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MATERNAL IMMUNE ACTIVATION AND ITS CONSEQUENCES ON OFFSPRING BEHAVIOR IN AN OVINE MODEL OF PRENATAL INFECTION

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I dedicate this thesis to Ewa

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LIST OF ABBREVIATIONS

Abbreviation	Definition
ADHD	Attention Deficit Hyperactivity Disorder
ASD	Autism Spectrum Disorder
B.W.M.	Body Weight Mass
CNS	Central Nervous System
DPC	Days *Post-coitum*
ID	Intellectual Disability
IL-1ß	Interleukin 1 beta
IL-6	Interleukin 6
LPS	Lipopolysaccharide
MIA	Maternal Immune Activation
NDDs	Neurodevelopmental Disorders
PND	Postnatal Day
Poly(I:C)	Polyinosinic:Polycytidylic Acid
SAL	Saline Solution
TNF-α	Tumor Necrosis Factor Alpha
USVs	Ultrasound Vocalizations
VPA	Valproic Acid

ABSTRACT

The prenatal period is crucial for brain development and its future functioning. Being highly vulnerable to environmental insults, the developing brain may be influenced by a wide range of genetic and environmental factors. Therefore, maternal health issues occurring during fetal life may lead to an increased risk of developing neurodevelopmental disorders (NDDs) in the offspring, such as for example autism spectrum disorder (ASD). In this context, maternal infection during pregnancy, which elicits the activation of an inflammatory response in the mother and the fetus, namely maternal immune activation (MIA), has been associated with a higher risk of developing NDDs in human offspring. Seemingly, rodent and non-human primate models showed that prenatal MIA profoundly affects brain development and induces long-term effects on the offspring's behavior. Despite being an efficient tool for studying neurodevelopmental disturbances, existing models display many limitations that restrict their translational value in terms of useful clinical applications. In this study, the effects of MIA on the offspring behavior were investigated in an unconventional species, the sheep (*Ovis aries*), which is more ethically and cost-affordable than non-human primates.

The main hypothesis of this study is that the LPS-challenge administered to pregnant ewes, leads to the development of behavioral impairments in the offspring, which are characteristic of ASD (social deficits, perseverative behaviors, and communication impairments). The specific hypotheses underlying this study assume that: (1) Immune challenge during different gestational time points may differently affect the behavioral phenotype of the offspring; (2) The intensity of maternal immune response to LPS may be correlated with the severity of behavioral disorders in the offspring.

The specific aims of the present study were the following: (1) to investigate the effects of MIA on the lamb behavior; (2) to examine the inflammatory responses of the ewe to endotoxin-lipopolysaccharide administration; (3) to study the association between the levels of maternal inflammatory responses and the severity of behavioral changes in the lamb.

The experiments were performed during three consecutive breeding seasons. Each year, thirty sheep of the świniarka breed were divided into two groups and mated with a single ram. The MIA groups were intravenously injected with the endotoxin -

lipopolysaccharide (LPS), while the control groups were injected with saline solution. Treatments were administered on the 125th day post coitum (experiment 1 - single dose) and 85th day post coitum (experiment 2 - single dose, experiment 3 - three doses). The physiological and molecular inflammatory responses of the ewes were monitored at different time points after the treatment. Obtained lambs were subjected to a battery of behavioral tests designed to evaluate specific behavioral domains, commonly affected in individuals with NDDs, including social skills, learning, memory as well as repetitive behaviors, and cognitive flexibility. The screening consisted of three tests: Isolation Test, V-detour test, and T-maze test. Video materials were analyzed with the use of video tracking software Noldus EthoVision® XT 11.5.

The results indicate that low doses of LPS efficiently induce MIA in pregnant ewes, leading to a rise of inflammatory cytokines expression and illness-like physiological responses, lasting a few hours after administration. Lambs prenatally exposed to MIA displayed subtle changes in specific behavioral domains, including learning, memory, and cognitive flexibility, whereas the social bonding with the mother, as well as the responses to isolation-induced stress, were not affected by MIA. Furthermore, the intensity of maternal immune response to the LPS challenge, especially concerning the plasma levels of IL-6, was associated with the severity of behavioral impairments, such as inhibitory control and perseverative behaviors, observed in the offspring.

Overall, the performed experiments allowed us to put a solid foundation to establish an ovine model of MIA in terms of resembling several specific behavioral symptoms of human NDDs. The proposed ovine model could potentially display higher translational validity than rodents and carry fewer ethical concerns than the use of non-human primates. Thus, it may become a useful tool to study the poorly known etiology and neuropathology of NDDs in the future and help to develop new therapeutic and preventive strategies.

POLISH ABSTRACT

AKTYWACJA UKŁADU IMMUNOLOGICZNEGO MATKI I JEJ WPŁYW NA ZACHOWANIE POTOMSTWA W OWCZYM MODELU INFEKCJI PRENATALNEJ

Okres prenatalny jest kluczowy dla rozwoju mózgu i jego przyszłego funkcjonowania. Rozwijający się mózg, będąc bardzo podatnym na czynniki zewnętrze, może być szczególnie wrażