecessary or weak synapses during brain development. The process of synaptogenesis and pruning is essential for proper neuronal connectivity and the establishment of functional neural networks in the brain (Olsen and Færgeman 2017). Synapse

formation and stability is affected by the composition and properties of the lipids that make up cell membranes, one of the most well-known functions of lipids. One important class of lipids involved in synaptogenesis and pruning is phospholipids. Phosphatidylserine, a type of phospholipid, has been shown to promote synaptogenesis and improve synaptic plasticity (Fagone and Jackowski 2009). In contrast, high levels of sphingomyelin, another type of phospholipid, have been associated with decreased synaptic density and impaired synaptic function (Mauch et al. 2001).

In addition to their role in cell membrane composition, lipids can also act as signalling molecules that regulate synaptogenesis and pruning. For example, arachidonic acid – an omega-6 fatty acid – has been shown to promote synapse formation and maturation by modulating NMDA receptor function (Bazan 2003). Docosahexaenoic acid (DHA) – another omega-3 fatty acid - has also been implicated in synapse formation and function, as well as cognitive development (Bazinet and Layé 2014). DHA is particularly enriched in the brain, and its deficiency has been linked to impaired synaptic plasticity and cognitive deficits (Hall 2016; Talamonti et al. 2020).

1.2.2 Role of lipid peroxidation and oxidative stress in brain development

During ART procedures, gametes and embryos are subjected to several non-physiological conditions such as temperature fluctuations, visible light exposure, atmospheric oxygen, CO_2 incubators, humidity levels, volatile organic compounds, and additives present in the culture media. Collectively these factors contribute to an environment that promotes increased oxidative stress (OS) (Agarwal et al. 2006) which can lead to damage of cellular lipids, organelles, and DNA in gametes and embryos (Combelles, Gupta, and Agarwal 2009; Mihalas et al. 2017; Musson et al. 2022). OS results from an imbalance between reactive oxygen species

(ROS) production and antioxidant defence mechanisms. Exposure to OS during preimplantation development may then further impair embryonic development during the post-implantation period as the embryo will be the exclusive source of building blocks (i.e lipids, proteins) for the first developmental stage before maternal contribution begins via placentation (Chen et al. 2017). OS therefore may have the potential to influence brain development, as a result of damage sustained to embryos during ART procedures.

Moreover, the developing brain itself is particularly vulnerable to oxidative stress due to its high oxygen consumption, high content of polyunsaturated fatty acids, and low antioxidant capacity. Lipid peroxidation, a major consequence of OS, is the process of oxidative degradation of polyunsaturated fatty acids in cellular membranes, leading to the formation of reactive aldehydes such as 4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA) (Dix and Aikens 1993). These lipid peroxidation products can alter cellular signalling pathways, modify protein structure and function, and induce DNA damage, leading to impaired neuronal function and neurodevelopmental disorders (Shichiri 2014). Additionally, 4-HNE and MDA act as potent inflammatory mediators. which can trigger the activation of pro-inflammatory signalling pathways and can damage cellular membranes, disrupting the integrity of the blood-brain barrier, and compromising the function of critical brain cells, contributing to neuroinflammation and impairing normal brain development (Adibhatla and Hatcher 2008; Cumaoğlu, Ağkaya, and Özkul 2019; Shichiri 2014; Wang et al. 2014).

During brain development, oxidative stress and lipid peroxidation can impact several critical processes, including neurogenesis, synaptogenesis, myelination, and neurotransmitter signalling. In particular, oxidative stress has been shown to impair neurogenesis by reducing the proliferation and differentiation of neural stem cells (NSCs) and inducing apoptosis of immature neurons (Semkova et al. 2022). Similarly, lipid peroxidation products such as HNE have been shown to alter the activity of synaptic proteins and impair synaptic plasticity and

function (Li et al. 2022). Furthermore, oxidative stress and lipid peroxidation can disrupt myelination by impairing the differentiation and maturation of oligodendrocytes, the cells that produce the myelin sheath that insulates neuronal axons (Haynes et al. 2006).

Excessive oxidative stress and lipid peroxidation have been implicated in the pathogenesis of several neurodevelopmental disorders, including ASD (Rose et al. 2012). For example, studies have shown increased levels of lipid peroxidation products in the brains of individuals with ASD, suggesting that lipid peroxidation may contribute to the pathogenesis of the disorder (Rossignol and Frye 2014).

The regulation of oxidative stress and lipid peroxidation is critical for normal brain development and function. Antioxidant defence mechanisms, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx), can scavenge ROS and prevent oxidative damage (Khan and Black 2003).

1.3 Brain structures

The human brain is a complex organ made up of numerous interconnected structures, each with unique functions and roles in regulating behaviour, cognition, and emotion. One of the most prominent structures is the cerebral cortex, which is responsible for processing sensory information, motor control, and higher cognitive functions such as language and decision-making. The cortex is divided into several lobes, including the frontal lobe, parietal lobe, temporal lobe, and occipital lobe, each of which has distinct functional specialisations (Gazzinga, Ivry, and Mangun 2018)

Other key structures in the brain include the limbic system, which is involved in emotion regulation and memory formation, and includes structures such as the hippocampus, amygdala, and hypothalamus (LeDoux 2003). The thalamus serves as a relay station for sensory information, while the basal ganglia play a crucial role in motor control and reward-based learning (Graybiel 2008).

In addition, the cerebellum is important for coordinating movement and balance, while the brainstem regulates essential functions such as breathing and heart rate (Swanson 2011).

1.3.1. Hippocampus: development, structure, and function

The hippocampus is a brain structure that is found in both humans and mice, and is known to play a critical role in learning and memory processes.

During early embryonic development, the hippocampus is derived from the neural ectoderm and arises from the dorsal portion of the telencephalon (Altman and Bayer 1990). As development proceeds, the hippocampus undergoes a series of complex morphological changes, including the formation of several subregions, such as the dentate gyrus, CA1, CA2, and CA3 (David and Pierre 2006).

The formation and differentiation of these subregions is regulated by a variety of genetic and environmental factors, including transcription factors, growth factors, and neurotransmitters (Khalaf-Nazzal and Francis 2013). For example, the transcription factor Pax6 is critical for the development of the dentate gyrus, while the growth factor FGF2 plays a key role in the differentiation of CA1 neurons (Li et al. 2013). As development continues into adolescence and adulthood, the hippocampus continues to undergo structural changes and reorganisation. For example, there is evidence to suggest that the number of synapses and dendritic spines in the

hippocampus increases during adolescence, leading to increased synaptic plasticity and learning ability (Andersen and Teicher 2004).

In adulthood, the hippocampus is characterised by a high degree of neuroplasticity, or the ability of neurons to change and adapt based on experience (Garthe and Kempermann 2012). This neuroplasticity is thought to underlie the formation of new memories and the ability to learn and adapt to new situations. However, the development of the hippocampus is not always smooth and can be affected by a variety of genetic and environmental factors. For example, exposure to stress during early development has been shown to have negative effects on hippocampal development, leading to reduced neurogenesis and impaired learning and memory processes (Smith and Pollak 2020).

There are some differences in the exact structure and function of the hippocampus between humans and mice, but there are also many similarities that have been observed through research.

In humans, the hippocampus is in the medial temporal lobe and is composed of several subregions, including the dentate gyrus, CA1, CA2, and CA3 (David and Pierre 2006). The hippocampus is involved in many aspects of memory formation and retrieval, including spatial memory, contextual memory, and episodic memory (Eichenbaum 2000). Damage to the hippocampus – such as in Alzheimer's disease or after a traumatic brain injury – can lead to severe memory deficits (Squire 1992).

In mice, the hippocampus is also located in the medial temporal lobe and is composed of similar subregions as in humans (Fanselow and Dong 2010). Studies in mice have been instrumental in elucidating the cellular and molecular mechanisms underlying hippocampal-dependent learning and memory processes. For example, it has been shown that the formation of new memories in the hippocampus is dependent on the plasticity of synapses, or the ability of neurons to strengthen or weaken their connections based on experience (Malenka and Bear 2004).

Only recently, some key differences in the structure and function of the hippocampus between humans and mice have been discovered; it has been shown that the human hippocampus is larger and more complex than the mouse hippocampus, with a greater number of cells and a more convoluted shape. Additionally, while the hippocampus is critical for spatial memory in both humans and mice, there is some evidence to suggest that the human hippocampus may be more involved in the integration of spatial and non-spatial information (Charvet and Finlay 2018).

1.3.2. Role of Hippocampus in NND

Abnormalities in hippocampal structure and function have been implicated in a range of neurodevelopmental disorders, including ASD, ADHD, and schizophrenia (Li et al. 2019). Studies have shown that individuals with ASD often have increased hippocampal volumes compared to normal, healthy brains, and that these volume alteration are associated with impairments in social communication and repetitive behaviours (Banker et al. 2021; Cloarec et al. 2019). In ADHD, the hippocampus has been found to be involved in working memory deficits, which are common in the disorder (Sagvolden and Sergeant 1998). In schizophrenia, there is evidence of reduced hippocampal volume and abnormalities in hippocampal function, which are associated with cognitive impairments and psychotic symptoms (Sun et al. 2023).

Animal studies have also provided further insights into the role of the hippocampus in neurodevelopmental disorders. Indeed, mice with genetic mutations that lead to ASD-like behaviours show abnormalities in hippocampal synaptic plasticity and impaired spatial learning and memory (Han et al. 2012).

1.3.3 Hypothalamus: development, structure, and function

The hypothalamus is a small but crucial region of the brain involved in a wide range of physiological processes, including the regulation of homeostasis, hormone secretion, circadian rhythms, and behaviour.

Its development begins during early embryogenesis through a complex series of events. It arises from the ventral part of the diencephalon and undergoes regionalisation into distinct nuclei and neuronal populations. The development of the hypothalamus is governed by precise spatio-temporal expression patterns of transcription factors which regulate the proliferation, differentiation, and migration of hypothalamic progenitor cells, contributing to the establishment of diverse hypothalamic nuclei (Shimogori et al. 2010).

The hypothalamus is a compact region located below the thalamus, forming the ventral part of the diencephalon. It consists of several distinct nuclei, including the paraventricular nucleus (PVN), arcuate nucleus (ARC), suprachiasmatic nucleus (SCN), and lateral hypothalamic area (LHA), among others (Bear, Reddy, and Bollu 2022).

The hypothalamus plays a vital role in maintaining homeostasis by integrating sensory information and orchestrating appropriate physiological responses. It regulates body temperature, thirst and hunger sensations, sleep-wake cycles, stress responses, and reproductive behaviours. Additionally, the hypothalamus controls the release of hormones from the pituitary gland, coordinating endocrine functions throughout the body. Relevantly, this region also interacts with other brain areas, including the limbic system, in modulating emotional and motivated behaviours. The intricate functions of the hypothalamus are mediated by various neurotransmitter systems and neuropeptides, such as dopamine, serotonin, orexins, and corticotropin-releasing hormone (CRH) (Shahid, Asuka, and Singh 2022).

1.3.4 Role of Hypothalamus in NND

The hypothalamus is increasingly recognised as playing a significant role in the pathogenesis of neurodevelopmental disorders. Abnormalities in hypothalamic structure, connectivity, and function can contribute to the core symptoms and associated features of these disorders. Dysregulation of hypothalamic circuits can impact vital processes such as social behaviour, emotion regulation, sleep-wake cycles, appetite, and hormone secretion.

Studies have identified alterations in hypothalamic structure and connectivity in individuals with ASD, and it has been associated with impaired social cognition and behaviour in ASD (Caria, Ciringione, and de Falco 2020).

1.4 *Mus Musculus* as a model for neurodevelopmental disorders

Neurodevelopmental disorders are a group of disorders that affect the development and function of the nervous system. These disorders are highly complex and have a multifactorial etiology, involving both genetic and environmental factors. One approach to understanding the underlying mechanisms of these disorders is to use animal models, such as *Mus musculus*, commonly known as the laboratory mouse. Humans and mice are similar under many aspects, like genetic, behaviour, and brain structure and function. Particularly, humans and mice share a high degree of genetic similarity, with approximately 85% homologous genomes. Many genes and pathways that are critical to neurodevelopment and neurological functions are conserved between the two species. For instance, genes involved in synapse formation, neural circuit development, and neurotransmitter signalling are shared between humans and mice (Rosenthal and Brown 2007; Silverman et al. 2010). Moreover, mice exhibit many of the same

behaviours and cognitive abilities as humans, and can be tested using similar behavioural assays. For example, both humans and mice exhibit social behaviours, and deficits in social behaviour are a hallmark feature of many neurodevelopmental disorders, including autism spectrum disorder (ASD). Mouse models of ASD exhibit similar deficits in social behaviour, as well as repetitive behaviours and communication deficits, which are also observed in humans with ASD (Crawley 2007; Kazdoba et al. 2012). Another similarity between human and mice is related to the structure and function of the brain which is also highly similar with some of the same brain regions and circuits involved in complex behaviours. For instance, studies have revealed similarities between human and mouse hippocampus, showing comparable neuronal organisation, such as the presence of pyramidal cells and granule cells, as well as distribution of neurotransmitter receptors in both human and mouse hippocampi (Clark and Squirea 2013). Moreover, both human and mouse hippocampi exhibit analogous synaptic plasticity mechanisms, including long-term potentiation and long-term depression, suggesting shared fundamental processes underlying learning and memory (Clark and Squirea 2013).

The remarkable resemblances between the structure, neuronal arrangement, synaptic plasticity, and functional connectivity of the human and mouse brains emphasise the utility of mice as model organisms in the study of neurodevelopmental disorders. Additionally, the shorter lifespan of mice compared to humans makes them a valuable tool for assessing the long-term impacts and transgenerational transmission of neurodevelopmental disorders in offspring conceived through assisted reproductive technologies (ART).

1.4.1 Impact of sex on mouse behaviour

It is increasingly recognised that male and female mice exhibit distinct behavioural patterns indicating that sex differences play an important role in mouse behavioural testing.

Various behavioural domains can be influenced by sex, including social behaviour, anxiety, cognitive function, and locomotor activity.

Female mice may display more pronounced maternal behaviour but hormonal fluctuations across the estrous cycle can impact female behaviour, necessitating careful consideration of the stage of the estrous cycle during testing. Using females in behavioural testing may constitute a challenging biological variable. This is one of the reasons why male mice are commonly used for behavioural testing and females are excluded. Moreover, male mice display more consistent and robust behaviours, making them suitable for assessing behavioural phenotypes in various neurobiological studies. Second, the hormonal profile of male mice is relatively stable compared to female mice, reducing the potential confounding effects of hormonal fluctuations on behaviour. Third, male mice show specific behavioural patterns, such as increased aggression and territoriality, which can be relevant to certain research questions (Eltokhi, Kurpiers, and Pitzer 2020; Saré, Lemons, and Smith 2021).

2. METHODS

2.1 Animals

All experimental procedures were conducted according to the guidelines of European Community Regulation 86/609 and conformed to the Polish Governmental Act for Animal Care. Animal procedures were conducted at the Małopolska Centre of Biotechnology and the Institute of Zoology and Biomedical Research of Jagiellonian University-Kraków (permission no. 329/2021 approved by II Local Ethical Commission of Kraków). Animal experiments were performed on C57BL/6 mice. Animals were maintained in a temperature- and light-controlled room (22 °C and 12-h light–dark cycle) and were provided with food and water *ad libitum*.

2.2 Embryo collection and culture

3-5 months old C57BL/6 females were injected with 5iu of PMSG, followed by 5iu of hCG after 45-48h, and mated with 3-5 months old C57BL/6 males for IVC, and ET; while for NM (natural mating), 3-5 months old C57BL/6 females were mated with 3-5 months old C57BL/6 males, and allowed to deliver naturally (fig.2 Experimental plan). For IVC group the embryos were collected at day 2 (morning) and placed in culture in KSOM medium (Merck) covered with mineral oil for 2 days at 37°C in atmosphere of 90% N_2, 5% CO_2 and 5% O_2. For ET group, the embryos were flushed from uterus with 2ml of M2 media for each horn at day 4 (morning).

2.3 Embryo transfer

Day 4 embryos (IVC and ET) were transferred to pseudo-pregnant asynchronous F1 females (at day 3). The females were anesthetised with Ketamine/Xylazine (0.1 ml/g bodyweight of 12.5% solutions). The transfer of embryos to asynchronous recipients was carefully timed for each pregnancy. Embryo transfers for IVC, and ET were performed at day 4 to recipient mice

at day 3 of pseudo-pregnancy. After cleaning with chlorohexidine and shaving the back of the animal, a small incision was made and the uterus exposed. A small hole was made to the uterine wall to transfer embryos with a glass capillary pipette. The procedure was performed in both horns of the uterus. The body cavity was sutured, and the recipient female transferred to a clean cage placed on warming pad until its recovery. Females were allowed to deliver spontaneously (Sampino et al. 2014).

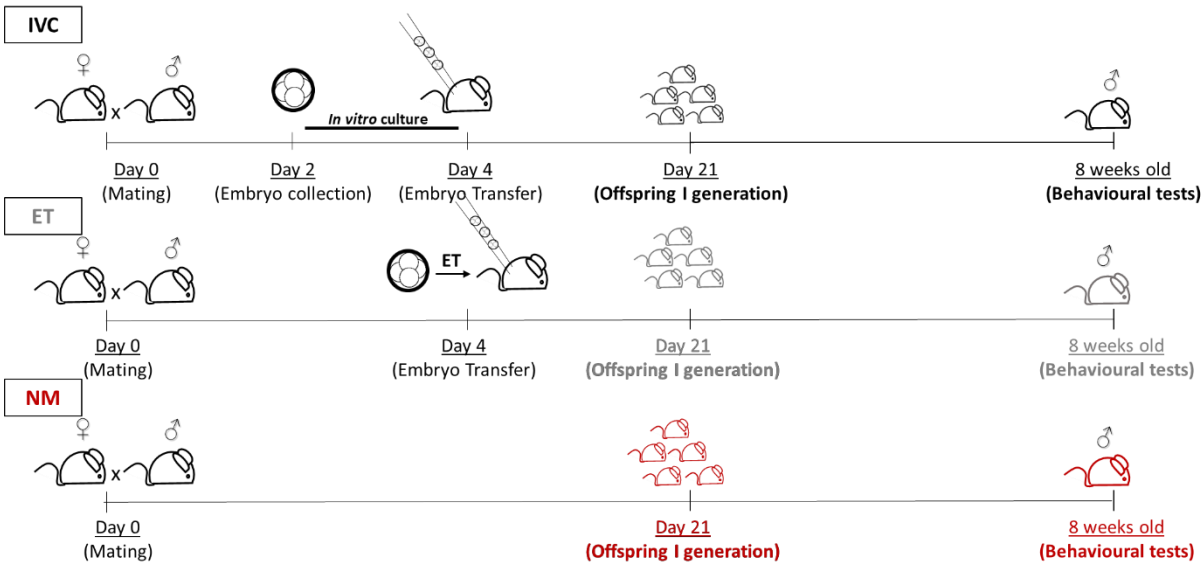


Figure 2: Experimental plan Offspring-I Generation. IVC= *in vitro* culture, ET= embryo transfer, NM= natural mating

2.4 Behavioural testing

8-9 weeks old IVC, ET, and NM males were subjected to Three Chambers recognition test, followed 1 week later by Light/Dark Box test.

2.4.1 Three Chamber Social recognition test

The test is conducted in an arena divided in 3 chambers with openings between them. The test consists of 3 Phases (Fig.3): I phase the animal is allowed to habituate to the experimental enviroment for 5 minutes; II Phase a stranger is placed in one of the compartments as a social stimuli and the activity of the experimental animal, being free to move throughout all

compartments, is recorded; III phase a second stranger as a novel stimuli is placed in the other compartment and the activity of the experimental animal, again free to move in all compartments, is recorded for 5 minutes (Rein, Ma, and Yan 2020).

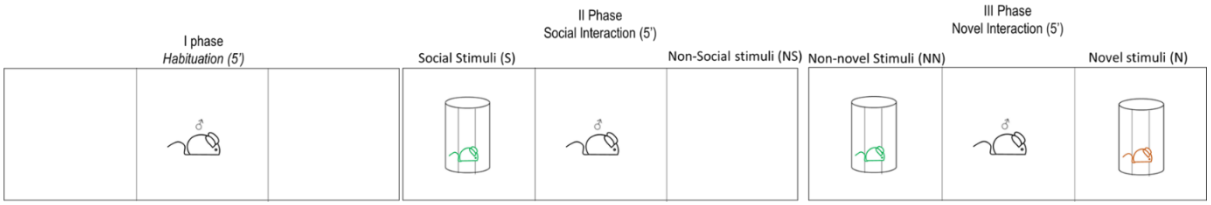


Figure 3: Schematic rapresentation of Three chamber social recognition test.

2.4.1 Light/Dark Box test

The test is performed in arena that consists of two compartments. The light compartment is ½ of the box and is brightly lit and open. The dark compartment is ½ of the total box and is covered and dark. A door of 7 cm connects the two compartments. Mice are placed in the dark compartment of the apparatus and are allowed to move freely between the two different compartments for 10 minutes. The length of time that the mouse spends before first entering the light box (i.e. latency time), the number of entries to each compartment, and the time spent in each zone – light or dark – were scored. Videos were recorded using Logitech camera and Clipchamp software, video analyses were assessed by an experimenter blind to the experimental conditions (Bourin and Hascoët 2003).

2.5 Animals- II generation

3 months old females from IVC and ET group were mated with 3-5 months old C57BL/6 males to obtain II generation IVC- female line and ET- female line; 14 days after completing behavioural testing 3 months old males from IVC and ET group were mated with 3-5 months C57BL/6 females to obtain II generation IVC-male line and II generation ET- male line. Additionally, IVC females and IVC males were crossed to obtain II generation IVC female x

IVC male line (Fig.4). The above-described paired animals were housed for 14 days to allow the mating, after that the females' bodyweight was recorded to ensure the established pregnancy (i.e. increase in bodyweight indicates pregnancy), then the females were separated from the males and allowed to deliver naturally.

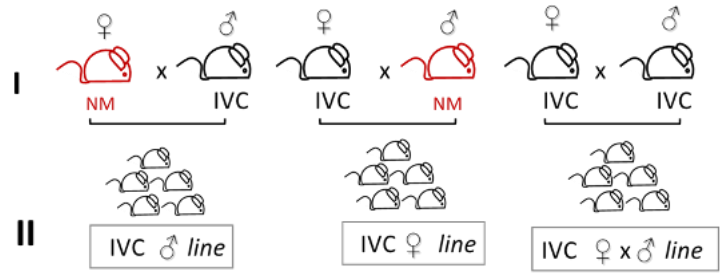


Figure 4: Schematic representation of II generation groups. IVC= *in vitro* Culture, NM=Natural Mating (CONTROL) I= First Generation, II= Second Generation.

2.6 Brain Collection

After completion of behavioural tests, and subsequent mating for II generation, adult mice offspring (I Generation) were ethically sacrificed by decapitation performed with fine scissors. The heads were placed on clean paper towel to remove remaining blood. Using the scissors, midline incision was made in the skin. Remaining muscles and skin were removed using curved scissors to reveal skull. After cutting of the optic nerve behind each eyeball, a cut was made in the skull between nasal and frontal area. Using Pean's forceps or spatula all bones were peeled off starting from occipital area. Finally, using spatula the brain was extracted by elevating it from the medulla bottom. The removed brain was weighed and immediately placed on ice-cold Petri dish where all brain regions of interest were dissected and snap frozen in liquid nitrogen (LN). The samples were stored in -80°C freezer or in LN for further use (Wager-Miller et al. 2020).

2.7 Prenatal collection at 19.5 dpc

Pregnant females were ethically sacrificed by cervical dislocation at 19 dpc; a midline incision of the ventral skin and abdominal wall muscles was performed to expose and remove the uterus containing embryos. Each embryo was isolated by firstly removing remaining uterine tissue, then each embryo was carefully extracted out of its yolk sac, and ethically euthanized. Prenatal brain was isolated using curved forceps (Clarkson, Martin, and McKeegan 2022) .

2.8 Histology- prenatal brain

After collection, foetuses at 19 dpc were fixed in 4% (w:v) paraformaldehyde and subsequently dehydrated into increasing ethanol solutions and cleared in xylene mixture. Finally, they were paraffin embedded. 8μm sections were used for haematoxylin eosin staining (Cardiff, Miller, and Munn 2014).

2.9 ELISA based assays - prenatal brain and adult Hypothalamus

Firstly, protein extraction was performed on prenatal brain and adult hypothalamus, the tissues were lysed in PBS supplemented with protease inhibitor cocktail (Sigma Aldrich Co). Samples were then centrifuged at 5000g for 5 minutes and supernatant was used immediately for analysis or aliquoted and stored at -20ºC for further measurements. Total protein content was determined by Coomassie Protein Assay for normalisation of values. Samples were subjected to ELISA-based assays.

Superoxide dismutase (SOD) activity was measured in prenatal brain and hippocampus homogenates, using a colorimetric assay (Invitrogen), following manufacturer's instructions. Levels of reduced (GSH)- and oxidised (GSSG)- glutathione were determined using a colorimetric assay in prenatal brain and hippocampus following homogenisation according to

manufacturer's instructions. MDA levels were determined by TBArs assay (Sigma Aldrich), following manufacturer's instructions.

2.10 qPCR- prenatal brain - Gene expression analysis

Gene expression analysis was performed on mouse prenatal brain tissues obtained from CTR, IVC, and ET groups. Total RNA was extracted with RNAzol (Sigma Aldrich Co.) following manufacturer's instructions. One microgram of total RNA was subjected to reverse transcription to obtain cDNA, using Omniscript reverse transcription kit (Qiagen). cDNA obtained were used for gene expression analysis using specific primers. PCR reactions were performed using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems) with a QuantStudio Real-Time PCR System (Applied Biosystem). Relative gene expression data was calculated using the comparative threshold cycle method (ΔΔCt) (Livak and Schmittgen 2001).

2.11 Liquid chromatography and tandem mass spectrometry- adult hippocampus

Proteomic analysis was performed on hippocampus collected from 4- month-old mice. 20mg of tissue (n. 5 / group) were homogenised in 200ml of ice-cold lysis buffer. The homogenates were sonicated in a Bioruptor UCD-200 (Diagenode, 320W, 30s/30s on/off, 10 min.) and centrifuged (20 000g, 12°C, 10 min.). The protein concentration was measured by Bradford assay. Then, samples were processed using filter-aided sample preparation protocol. The peptides were analysed on a Q-Exactive mass spectrometer (Thermo Fisher Scientific) coupled to an UltiMate 3000 RSLCnano System (Thermo Fisher Scientific). Samples were loaded into a trap column (Thermo Fisher Scientific) in 2% acetonitrile with 0.05% trifluoracetic acid at a flow rate of 5ml/min and were further separated on an analytical column (Thermo Fisher Scientific) with a 240-min gradient from 2% to 40% acetonitrile in 0.05% formic acid at a flow

rate of 250nl/min. Eluting peptides were ionised using a Digital PicoView 550 nanospray source (New Objective). The mass spectrometer was operated in data-dependent acquisition mode. Full-scan MS spectra were acquired at a resolution of 70 000 over a mass range of 300 to 2000m/z, with an automatic gain control (AGC) target of 1e6. After each survey scan, the top twelve most intense, multiply charged ions were selected for fragmentation with 30s of dynamic exclusion. The MS/MS spectra were acquired at a resolution of 17 500 with an AGC target of 5e5. The maximum ion accumulation times for the full MS and the MS/MS scans were 120 and 60ms, respectively. Instrument performance was controlled with QCloud. MS data processing: raw mass spectra from individual samples were processed using MaxQuant 1.6.5.0. Peak lists were searched against the forward and reverse Swissprot_201 902 database (Rodentia, 26 822 sequences) using the integrated Andromeda search engine (Cox et al. 2011). Only fully tryptic peptides with up to two missed cleavages and a minimum length of seven amino acids were considered. Cysteine carbamidomethylation was set as a fixed modification. Methionine oxidation and protein N-terminal acetylation were searched as variable modifications. The precursor mass tolerance in the first search used for mass recalibration was set to 20ppm. The main search was performed with precursor and fragment mass tolerances of 4.5ppm and 20ppm, respectively. The match between runs option was enabled within a time window of 0.7min. The maximum false discovery rate for both peptide and protein identifications were set to 1%. All proteins that could not be distinguished based on the identified peptides were merged to one protein group. Relative quantification and normalisation were performed with the MaxLFQ label-free algorithm using a minimum ratio count of 2. Both razor and unique peptides were used for protein quantitation. Samples from males and females were analysed in separate batches. Bioinformatic and statistical analysis: for bioinformatic analysis and visualisation, Perseus 1.6.5.0, which is part of MaxQuant and the R framework, was used (Tyanova et al. 2016). Contaminants, proteins from the reverse

database, and proteins identified only with modified peptides were excluded from the study. All bioinformatic analyses were executed on label-free quantification intensities transformed to logarithmic scale to the base two. Proteins that were identified in at least three replicates in each group were included in the quantitative analysis. Missing values were imputed with a normal distribution (width ¼ 0.3; shift ¼ 1.8). Overall group comparisons were performed using one-way ANOVA; significant results were further examined using Tukey's adjustment for *post hoc* comparisons. $P < 0.05$ was considered significant. Enrichment analysis was performed using online tool g:Profiler (http://biit.cs.ut.ee/gprofiler/) (Reimand et al. 2007).

2.12 Coherent Anti-Stokes Raman spectroscopy- oocytes and embryos

The multimodal nonlinear microscope used in this study consisted of a Leica DMi8 inverted microscope equipped with the Leica TCS SP8 CARS module and the Leica SP8 confocal module (Leica Microsystems). The Leica TCS SP8 CARS uses a tuneable pump laser with a tuning range of 780 nm to 940 nm, combined with a Stokes laser at 1064 nm, provided by Laser picoEmerald and integrated with a 750mW power optical parametric oscillator. *Samples preparation:* Samples were measured in drops of 200µl of 0.4% PBS-PVP placed on a Lab-Tek chamber (Thermo Scientific) after fixation in 4% paraformaldehyde for 20min. *LDs signal detection:* to image the LDs, the optical parametric oscillator's wavelength was tuned to 816.7nm to serve as the pump beam, in combination with the 1064nm Stokes beam to probe the CH_2 stretch vibration. All signals were detected using a photomultiplier tube by collecting the photons in a forward direction through a 40X objective. Each sample was scanned across all of its sizes in the Z-axis using a z-step of 1.5µm. For the analysis, all stacks from a single sample were processed to obtain a maximum projection image. The threshold was adjusted specifically for all samples to collect all positive signals. When necessary, LDs were separated

manually in cases of obvious pixel overlay. Statistics regarding the LDs area and number were generated automatically by LAS X software (Leica Microsystems). The LAS X software was used to apply colour-coded LD size masks to the maximum projection images for graphical observation of the LDs' size distribution of the analysed samples. The oocytes and blastocyst area was calculated using ImageJ (NIH) due to limitations of the LAS X software (Arena et al. 2021).

2.13 Nile Red-Bodipy staining

LDs were stained on blastocysts previously fixed in 4% paraformaldehyde for 20min. The fixed blastocysts were incubated with 1µg/ml BODIPY 493/503 (Invitrogen™) and 10µg/ml Nile Red in 0.4% PBS-PVP for 1h and then washed three times in 0.4% PBS-PVP. Mounted specimens were analysed with confocal a ZEISS LSM 880 Confocal Laser Scanning Microscope using a 20X Zeiss Plan-Apochromat Infinitive corrected objective with a numerical aperture of 0.8. Relative fluorescence signal intensities were analysed using ImageJ and normalised to control (young) (Bisogno et al. 2023) .

Chapter I. Effect of *in vitro* culture of embryos on offspring behaviour and development.

I.I Behaviour

In order to verify the extent of ART-induced neurodevelopmental alterations, the effect of *in vitro* culture of embryos on offspring behaviour was evaluated.

In vitro cultured embryos (IVC) were transferred to pseudo-pregnant females. Control groups consist of embryos obtained by natural mating (NM), developed *in vivo*. As an additional control, part of those embryos were transferred to pseudo-pregnant females (embryo transfer, ET), similarly to IVC group. Offspring born from IVC, ET, and NM was subjected to behavioural testing to assess sociability and anxiety like-behaviour.

1. Social recognition -Three chamber social recognition test

IVC adult males showed impairment in behaviour, particularly reduced sociability, and novel interaction.

First generation of IVC males showed significant reduction of social interaction time in comparison to NM and reduced social preference index (Fig 5A) in comparison to both control groups NM and ET which is a consistent indication of social deficits, including impairment in social engagement, social interest, social interaction and social preference.

In regards to novelty interaction, IVC males are characterised by decreased social motivation and novelty in comparison to NM, as shown by lower interaction time with the novel stimuli (N) (Fig.5B), although IVC males still showed higher Novel interaction (N) than Non-Novel interaction (NN), a sign of intact social memory.

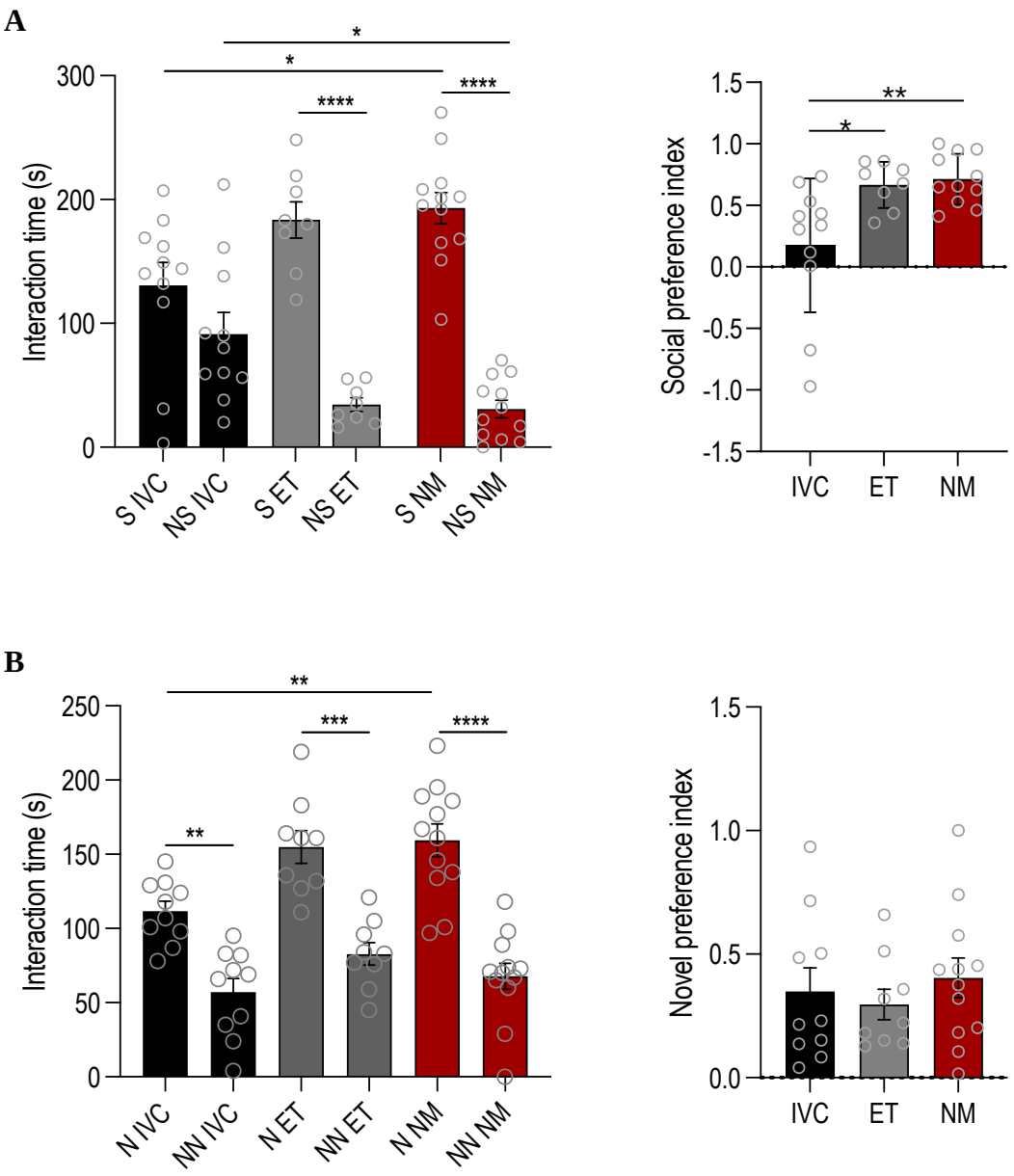


Figure 5: Sociability and novel interaction are reduced in IVC males (Three chamber social recognition test). A Social interaction. Left graph shows interaction time (in seconds) with the social stimuli (S), and Non-Social stimuli (NS) during the II phase of the test. ANOVA- followed by *post hoc* Bonferroni test multiple comparison. * $p < 0.05$ ** $p< 0.01$ *** $p< 0.001$ **** $p< 0.0001$. mean ± SEM. Right graph shows social preference index calculated as $ISP = (T_S - T_{NS})/(T_S + T_{NS})$. T (time), I_{SP} (Index of Social Preference) Non-parametric ANOVA- followed by Kruskal-Wallis test multiple comparison. * $p < 0.05$ ** $p< 0.01$, mean ± SEM. Number of samples ≥ 9 **B. Novel interaction** Left graph shows interaction time (in seconds) with the novel stimuli (N), and Non-novel stimuli (NN) during the III phase of the test. ANOVA- followed by post hoc Bonferroni test multiple comparison. ** $p< 0.01$ *** $p< 0.001$ **** $p< 0.0001$. mean ± SEM. Right graph shows novel preference

index calculated as $I_{NP} = (T_N - T_{NN})/(T_N + T_{NN})$. One way ANOVA- followed by *post hoc* Bonferroni test multiple comparison. * $p < 0.05$ mean ± SEM. Number of samples ≥ 9.

2. Anxiety-like behaviour (Light/Dark Box test)

In order to reveal if IVC offspring show any anxiety-like behaviour, animals from IVC group were subjected to Light/dark box test, a behavioural assay commonly used for this purpose. IVC males displayed normal spontaneous exploratory behaviour in novel environments as demonstrated by similar values compared to NM males in latency time (s) – the length of time the mouse spends to first enter the light box – and in the number (n) of transitions, which indicates how many times the mouse moved from the dark to the light box (Fig. 6). Additionally, IVC males present natural aversion to the brightly illuminated area. Indeed, IVC males as well as ET and NM spent majority of their time in the dark box, and no differences in time spent in the dark box were found between groups (Fig. 6).

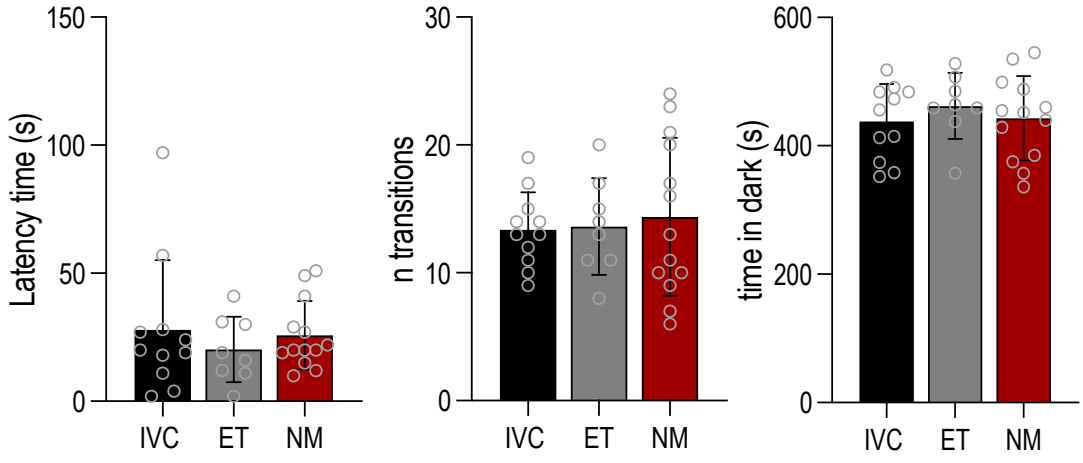


Figure 6: Anxiety-like behaviour is not altered in IVC offspring (Light/Dark Box test). The graphs show latency time in seconds (s), the number (n) of transition from dark to light box, and time in the light box for IVC, ET, and NM animals. n of samples ≥8. mean ± SEM. ANOVA- followed by Kruskal-Wallis test multiple comparison, $p > 0.05$.

I.II General health assessment

To reveal the effect of *in vitro* culture of embryos on offspring development, the general health of offspring born from IVC embryos was assessed by evaluating bodyweight and survival rate from pre-weaning period to adulthood. Pregnancy rate, offspring rate, and litter size were also evaluated to assess embryo survival and subsequent development to term.

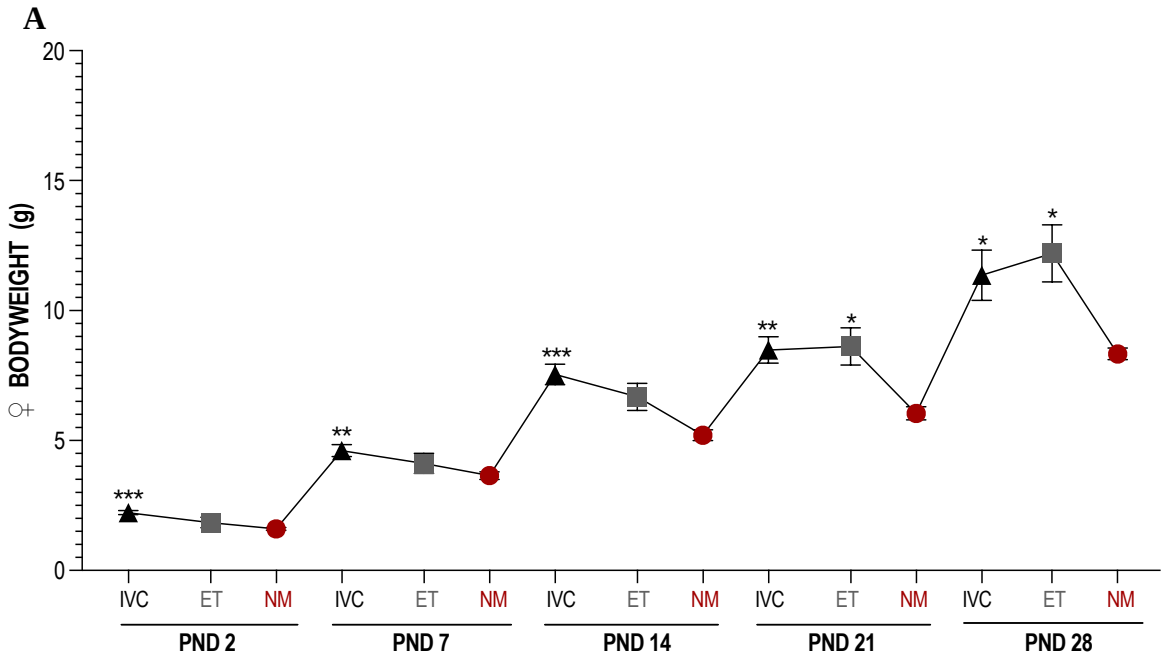
1. Bodyweight

First generation progeny born after *in vitro* culture present increased bodyweight in pre-weaning period which persists in adulthood.

Progeny born after *in vitro* culture (IVC) showed increased bodyweight in comparison to naturally-conceived (NM) offspring at birth and during preweaning age. This alteration is present in both males and females.

Specifically, IVC males present higher bodyweight at PND 2, 14, 21, and 28 in comparison to NM (Fig.7A). Interestingly, this increased bodyweight is not correlated with litter size ($r < 0.3$ or $r > -0.3$ p value > 0.05 after Pearson r correlation analysis). Additionally, ET males also present increased bodyweight in comparison to NM, at PND 14, 21, and 28 (Fig.7A) but at PND 14 the increased bodyweight is moderately correlated to the litter size ($r = -0.6895$ p value= 0.0399 after Pearson r correlation).

Similarly, IVC females showed higher bodyweight in each stage of pre-weaning period (PND 2, 7, 14, 21, 28) in comparison to NM females while ET females show increased bodyweight only at PND 21 and 28 (Fig.7B). The increased bodyweight for IVC and ET females is not correlated to the litter size ($r < 0.3$ or $r > -0.3$ p value > 0.05 after Pearson r correlation analysis). The elevated bodyweight persists in adulthood for both IVC males and IVC females (Fig.7 C).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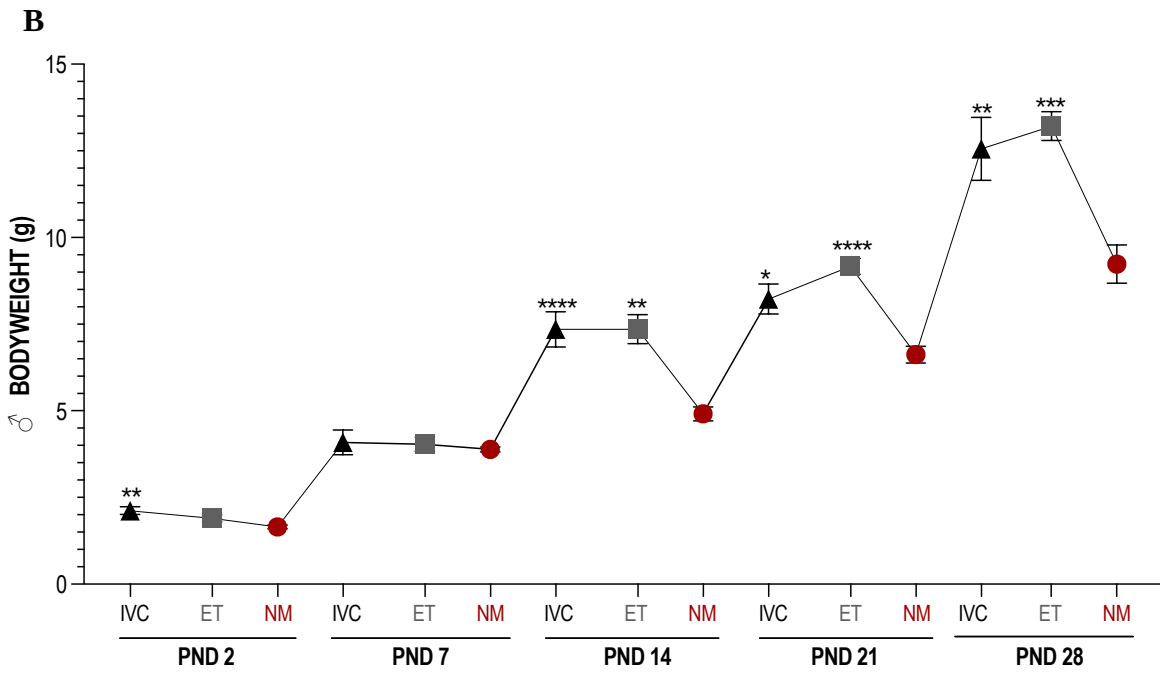
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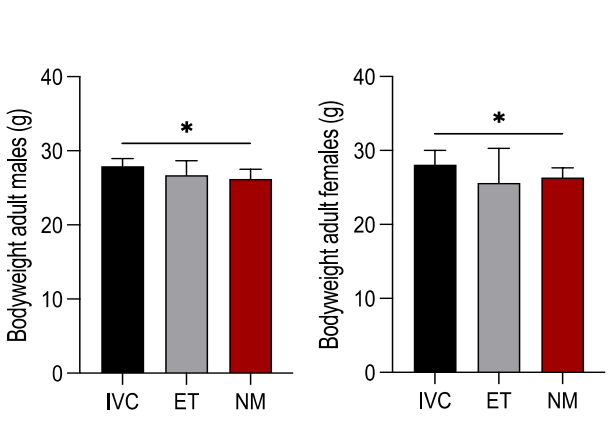
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30
20
10
0
*
IVC ET NM
Bodyweight adult females (g)
40
30
20
10
0
*
IVC ET NM

Figure 7: Pre-weaning growth curve and adulthood bodyweight perturbations in IVC offspring. A. Pre-weaning bodyweight (Males). The graph shows bodyweight at PND 2, 7, 14, 21, and 28 males progeny (IVC, ET, and NM). Mean ± SEM, ANOVA- followed by Kruskal-Wallis test multiple comparison, * $p < 0.05$ ** $p< 0.01$, $p< 0.01$ *** $p< 0.001$ **** $p< 0.0001$. Number of samples NM ≥18, ET=9, IVC ≤10 **B. Pre-weaning bodyweight (females)** The graph shows bodyweight at PND 2, 7, 14, 21, and 28 female progeny (IVC, ET, and NM). Mean ± SEM, ANOVA- followed by Kruskal-Wallis test multiple comparison, * $p < 0.05$ ** $p< 0.01$, $p< 0.01$ *** $p< 0.001$. Number of samples NM ≥16, ET=5, IVC=8 **C. Adult bodyweight (males and females).** The graphs show bodyweight in adult males and females (3-4 months old). Mean ± SEM, ANOVA- followed by Kruskal-Wallis test multiple comparison, * $p < 0.05$ n samples males IVC=9, ET=5, NM=11, n samples females IVC=12, ET=4, NM=9.

2. Offspring survival

IVC progeny show a survival rate comparable to NM offspring.

Survival rate of the progenies has been recorded during preweaning time (PND 2, and PND 28), at weaning (5 weeks), and at 6 weeks (1 week after weaning). IVC progeny doesn't show any significant difference in survival rate in comparison with ET and NM group.

During pre-weaning time at PND 2, 90.91% (30 pups/33 pups born), 87.40% (14 pups/ 16 pups born), and 97.30% (36 pups/37 pups born) of the pups born respectively from IVC, ET, and NM group were still alive. Similarly, at PND 28, 72.73% of IVC progeny (24 pups/33 pups born), 87.50% of ET progeny (14 pups/16 pups born), and 86.49% of NM progeny (32 pups/37 pups born) survived (Fig.8A).

IVC, ET, and NM pups equally survived until weaning (5 weeks) and beyond (6 weeks). At 5 weeks 72.73% of IVC group, 87.50% of ET group, and 78.38% of NM pups survived; similarly at 6 weeks, 63.64% of IVC, 81.25% of ET, and 78.38% of NM pups survived (Fig.8B).

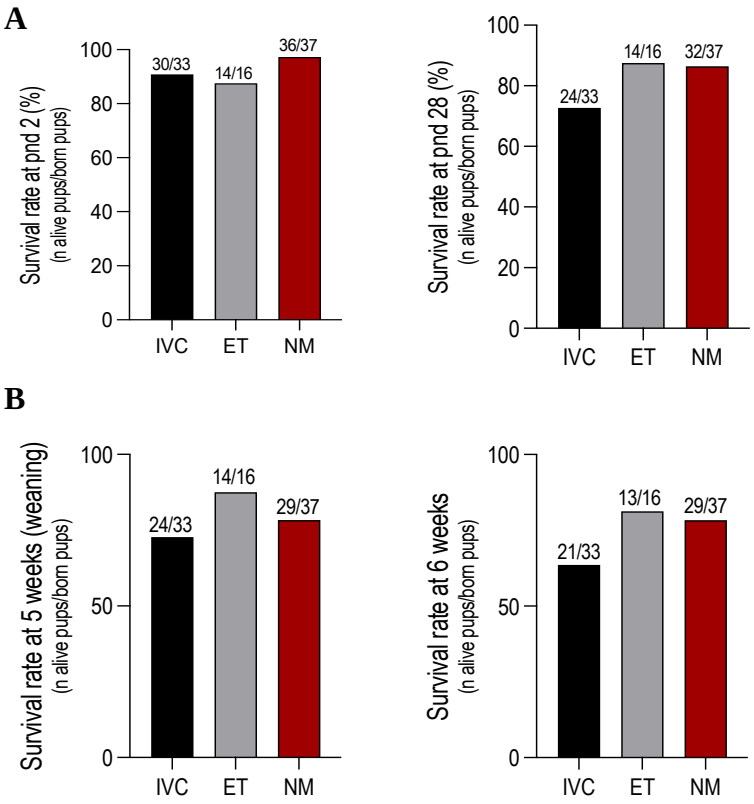


Figure 8: Offspring survival I generation. A. Survival rate at pre-weaning age. The graphs show survival rate in percentage (%) at PND 2 and PND 28; numbers above the columns indicates n of alive pups out of n of born pups. $p>0.05$ after χ-square test **B. Survival rate at weaning and after weaning.** The graphs show survival rate in percentage (%) at 5 weeks old and 6 weeks old; numbers above the columns indicates n of alive pups out of n of born pups. $p>0.05$ after χ-square test.

3. Embryo survival and development to term

The use of assisted reproductive technologies negatively impact on embryo survival and development to term.

The majority of the transferred IVC embryos did not develop into alive offspring. Indeed only 21.85% IVC embryos (33 pups born/151 transferred embryos) successfully developed to term

resulting in alive offspring. Similarly, 24.62% of ET embryos developed to term (16 pups born/65 transferred embryos) (Fig.9).

Additionally, the survival of IVC and ET embryos was lower than NM. IVC litter size and ET litter size were significantly smaller than NM (Fig. 9).

However, pregnancy was not affected by ART. Specifically, 9 out of 11 embryo transfers (81.82%) performed using IVC embryos successfully resulted in pregnancy, and similarly 5 out 6 females (83.33%) resulted pregnant after transfer of ET embryos. Naturally-conceived embryos showed 100% of pregnancy success: 7 out of 7 mated females became pregnant.

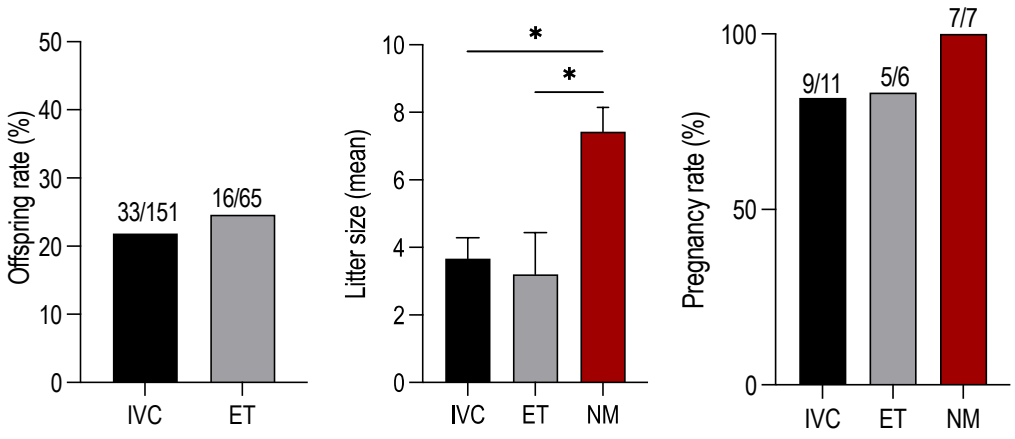


Figure 9: ART impacts on embryo survival and development to term. Left graph shows offspring rate in percentage; n above the columns indicate n pups born out of transferred embryos. $p>0.05$ after χ-square test. Middle graph shows the litter size of IVC, ET, and NM group. Mean ± SEM, ANOVA- followed by Kruskal-Wallis test multiple comparison. * $p < 0.05$; n litters per group: IVC=9, ET=5, NM=7. Right graph shows percentage of pregnant females per group. Numbers above the columns indicate respectively, number of pregnant females out of number of females in which IVC and ET embryos have been transferred to, and number of pregnant females out of number of mated females (NM). $p>0.05$ after χ-square test.

4. Fertility

Progeny conceived by ART does not show fertility impairments.

IVC males and females from I generation progeny were mated in order to obtain II generation male and female line (IVC ♂ line, and IVC ♀ line); moreover IVC females and males were crossed in order to obtain IVC cross line (IVC ♂ x ♀ line).

The pregnancy rate for IVC cross line, male line, and female line is respectively 88.89% of pregnancy success (8 pregnant females out of 9 mated females), 66.66% (6 pregnant females out of 9 mated females), and 100% (5 pregnant females out of 5 mated females) (Fig.10).

Similarly, the litter size of the successful pregnancies did not show any difference in comparison to control group (NM) (Fig.10).

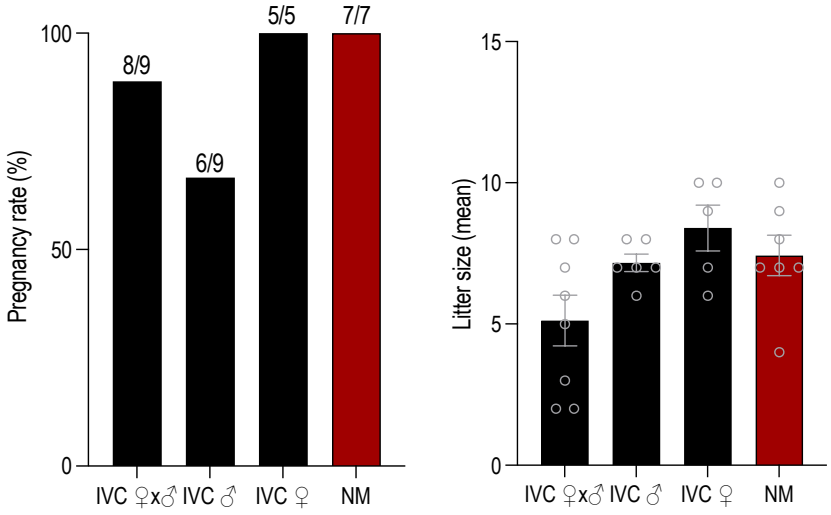


Figure 10. Fertility is not impaired in IVC offspring-I generation. Left graph shows percentage of pregnant females per group. Numbers above the columns indicate n of pregnant females out of n females mated. $p>0.05$ after χ-square test. Right graph show the litter size (n pups per litter). Mean ± SEM, ANOVA- followed by Kruskal-Wallis test multiple comparison. $p> 0.05$.

Chapter II. Intergenerational transmission of ART effects and effects of crossbreeding ART progenies

II.I Survival Rate

Survival was reduced in the second generation of ART offspring.

In order to evaluate the intergenerational transmission of ART effects, IVC males and females from I generation progeny were mated to obtain II generation male and female line (IVC ♂ line, and IVC ♀ line); additionally, in order to establish the level of penetrance of ART aberrancies, IVC males and females were crossed to obtain IVC cross line (IVC ♂ x ♀ line). Survival rate of the progenies was recorded during preweaning time (PND 2, and PND 28), at weaning (5 weeks), and at 6 weeks (1 week after weaning).

II generation progeny born from IVC parents showed higher mortality in comparison to control group (NM). In particular, IVC cross line (IVC ♂ x ♀ line) had the lowest survival between all experimental groups; indeed, only 24.39% of the born pups survived until 6 weeks of age (10 alive pups/41 pups born) (Fig. 11B). Similarly, during pre-weaning period IVC cross line exhibited lower survival rate than NM with 75.6% of survival rate (31 pups alive/41 born pups) at PND-2, 51.22% (21 alive pups/41 born pups) at PND-28, and 34.14% (14 alive pups/41 born pups) at 5-weeks of age (Fig.11A-B).

IVC male line progeny (IVC ♂ line) also showed lower survival rate than NM but only after pre-weaning time when survival decreased, respectively, to 58.6% at 5-weeks of age (17 pups alive/36 pups born), and finally to 33.33% at 6-weeks (12 pups alive/36 pups born) (Fig.11B). During the pre-weaning time at PND 2 and PND-28, respectively 97,22% (35 pups/36 pups born), and 88.89% (32 pups/ 36 pups born) of the pups born were alive (Fig.11A).

IVC female line progeny (IVC ♀ line) showed lower survival rate than control group (NM) at 6 weeks when only 50% of the pups were alive (16 pups/ 32 pups born) (Fig.11 B). At PND-2, -28, and 5 weeks of age, the survival rate remained steady at 90.63% (29 pups alive/32 pups born) and comparable to control group (Fig. 11 A-B).

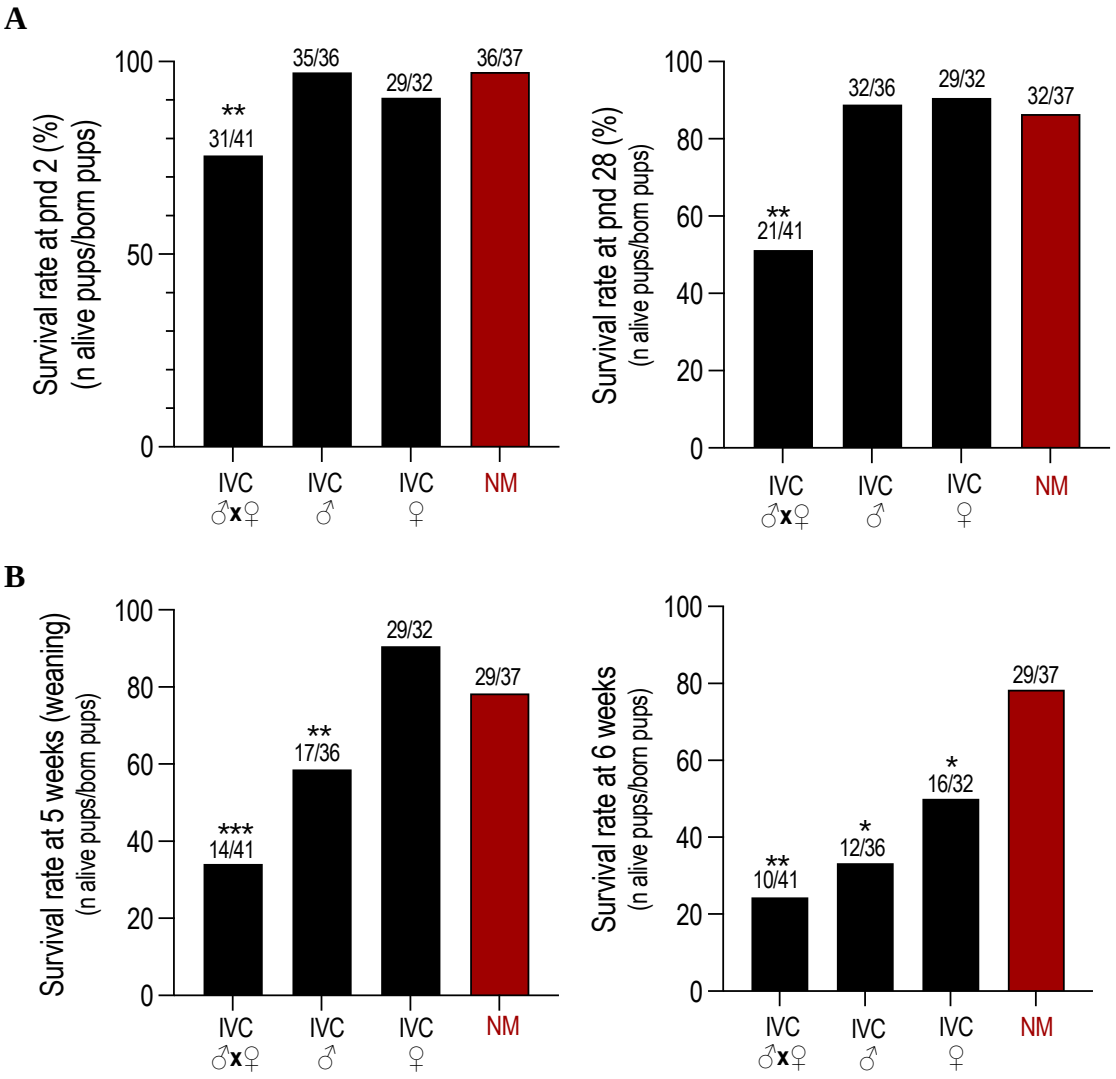


Figure 11: II generation survival rate decreases at pre-weaning age and after weaning A. Survival rate at pre-weaning age. The graphs show survival rate in percentage (%) at PND 2 and PND 28, numbers above the columns indicates n of alive pups out of n of born pups. ** $p < 0.01$ vs NM, χ-square test **B. Survival rate after weaning.** The graphs show survival rate in percentage (%) at 5 weeks old and 6 weeks old, numbers above the columns indicates n of alive pups out of n of born pups. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ vs NM, χ-Square test.

II.II Behaviour

1. Social recognition -Three chamber social recognition test

II generation ART offspring (IVC male line and female line) show impaired social recognition memory and reduced motivation for novelty.

In order to assess if social and novelty impairments in behaviour have been transmitted to the II generation of ART offspring, three chamber social recognition test was performed for all survived male progeny (5 from IVC ♀x♂, 7 from IVC ♂, and 8 from IVC ♀).

Males from IVC male line (IVC ♂) and IVC female line (IVC ♀) showed no predilection for the novel stimuli (N) in comparison to the non-novel stimuli (NN) in III phase of the test, spending equal time to interact with the newly-introduced mice and already-known mice introduced in II phase (Fig. 12B-C right graphs). However, males from IVC ♀ and IVC ♂ group showed lower interaction time with the novel stimuli (N) in comparison to the control group (NM) (Fig. 12B-C right graphs).

Males from IVC ♀x♂ showed higher interaction time with the novel stimuli (N) in comparison to Non-Novel stimuli (NN), as well as similar N and NN interaction time compared to the control group (NM) (Fig. 12 A- right graphs).

No alterations in social interaction were recorded for all 3 ART groups, as the interaction time spent with the social stimuli (S) resulted similar to NM, as well as the Social preference index (Fig. 12A-B-C left graphs)

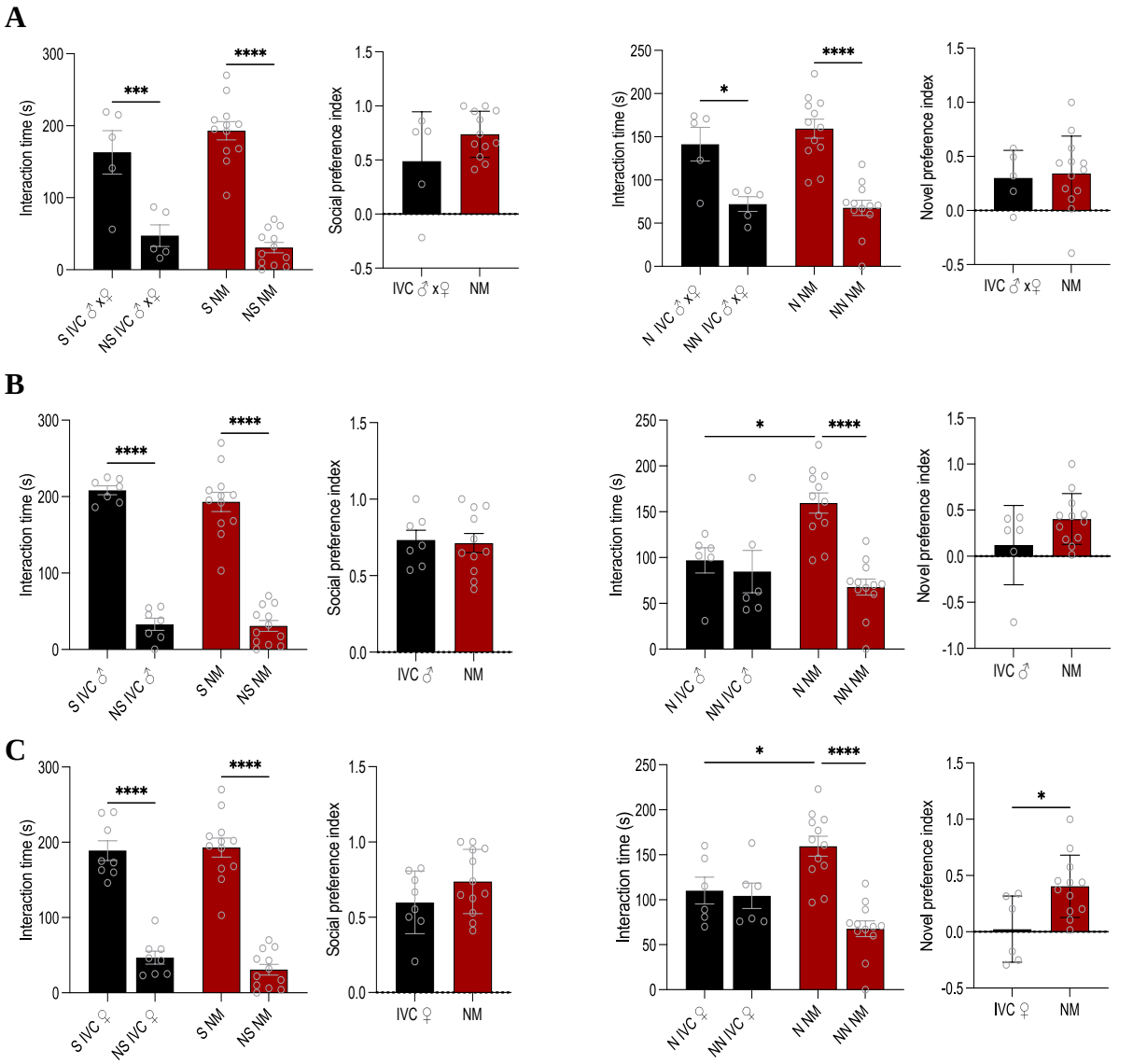


Figure 12: Social recognition memory and motivation for novelty in II generation IVC (Three-Chamber social recognition test). A. IVC female line x IVC male line (IVC ♀x♂). B. IVC male line (IVC ♂). C. IVC female line (IVC ♀). Left graphs show interaction time (in seconds) with the social stimuli (S), and Non-Social stimuli (NS) during the II phase of the test, followed by social preference index. ANOVA- followed by *post hoc* Bonferroni test multiple comparison. *** $p< 0.001$ **** $p< 0.0001$. mean ± SEM. social preference index calculated as $ISP = (T_S - T_{NS})/(T_S + T_{NS})$. T (time), I_{SP} (Index of Social Preference) Non-parametric ANOVA- followed by Kruskal-Wallis test multiple comparison, mean ± SEM. Right graphs show interaction time (in seconds) with the novel stimuli (N), and Non-novel stimuli (NN) during the III phase of the test, and preference index calculated as $I_{NP}= (T_N - T_{NN})/(T_N + T_{NN})$. One way ANOVA- followed by *post hoc* Bonferroni test multiple comparison. * $p < 0.05$ **** $p< 0.0001$ mean ± SEM.

1. Anxiety-like behaviour (Light/Dark Box test)

II generation ART offspring (IVC female line) is characterised by higher levels of anxiety-like behaviour.

Males from II generation were tested for anxiety-like behaviour by Light/Dark test which revealed that IVC males from females line (IVC ♀) spent less time than control (NM) to enter the light box for the first time (latency time), and spent more time than control group in the light box (time in light); while males from IVC ♂ and IVC ♀x♂ group showed similar latency time and time spent in light box than NM (Fig 13). All experimental groups showed similar number of transitions (n transitions) between dark and light box.

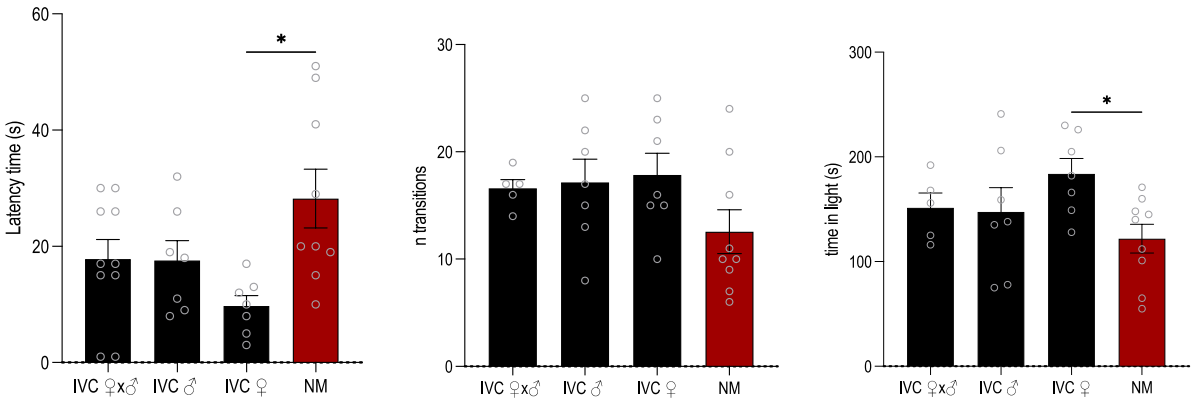


Figure 13: Anxiety-like behaviour in II generation IVC (Light/dark box test). The graphs show latency time in seconds (s), the number (n) of transition from dark to light box, and time in the light box. mean ± SEM. ANOVA- followed by Kruskal-Wallis test multiple comparison, ** $p < 0.01$.

II.III Bodyweight

The survived II generation ART offspring don't show any growth perturbation in pre-weaning period.

Bodyweight was recorded from PND 2 until PND 28 (every 7 days). Males from , IVC ♂ line, IVC ♀ line showed lower bodyweight at PND-7 in comparison to NM males. No other

differences have been noticed from PND-14 to PND-28 (Fig.14A). Females from the 3 experimental groups of II generation did not show any variation in bodyweight when compared to NM females Fig. 14B).

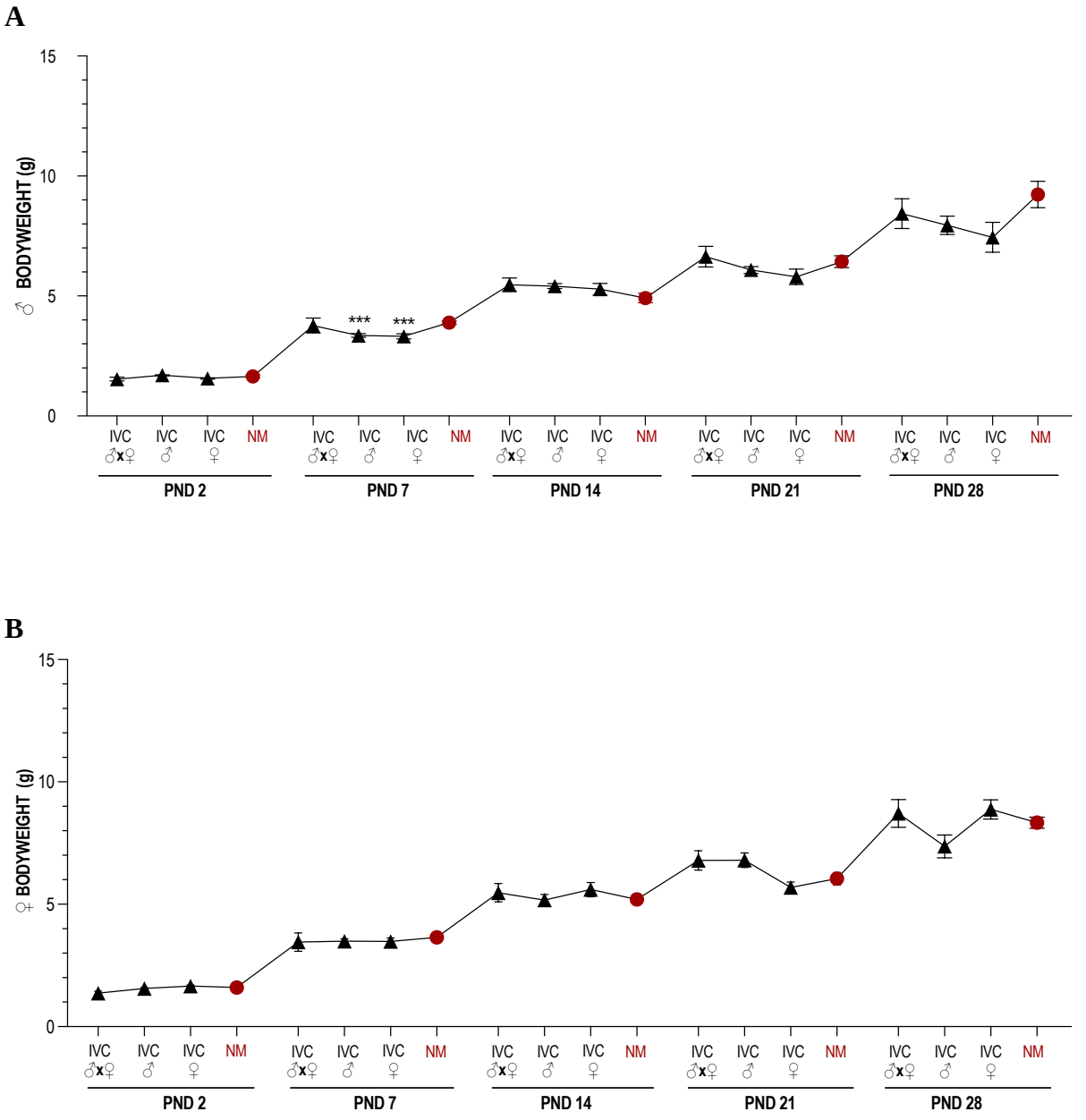


Figure 14: Pre-weaning growth curve of II generation IVC. A The graph shows the bodyweight at PND 2, 7, 14, 21, and 28 related to males. Mean ± SEM, ANOVA- Kruskal Wallis test multiple comparison, * $p < 0.05$ ** $p< 0.01$ *** $p< 0.001$ **** $p< 0.0001$ vs NM **B** The graph shows the bodyweight at PND 2, 7, 14, 21, and 28 related to females, n for Mean ± SEM, ANOVA- Kruskal Wallis test multiple comparison, * $p < 0.05$ ** $p< 0.01$ *** $p< 0.001$ **** $p< 0.0001$ vs NM.

Chapter III. Brain alterations in ART offspring and foetuses collected at late pregnancy.

III.I Adult brain- Hypothalamus Oxidative stress

Hypothalamus of IVC males showed impaired antioxidant activity resulting in increased oxidative stress and elevated level of lipid peroxidation byproducts.

The regulation of oxidative stress and lipid peroxidation, critical for normal brain development and function, were observed to be altered in hypothalamus of IVC offspring as shown by lower level of reduced glutathione (GSH) – a crucial antioxidant that helps protect cells from oxidative damage – and lower levels of oxidised glutathione (GSGG) in comparison to control group (NM) (Fig.15). Moreover, GSH/GSSG ratio, an important indicator of cellular redox status and balance between antioxidant defence and oxidative stress, is increased in IVC hypothalamus, indicating an increased OS level, ulteriorly evidenced by increased level of Malondialdehyde (MDA), a lipid peroxidation byproduct (Fig 15).

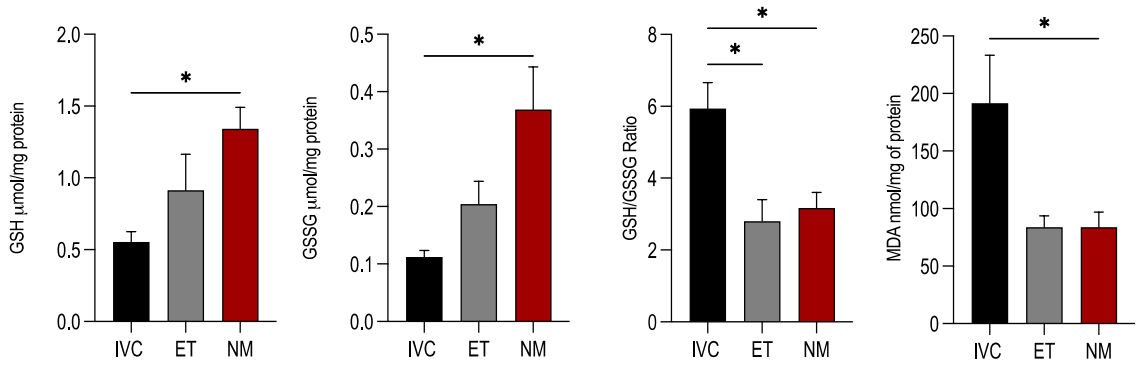


Figure 15: Impaired antioxidant activity in Hypothalamus of adult IVC males – I generation. The graphs show GSSG, GSH in µmol/mg, GSH/GSSG ratio, and MDA in nmol/mg. Mean ± SEM, ANOVA- Kruskal Wallis test multiple comparison, * $p < 0.05$; n samples per group =8.

III.II Adult brain- Hippocampus Proteomic

Proteins involved in pathways of neurodegeneration/neurological diseases and oxidative stress are differentially expressed in hippocampus of adult IVC progeny.

Proteomic analysis revealed alterations of protein expression in IVC mice hippocampi in comparison to NM groups. The differently-expressed proteins are involved in several pathways of neurodegeneration and neurodevelopmental disorders. Also, proteins involved in pathways related to oxidative stress, such as glutathione metabolism and oxidative phosphorylation, were differentially expressed in IVC hippocampus (Fig. 16).

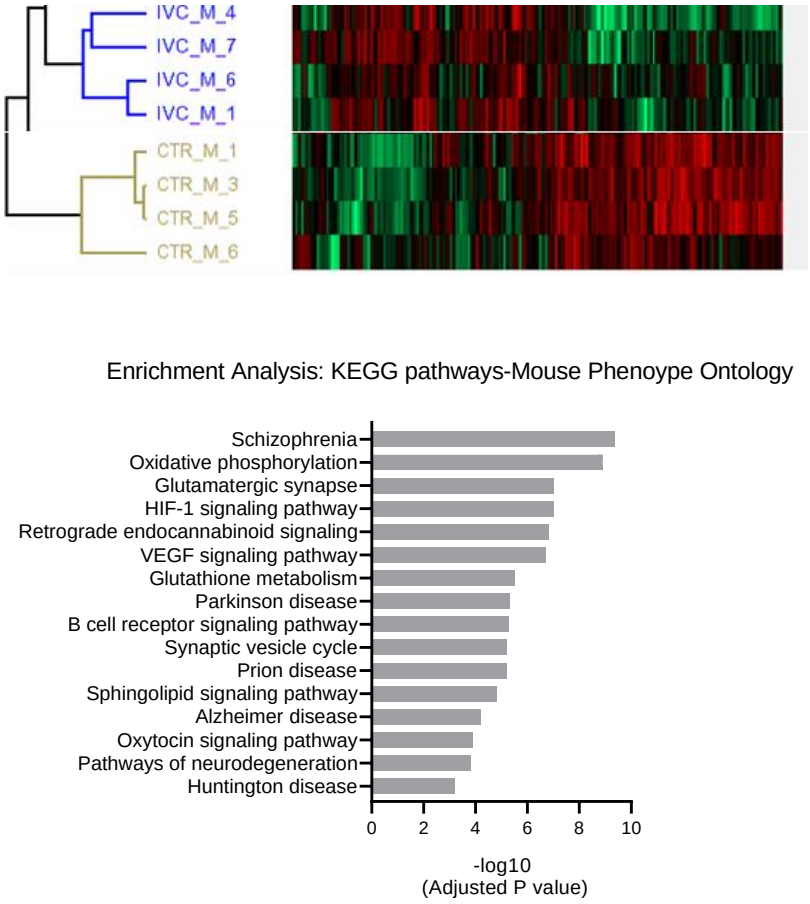


Figure 16: Pathways involved in neurodegeneration/neurodevelopmental disorders and oxidative stress are altered in IVC adult hippocampus (Proteomic) . Heatmap of differentially-expressed proteins in Hippocampus

from IVC adult male compared to NM *(upper graph)*; enrichment analysis of differentially expressed proteins in hippocampus from IVC group related to several pathways *(bottom graph)* n samples= 5.

III.I Adult brain- weight

Adult IVC progeny showed increased brain weight which may indicate macrocephaly.

IVC adult brain weight is higher in comparison to NM adult brain weight (Fig. 17) and it is not correlated to the increased bodyweight ($r < 0.3$, p value > 0.05 after Pearson r correlation analysis). The increased brain weight could be an indication of macrocephaly or brain overgrowth, linked to autism-associated behaviours (Wang et al. 2020).

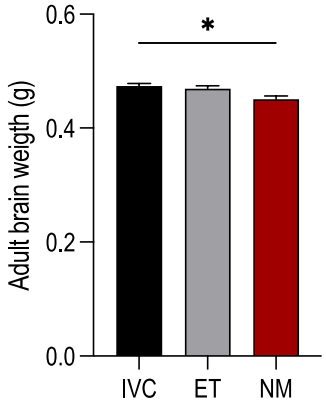


Figure 17: Brain weight increasing in adult IVC males. Brain weight in grams (g) of adult males for I generation IVC, ET, and NM. n samples IVC=5, ET=5, NM=11. Mean ± SEM, ANOVA- Kruskal Wallis test multiple comparison, * $p < 0.05$ vs NM.

III.IV Prenatal brain - Oxidative stress

Oxidative stress level and consequent lipid peroxidation activity is considerably increased in IVC prenatal brain.

IVC prenatal brain showed increased level of GSH, GSSG, SOD activity, and MDA (Fig.18A). Elevated GSH levels is an adaptive response to counteract increased ROS production, while

elevated GSSG levels reflect the accumulation of oxidised glutathione due to excessive ROS exposure. Also, SOD activity increases in order to mitigate the harmful effects of ROS by converting superoxide radicals into less reactive species. As a consequence of excessive oxidative stress, lipid peroxidation occurs and an elevated level of lipid peroxidation byproducts like MDA can be observed, likely resulting in cell membrane damage and cellular dysfunction.

Moreover, IVC prenatal brain showed decreased mRNA expression of HMOX1 (Heme Oxigenase 1) – a key component of the cellular antioxidant defence system – as well as decreased mRNA expression of PPAR-α (Peroxisome proliferator-activated receptor alpha), a regulator of anti-oxidative processes and essential element in fatty acid transport and lipid metabolism (Fig.18B).

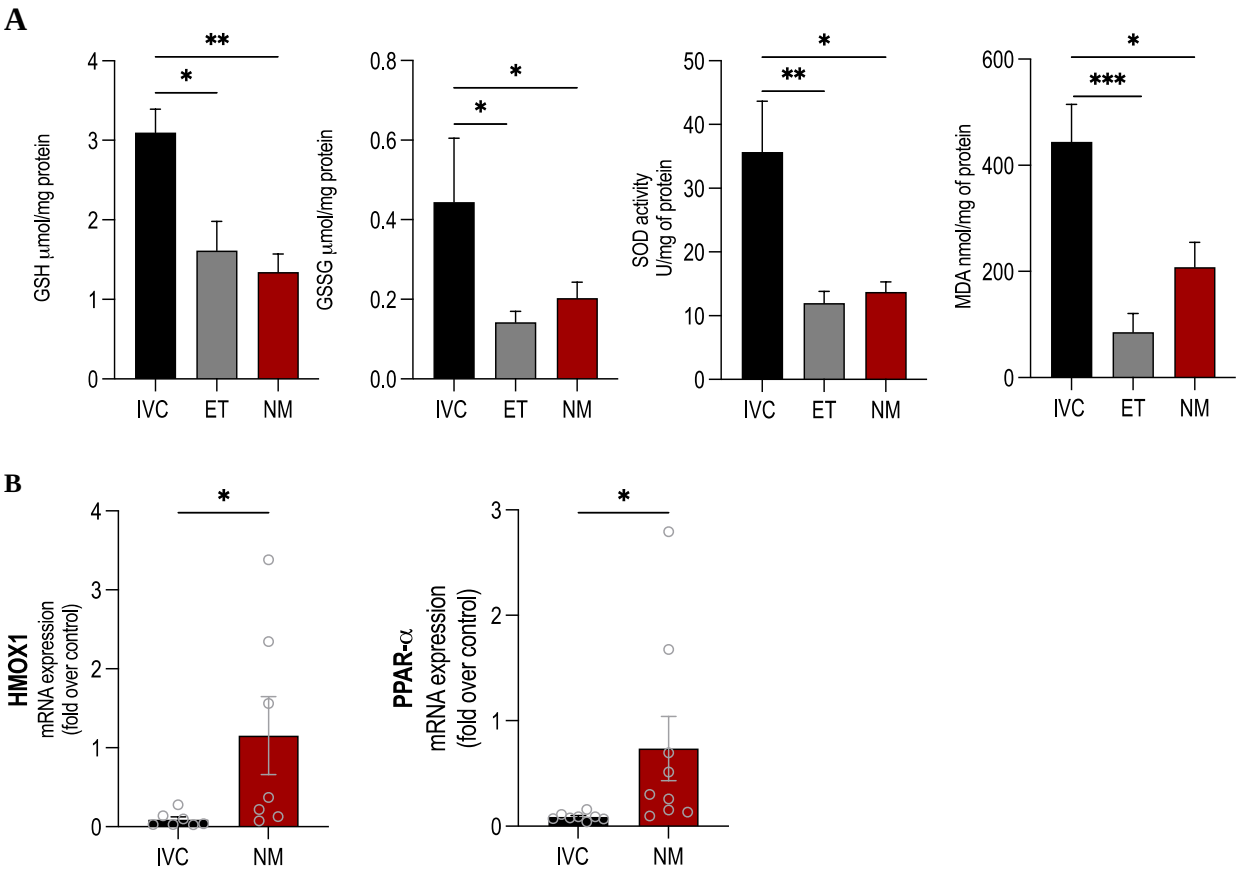


Figure 18: Oxidative stress and lipid peroxidation increases in IVC prenatal brain. **A.** The graphs show GSSG, GSH in µmol/mg, SOD activity in units/mg, and MDA in nmol/mg. Mean ± SEM, ANOVA- Kruskal Wallis test multiple comparison, * $p < 0.05$ ** $p< 0.01$ *** $p< 0.001$.n samples =8; **B.** The graphs show mRNA expression of HMOX1 and, PPAR-α, values are depicted as mRNA abundance relative to housekeeping gene eEF2, Student-t test , mean ± SEM * < 0.05.

III.V Prenatal brain- Neurogenesis

Genes involved in crucial steps of neurogenesis have decreased expression in IVC prenatal brains.

In order to establish if brain structure and function develop correctly in ART foetuses, several genes crucial in neurogenesis process and in neurodevelopmental diseases insurgence were analysed.

The expression of GABRA1 (Gamma-aminobutyric acid receptor subunit alpha-1) gene – which contributes to the proper balance of excitatory and inhibitory neurotransmission during prenatal brain development and is involved neuronal circuit formation – was lower in IVC prenatal brain in comparison to control group. Moreover, Cntnap2 (Contactin-associated protein-like 2) – important for proper neuronal migration, axon guidance, and the establishment of neural circuits – showed a decreased expression in IVC prenatal brain. Finally, expression of MapK3 (Mitogen-activated protein kinase 3) was lower in IVC group than control (Fig.19). MapK3 plays a crucial role in neuronal development and synaptic maturation in prenatal brain, and is involved in neurogenesis regulation, axon growth, and synapse formation.

The disruption in GABRA1, Cntnap2, and MapK3 can cause abnormal brain development leading to social and cognitive deficits.

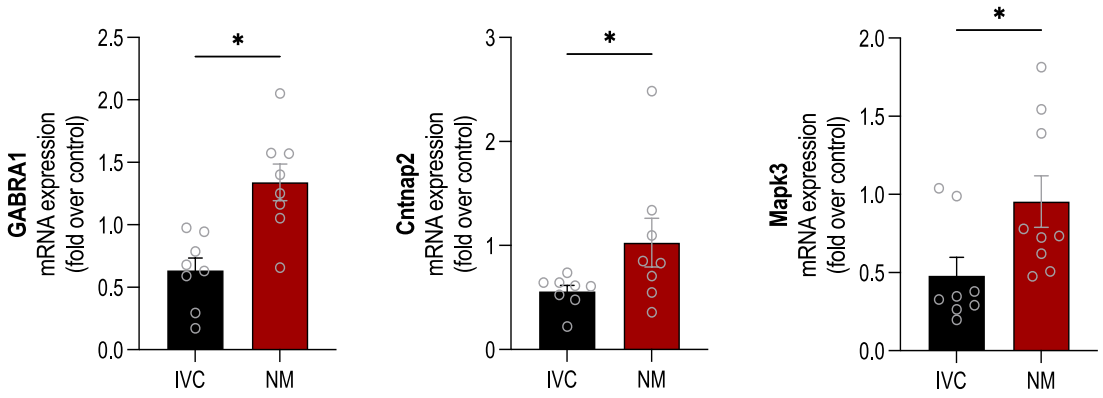


Figure 19. mRNA expression of genes involved in neurogenesis are impaired in IVC prenatal brain (qPCR). mRNA expression of Gabra1, Cntnap2, Mapk3 in prenatal brain homogenates. Values are depicted as mRNA abundance relative to housekeeping gene eEF2. Student-t test, mean ± SEM * < 0.05 ** p < 0.01.

III.V Prenatal brain morphology

IVC prenatal brain is characterised by altered morphology indicative of inflammation.

In order to investigate the consequences of increased lipid peroxidation and altered lipid metabolism, the inflammation state of prenatal brain was evaluated. Histology of IVC prenatal brain was analysed, particularly Medulla Oblongata (MO), Hypothalamus (HT), and neopallial cortex (NPC).

IVC prenatal brain showed altered MO structure characterised by increased presence of high diameter vacuoles, high presence of vacuole/spongiforms (Fig 20A), and distinctive signs of brain swelling/inflammation which were confirmed by elevated levels of Cox2 (Cyclooxygenase-2) expression (Fig. 20B), the main regulator of PGE2 (Prostaglandin-E_2) synthesis and inflammatory processes.

A

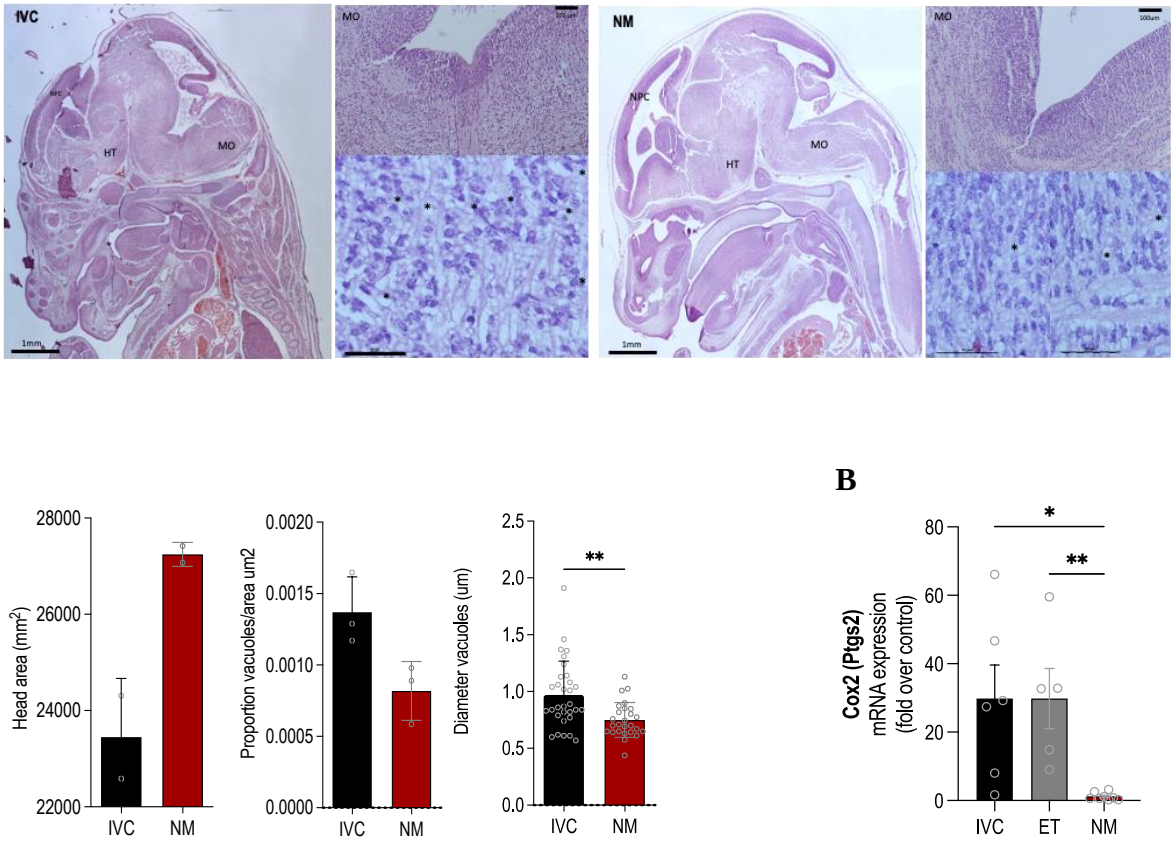


Figure 20: Altered morphology indicating inflammation in IVC prenatal brain. A. Representative images of foetuses from IVC group showing signs of swelling/inflammation and brain injury; and normal morphology of NM foetus (scale bar 1mm). MO: Medulla Oblongata; HT: hypothalamus; NPC: neopallial cortex. On the top right of the panel is a detail of medulla oblonga (MO), scale bar 100um; bottom right is higher magnification MO (scale bar 50um). MO from IVC group shows disarrangement and high presence of vacuoles/spongiform (star) Histograms show decreased head size; elevated proportion of vacuoles and higher vacuole diameter in IVC brains. Mean ± SEM; Student´s t test, * p< 0.05, ** p< 0.01. n samples =3. **B.** mRNA expression of Cox2 (in prenatal brain homogenates. Values are depicted as mRNA abundance relative to housekeeping gene eEF2. Student-t test, mean ± SEM * < 0.05 ** p < 0.01.

Chapter IV. Altered lipid profile and lipid peroxidation in ART embryos and oocytes.

IV.I Lipid profile in embryos after IVC

Altered lipid profile of IVC embryos showing increased lipid droplets (LDs) size, sign of LDs peroxidation.

To examine the source of the observed changes in lipid peroxidation and compositional alterations in the IVC prenatal brain, we analysed the LDs profile of IVC embryos.

Since the IVC embryos were transferred to healthy and young females, we can reasonably exclude the maternal side as cause of lipid damage in the prenatal brain. Therefore, it is likely that the origin of the lipid damage in the prenatal brain occurred prior to implantation (i.e. an embryonic origin).

IVC embryos were analysed by Coherent Anti-Stokes Raman Spectroscopy (CARs).

Embryos after *in vitro* culture (IVC) showed bigger lipid droplets mean size (clustering) and higher amount of LDs in the biggest size detectable by CARs (7.5-10.0 μm^2 and 10.0-100.0μm^2) compared to embryos in vivo produced (ET, and NM) (Fig. 21A-B). Moreover, IVC embryos have lower amount of LDs in the smallest size (0-0-250 μm^2) than ET and IVC group (Fig.21B).

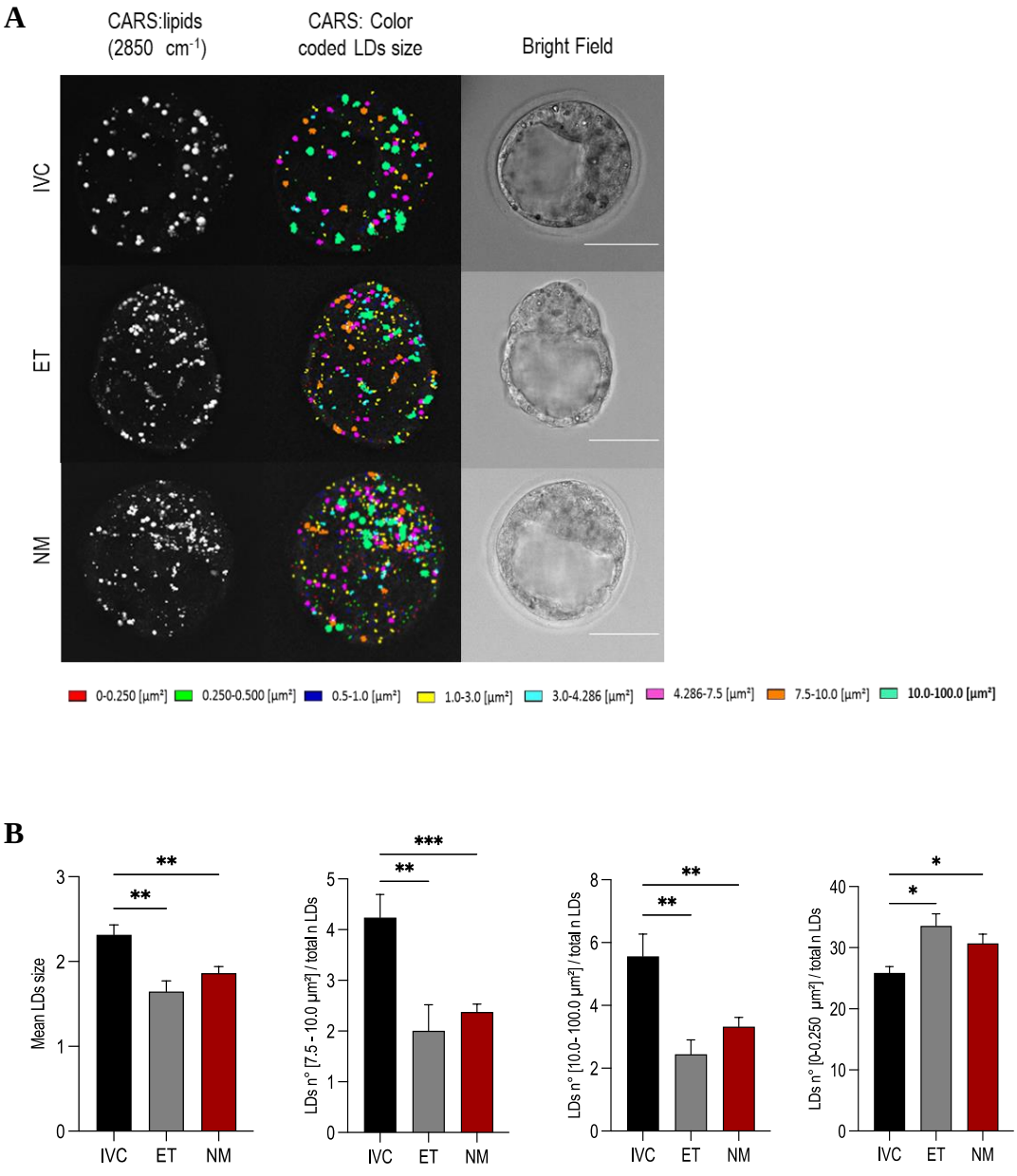


Fig. 21: LDs profile is altered in IVC embryos. Coherent anti-Stokes Raman spectroscopy (CARs)-mouse blastocyst. A. (Representative images of IVC, ET, and NM embryos CARs images**,** n samples IVC=12, ET=6, NM=15.Legend below the images indicates the colours associated with each LDs size; Scale bar 50 µm. **B.CARs LDs size analysis.** Graphs show mean LDs size, percentage of LDs in 7.5-10.0 μm^2 size, percentage of LDs in 10.0-100.0 μm^2 size, and percentage of lipids 0.0-0.250 μm^2 size. Mean ± SEM, ANOVA- Kruskal Wallis test multiple comparison, ** p< 0.01 *** p< 0.001 **** p< 0.0001. n samples IVC=12, ET=6, NM=15

IV.II LDs profile of AMA oocytes and embryos

LDs profile of AMA oocytes is also altered and shows similar LDs clustering in mouse model and human.

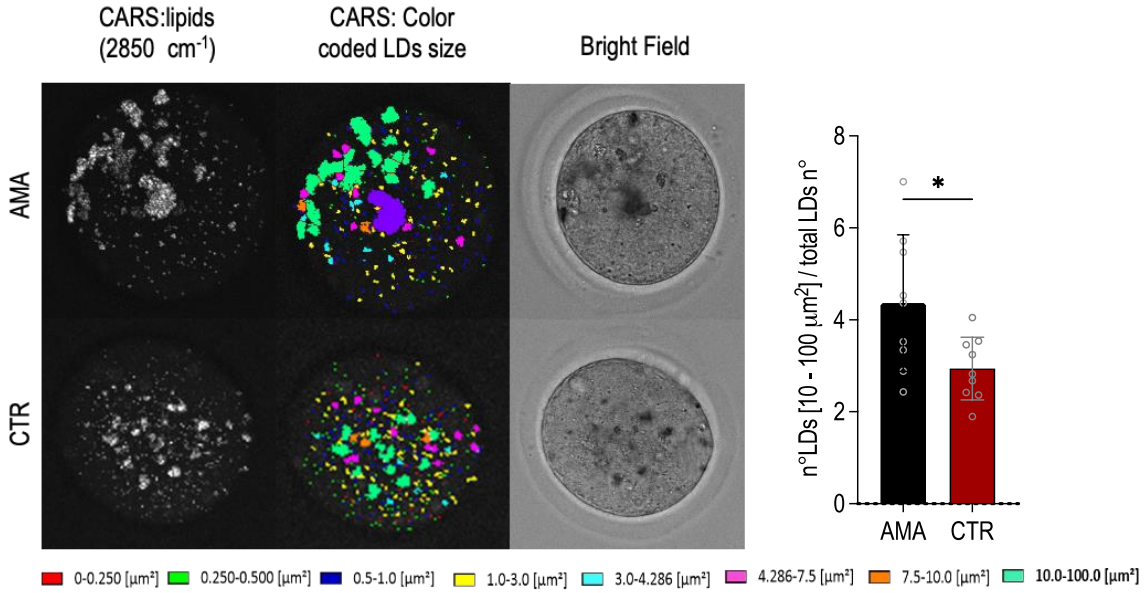
In order to elucidate the origin of increased risk of neurodevelopmental disorders (NDD) in the offspring that are associated with both ART and AMA (which is also frequently linked with ART), AMA oocytes and embryos from mouse and human model were analysed.

CARs analysis revealed that mouse AMA oocytes are characterised by agglomeration of lipids evidenced by large LDs droplets (10.0 - 100.0μm^2). Indeed, AMA oocytes have higher amount of LDs in the biggest size detectable by CARs (10.0 - 100.0μm^2) in comparison to CTR group (oocytes from mouse young donors) (Fig. 22.A). Interestingly, a similar LDs profile was shown by oocytes after OS treatment (+ OS) (Fig.22B)

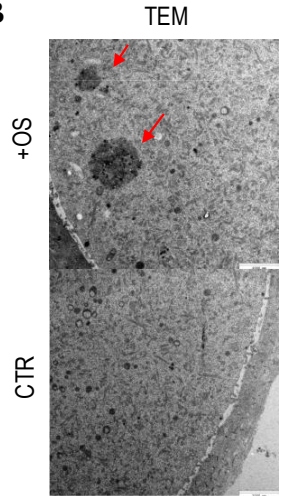
Similarly, in human – which is the mammalian species with the least amount of LDs – lipid droplet clustering occurs with advancement of age. Oocytes from donors of 3 different age groups – 19-23, 25-28, and 30-33 years old (Y) – were analysed by CARs. Oocytes from 30-33 Y and 25-28 Y had higher lipid total area and higher lipids mean size than oocytes from younger donors (19-23 Y) (Fig.22 C-D). Moreover, 30-33 Y and 25-28 Y oocytes showed higher LDs amount in the size of 1.0-3.0μm^2 in comparison to 19-23 Y oocytes. 1.0-3.0μm^2 LDs constitute the biggest LDs size represented in human (Fig.22 C); although a very low fraction (0.5-1.0%) of even larger size LDs – 3.0-4.286μm^2, and 4.286-7.5 μm^2 – have also been found in some human oocytes. Additionally, phospholipids structure changes, and lipid peroxidation is increased in 30-33 Y group (Fig.22D).

Relevantly, this altered LDs profile in AMA oocytes is maintained in embryos (Fig.22E). LDs have been labelled with Nile Red and BODIPY, and the analysis revealed that AMA blastocysts have higher amount of LDs than control (Fig.22E).

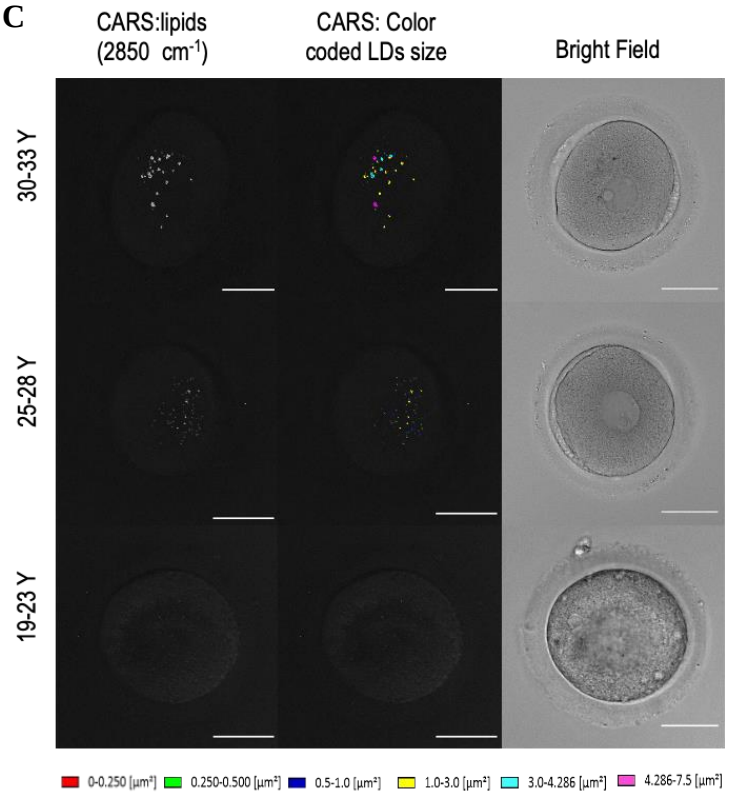
A

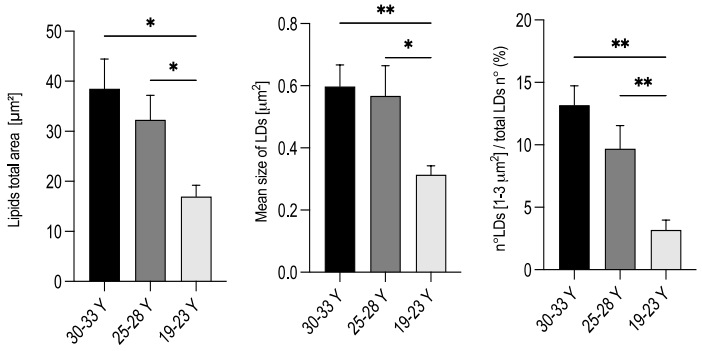

CARS:lipids
(2850 cm-1)
CARS: Color
coded LDs size
Bright Field
AMA
CTR
n°LDs [10 - 100 µm2] / total LDs n°
8
6
4
2
0
*
AMA
CTR
0-0.250 [µm²]
0.250-0.500 [µm²]
0.5-1.0 [µm²]
1.0-3.0 [µm²]
3.0-4.286 [µm²]
4.286-7.5 [µm²]
7.5-10.0 [µm²]
10.0-100.0 [µm²]

B

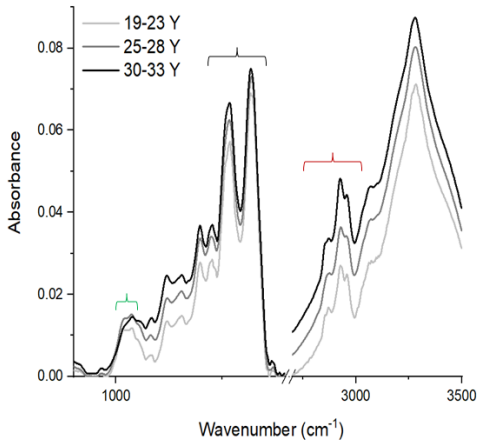

TEM
+OS
CTR

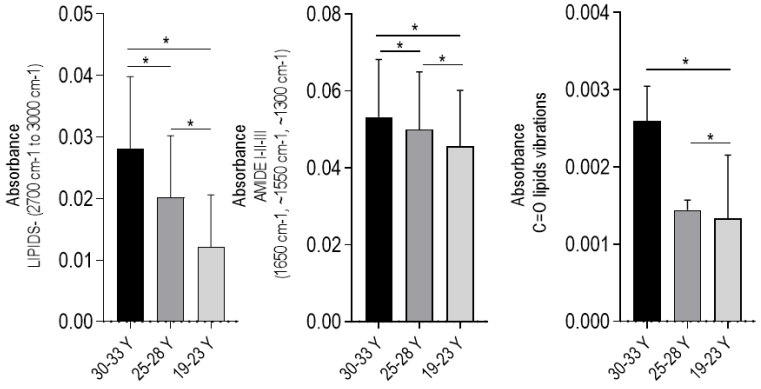
C


CARS:lipids (2850 cm-1)
CARS: Color coded LDs size
Bright Field
30-33 Y
25-28 Y
19-23 Y
0-0.250 [µm²]
0.250-0.500 [µm²]
0.5-1.0 [µm²]
1.0-3.0 [µm²]
3.0-4.286 [µm²]
4.286-7.5 [µm²]


Lipids total area [µm²]
Mean size of LDs [µm²]
n°LDs [1-3 µm²] / total LDs n° (%)
30-33 Y
25-28 Y
19-23 Y

D


19-23 Y
25-28 Y
30-33 Y
Absorbance
Wavenumber (cm-1)


Absorbance
LIPIDS- (2700 cm-1 to 3000 cm-1)
Absorbance
AMIDE I-II-III
(1650 cm-1, ~1550 cm-1, ~1300 cm-1)
Absorbance
C=O lipids vibrations
30-33 Y
25-28 Y
19-23 Y

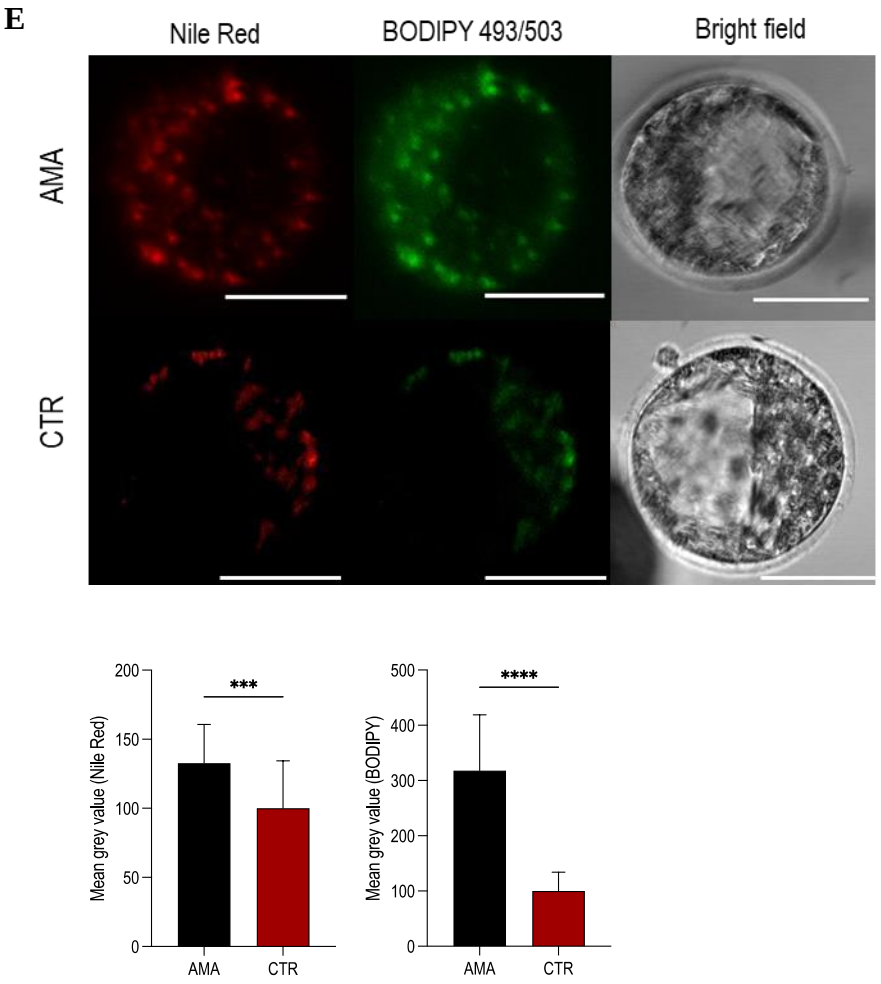


Figure 22: Altered lipid profile and lipid peroxidation in AMA oocytes and embryos (mouse and human). A. CARs analysis mouse oocytes. The panel shows representative images AMA and CTR oocytes at MII stage. The graph shows percentage of LDs in the size of 10.0-100.0μm^2. The legend below the images indicates the colour associated with each of the five LDs range/size. Mean ± SEM, t-Student test, * p< 0.05 n samples=10. **B. Lipid Profile of mouse oocytes after oxidative stress treatment (+OS).** The panel shows representative images of mouse oocytes +OS. Red arrows indicate the LDs agglomerations. **C. CARs analysis of human oocytes.** The panel shows representative images of human oocytes from 30-33 Y, 25-28 Y, and 19-23 Y groups. The legend below the images indicates the colour associated with each of the five LDs range/size. The graphs show Lipid total are in μm^2, mean LDs size in μm^2, percentage of LDs in 1.0-3.0μm^2 size. Mean ± SEM, ANOVA- Kruskal Wallis test multiple comparison, * p< 0.05, ** p< 0.01. n samples 30-33 Y=7, 25-28 Y= 18, 19-23 Y=14. **D FTIR analysis human oocytes.** Lipids amount and lipid peroxidation increases, phospholipid structure changes, as age advances. Graph shows Fourier transform infrared (FTIR) spectra of oocytes from 3 age ranges. Average FTIR spectra and relative graphs of all chemical compounds detectable in human oocytes shows that absorbance of

phospholipids (~1080cm^{-1}, ~1250cm-^{1})- green bracket, amide III (~1300cm^{-1}) amide II (~1550cm^{-1}), amide I vibrations (1650cm^{-1}) - red bracket and lipids functional groups (2700cm^{-1} and 3000cm^{-1})- black bracket increases as age advances *p<0.05, mean ± SD of the mean, n samples ≤ 16; ANOVA, Kruskal-Wallis, Tukey's *post hoc*. Moreover, phospholipids structural changes in oocytes from donors > 23 years of age are indicated by the modification of peaks relative to phospholipids (green bracket). **E. LD profile in mouse embryos.** The panel shows representative images of oocytes from AMA and CTR, stained with Nile Red, BODIPY 493/503, and in Bright Field scale bar 50µm, n= number of samples. The graphs show mean grey values of Nile Red and BODIPY, Mean ± SEM, t-Student test, *** p< 0.00. n samples, AMA=17, CTR=20.

4. DISCUSSION

Assisted Reproductive Technologies (ART) have become increasingly common over the past few decades, providing the solution for the fertility problems that millions of couples are facing worldwide. The results of the extensive use of ART is more than 8 million babies born, of which more than 2 million in Europe alone (Gliozheni et al. 2022).

The use of ART is often connected with advanced maternal age (AMA), as it is one of the main reasons of reduced fertility, especially nowadays due to the postponement of childbearing. ART and AMA have been often associated with higher risk of neurodevelopmental disorders (NDD) in the offspring. Numerous studies have investigated this relationship, but not only have the findings been somewhat controversial and inconclusive, but also it remains unclear whether the higher risk of developing NND originates from the ART process itself, or from underlying factors such as parental subfertility.

In this study, we hypothesised that embryo modifications caused by ART procedures are associated with an increased risk of neurodevelopmental afflictions.

To this end we first investigated ART-induced neurodevelopmental alterations and their intergenerational transmissibility, and then explored the origin of revealed neurodevelopmental traits in embryos and offspring.

4.1 Effect of *in vitro* culture of embryos on offspring behaviour and development.

The main findings of this chapter are related to the effect of *in vitro* culture of embryos on offspring development as well as social and anxiety-like behaviour.

Our results proved that offspring generated by ART exhibits social deficits and decreased social motivation, as evidenced by reduced social interaction time and social preference index. Moreover, ART offspring showed decreased social motivation and novelty-seeking behaviour, as suggested by reduced time spent with the novel stimuli during social recognition test. These findings suggest that the *in vitro* culture procedures used to generate IVC males may lead to deficits in social behaviour and motivation which are a core symptom of NND and a widely recognised characteristic of ASD phenotype (Moy et al. 2004, 2007). Additionally, ART offspring did not show any sign of anxiety-related behaviour, which have been previously linked with NND in several studies, particularly with ASD (van Steensel, Bögels, and Perrin 2011). However, the absence of anxiety-like behaviour may also suggest that ART males display highly selective behavioural abnormalities which model the defining symptoms of autism (McFarlane et al. 2008), or simply that anxiety-like behaviour is shown later in life as described by Qin et al. which showed that increased anxiety is more frequent in aged male offspring (18 months old) conceived by IVF than in young male offspring (3 months old) (Qin et al. 2021b).

Our results also indicate that the use of ART not only negatively affects the offspring sociability but also impairs the growth curve during pre-weaning period in both females and males, leading to increased bodyweight in adulthood. Indeed, IVC males and females presented increased bodyweight during all pre-weaning time, which persists during adulthood. Previous studies have exclusively associated ART to reduced bodyweight at birth and during childhood in humans (Chang et al. 2020; Kondapalli and Perales-Puchalt 2013; McDonald et al. 2010); while in ruminants famously is documented increased bodyweight in offspring conceived by ART, the so called “large offspring syndrome” (Chen et al. 2013; Young, Sinclair, and Wilmut 1998). In mouse model, increased bodyweight has been reported in male and female offspring born after IVF (Feuer et al. 2014), and it has been associated with impaired glucose tolerance

in IVF male (but not female) offspring (Donjacour et al. 2014). Additionally, it is known that perturbed growth during early stage of life has significant implications for bodyweight and overall development in adulthood (Leunissen, Stijnen, and Hokken-Koelega 2009; Stettler and Iotova 2010). Moreover, ART procedures often require *in vitro* manipulations of gametes or embryos in critical preimplantation period, in which embryos are highly sensitive and experience developmental plasticity; this may alter the embryonic developmental trajectory and can exert a long-lasting influence on health leading to adult-onset chronic diseases, according to the 'developmental origins of adult disease' (DOHaD) hypothesis (Barker 2004). Thus, the increased bodyweight of IVC offspring may be linked to ART, and therefore to the incidence of neurodevelopmental disorders which are highly over-expressed in children with obesity (Wentz, Björk, and Dahlgren 2017).

Additionally, the exposure of IVC embryos to supraphysiological ART-induced OS (Agarwal et al. 2022) may also have an influence on embryo development and implantation (Bedaiwy et al. 2004). Indeed, our results showed that IVC embryos have lower survival than naturally-conceived ones resulting in fewer embryos developed to term/alive pups. It is commonly estimated in mice that an average of 7 embryos per estrous cycle develops and can be recovered at day 4 from a range of 1 to 12 fertilised oocytes after natural mating (Nagy, A., Gertsenstein, M., Behringer, R. R., & Vintersten 2003). Even when for ET and IVC groups the number of embryos transferred were doubled in comparison to naturally-conceived cycle, the survival of ET and IVC embryos is evidently decreased in comparison to NM embryos. Indeed, ET litter sizes vary from 1 to 8 with an average of 3.2, similarly IVC litter size vary from 1 to 7 with an average of 3.7, while NM litter size ranges from 4 to 10 with an average of 7.8, as is commonly reported for C57BL/6 strain (Lee M. Silver 1995). Our results indicate that even the least invasive ART procedures (e.g. embryo transfer) can negatively impact the survival and development to term of embryos either *in vivo* (ET) or *in vitro* (IVC) produced. And at this

early developmental stage, the combination of IVC and embryo transfer doesn't exacerbate the effect of ET (no difference between ET and IVC offspring rate and litter size), but this doesn't exclude possibly differentiated impact in later development – but not on offspring survival and fertility which appeared to be comparable to control. Additionally, the survival of IVC offspring is in line with C57BL/6 strain mortality which ranges from a minimum of 10% (Gaskill et al. 2013) to a maximum of 49% (Inglis et al. 2014), and also the fertility was not impaired as after mating of females and males born by ART, the pregnancy rate resulted similarly to control. In humans, however, it has been demonstrated that progeny born after ART exhibit impaired infertility (Belva et al. 2017). It is important to underline that ART-conceived children in human studies are born from sub-fertile parents while the ART offspring in this study was born from young, fertile parents and only received stress IVC-induced during their development.

Taken together, our results suggest that *in vitro* culture of embryos reduce embryo survival leading to small litter size, negatively impact offspring development resulting in increased bodyweight from pre-weaning to adulthood, and leads later in life to neurodevelopmental disorders characterised by reduced sociability and novelty.

4.2 Intergenerational transmission of ART effects and effects of crossbreeding ART progenies

The findings of this chapter are related to the intergenerational transmissibility of ART-induced effects.

Our data show that progeny born from ART-conceived parents (IVC) exhibit increased mortality. Particularly, offspring born from crossing of IVC females and IVC males showed the highest mortality from PND-2 to week 6 reaching only 24.39% of survival at week 6 with

only 10 pups alive out of 41 born. IVC male and female line showed lower survival between week-5, and week-6 of age in which the survival rate was 33.33% for IVC male line and 50% for IVC female line.

5-6 weeks is a critical time of development in mice as several important physiological process are still ongoing. Specifically, hormonal changes and immune system development occur in mouse at this age together with organ development and maturation. Importantly, the hypothalamus-pituitary-gonadal axis becomes more and more active during development, regulating hormonal balance (Fu et al. 2013). Additionally, the immune system continues to develop and mature which is crucial for preventing infections and diseases (Ghia et al. 1998; Holladay and Smialowicz 2000). Impairments in the above described mechanisms could lead to elevated risk of death. The cause of elevated mortality in II generation born from IVC parents was not investigated and remains vague, although these data have high relevance and raise important concerns regarding the possible health status of children to be born in the near future from ART-conceived parents. These concerns are magnified by the intergenerational transmissibility of impaired social behaviour showed in this study. Indeed, second generation males born from IVC male line and female line showed impaired social recognition memory and reduced motivation for novelty, as they displayed altered behaviour to control group animals in that they did not prefer the novel object over the familiar one and they spent shorter time with the novel object. Moreover, male offspring from IVC female line showed increased levels of anxiety like-behaviour and “risk taking” phenotype. Indeed, the latency time to cross from the dark box to light one was reduced in comparison to control which may indicated lack of fear for exploring; more importantly, the time spent in the light box is increased in IVC female line indicating that the animals are less willing to explore the dark, enclosed space and natural condition, which can be interpreted as an indication of higher levels of anxiety-like behaviour (Bourin and Hascoët 2003b; Crawley 1985).

Similar traits in social impairments were also shown in the I generation IVC which may indicate intergenerational transmission of IVC effect through female and male line.

It is important to note that the IVC cross line did not show any impairments in social behaviour or novelty preference. However, we should consider that the animals were tested at 8-9 weeks of age, and behavioural phenotypes may be expressed differently across their life span (Brust, Schindler, and Lewejohann 2015). It is possible that these mice may exhibit altered behavioural profiles later in life, as certain brain regions continue to develop throughout the lifespan (Andersen and Teicher 2004). Alternatively, it is also possible that the IVC cross line mice do not show phenotypes of neurodevelopmental disorders but may possess genetic modifications related to such disorders, which could be manifested and visible in the next generation.

Similarly, the survived II generation ART offspring didn't show any growth perturbation in pre-weaning period, meaning the increased bodyweight from I generation was not inherited. Majority of disorders associated with increased bodyweight can be transmitted from either the maternal or paternal side to the next generation, and it is less common for these disorders to manifest in the third generation of offspring without affecting the second generation. However, there are a few examples where certain metabolic conditions have been shown to exhibit the so called 'delayed transgenerational' effect. For instance, it has been shown that exposure to a high-fat diet during pregnancy and lactation resulted in the development of metabolic syndrome-like features in the second generation of female offspring, but more pronounced metabolic abnormalities were observed in the third generation of female offspring, suggesting a delayed impact on bodyweight regulation (Dunn and Bale 2011). Similarly, paternally-induced transgenerational inheritance can also show a delayed transgenerational effect; researchers found also that male mice exposed to a HFD exhibited impaired glucose metabolism and increased bodyweight. Surprisingly, these metabolic disturbances were not evident in the first- or second-generation offspring. However, the third-generation male

offspring showed altered glucose tolerance and increased bodyweight (Wei et al. 2014). Therefore, the lack of any growth perturbation in second IVC generation could then suggest a delayed transgenerational effect.

Our study showed that there is an intergenerational transmission of ART effects evident particularly in the high mortality of II generation progeny born from crossing IVC males and females, and then similarly in II generation born from both IVC males and IVC females. Moreover, decreased social behaviour is a transgenerational ART effect as it is also shown in II generation born from IVC males and females, and additionally we also observed increased anxiety-like behaviour in offspring born from IVC females. The mechanism of transmission of reduced sociability and novelty are not yet investigated, and the causes of mortality remain still elusive. Only a few transgenerational studies on animal models have been done so far: most notably reporting possible transmissibility of IVF-induced aberrant methylation patterns to the subsequent generations in mouse (Li et al. 2011); or long-term transgenerational effects of weakened zinc and fatty acid metabolism associated with alterations in hepatic metabolism of rabbits born after cryopreservation-transfer (Garcia-Dominguez et al. 2020). Similar research on transgenerational effect of ART in humans is limited as the oldest cohort of ART-conceived humans are still in their 30s and 40s .

The data presented is of high relevance in the research field considering the lack of transgenerational studies; moreover, the presented research highlights the importance of carrying out animal studies to investigate possible outcomes and raise awareness on the potential health status of children who will be born in the near future from parents who are themselves ART-conceived.

4.3 Brain alterations in ART offspring and, foetuses collected at late pregnancy.

The findings of this chapter are related to the brain alterations in ART offspring at different developmental stage, adult and prenatal.

Our results showed altered antioxidant activity in Hypothalamus and Hippocampus of adult IVC males, indicated respectively by increased oxidative stress and elevated lipid peroxidation byproducts in Hypothalamus and by differently expressed proteins involved in pathways of oxidative stress and neurological diseases in Hippocampus.

The brain is particularly vulnerable to oxidative stress for several reasons, particularly for its modest antioxidant defence and its lipid-rich constitution (Lee, Cha, and Lee 2020). There is evidence that alterations in oxidative stress as well as in lipid peroxidation are linked with impairment in sociability and anxiety-like behaviour in mice, and as well as connected to neurodegenerative disorders in mouse, rats, and human (Halliwell 2006; Patki et al. 2013; Rammal et al. 2008; Rossignol and Frye 2014). Hypothalamus and Hippocampus are brain structures highly involved in social behaviour regulation (Alexander et al., 2016; Lo et al., 2019). Our results suggest that IVC causes alterations in adult hypothalamic and hippocampal oxidative state leading to impairments in social behaviour.

Similarly, in IVC offspring prenatal brain OS levels and consequent lipid peroxidation activity were considerably increased, as indicated by higher levels of GSH, GSSG, SOD activity, as well as MDA. We also demonstrated decreased mRNA expression of HMOX1 (Heme Oxygenase 1), a key component of the cellular antioxidant defence system, and reduced mRNA expression of PPAR-α (Peroxisome proliferator-activated receptor alpha), a regulator of anti-oxidative processes and an essential element in fatty acid transport and lipid metabolism.

Moreover, IVC prenatal brain showed decreased expression of GABRA1 Cntnap2, MapK3: genes involved in crucial steps of neurogenesis.

During prenatal brain development, OS can disrupt critical processes such as neurogenesis, synaptogenesis, and myelination. This can result in altered connectivity and impaired functional development of brain regions leading to neurodevelopmental disorders in adulthood (Lanté et al. 2007). The antioxidant defence system plays an important role in neuroprotection in the prenatal and neonatal brain. It has been reported that PPAR-α agonists have well-documented anti-inflammatory and neuroprotective roles in the central nervous system. PPAR-α downregulation has been associated with decreased anti-oxidative and anti-inflammatory processes and alterations of fatty acid transport, lipid metabolism and disturbances of mitochondrial function in the brain of AD patients (Wójtowicz et al. 2020).

Our results showed increased OS level, decreased antioxidant defence, and reduced expression of genes involved in crucial steps of neurogenesis. These results taken together may suggest that IVC causes OS-induced damage in prenatal brain, which is conserved in adult brain and may be the base of revealed neurodevelopmental traits in offspring.

4.4 Altered lipid profile and lipid peroxidation in ART embryos and oocytes.

The findings of this chapter are related to ART-induced alteration in embryos, and the base of revealed neurodevelopmental traits in the offspring.

Our results show that lipid profile of IVC embryos is altered, clearly evidenced by increased LDs size (LD clustering). Relevantly, similar LD profiles were found in AMA oocytes (mouse and human) and this alteration is preserved in mouse AMA embryos.

During ART procedures embryos are subjected to non-physiological exposure to oxidative stress (Agarwal et al. 2006; Goto et al. 1993) which induces lipid peroxidation though reaction of ROS with lipids generating highly electrophilic aldehydes (MDA and 4-HNE). This process has been suggested as a potential mechanism by which ROS can inflict damage in the ageing oocyte (Mihalas et al. 2017). Lipid peroxidation byproducts can interact with proteins and DNA creating adducts and leading to oxidative stress-induced aneuploidy, as occurs in oocytes from aged mice (Mihalas et al. 2018). Lipids are not utilised by embryos during the pre-implantation time but they are crucial for embryo survival during post-implantation time (Arena et al. 2021). Before placental nursing via the bloodstream starts, the LDs contained in the embryos are the only source of lipids during prenatal development. Without the supply of new building blocks for lipid bilayers, any embryonic damage – nuclear or cytoplasmic – cannot be repaired. This explains why the likelihood of aberrances in early-developing central nervous system and heart is higher than for organs which develop later on, once fresh maternal resources can be delivered to the embryo through placental bloodstream. The altered LDs profile constituted by oxidised lipids will likely not properly support the development of IVC prenatal brain, suggesting that OS-induced alteration in embryos may be the cause of impaired prenatal brain development and have long-term health consequences.

Additionally, considering that lipid peroxidation can also cause epigenetic reprogramming (Legoff et al. 2021) and inadequate histone deacetylation through lipid-derived acetyl-CoA – which causes aneuploidy in mice oocytes (Akiyama et al., 2006) – our results may indicate that oxidative damage of lipids increases the risk of genetic instability leading to DNA modifications in IVC embryos, as they do in AMA. Thus, OS-driven lipid modifications in IVC embryos may be the origin of revealed neurodevelopmental traits in offspring.

5. Final Conclusions

The step by-step analysis of ART effects from embryonic-stage to adulthood, and their intergenerational transmissibility presented in this thesis allows a better understanding of NND etiology. The main findings of study are:

1) *In vitro* culture has a detrimental effect on offspring behaviour resulting in reduced sociability and novelty; has a negative effect on offspring development causing increase in bodyweight from pre-weaning to adulthood; and furthermore causes reduced embryo survival leading to small litter size.
2) Crossbreeding of ART-conceived parents leads to high progeny mortality; and relevantly, ART effects are intergenerationally transmitted as reduced sociability in adult male mice in the first ART generation and then later in II generation born from ART mothers and ART fathers.
3) ART prenatal brain development is impaired due to increased oxidative stress, elevated lipid peroxidation, and reduced expression of genes associated with neurogenesis which might cause changes in brain functionality and connectivity. Furthermore, these impairments persist until adulthood.
4) IVC-induced OS alters the LD profile of IVC embryos, causing impaired prenatal brain development and altered adult brain function which results in decreased sociability in the adult male offspring later in life. Likewise, ageing-induced OS alters the LD profile of AMA oocytes and embryos leading to higher risk of NDD in the offspring.

These findings enhance our understanding on the causes of neurodevelopmental disorders and more importantly paves the way for further exploration through lipidomic studies to identify

the specific mechanisms that contribute to NDD, thereby expanding the scientific frontiers in this field.

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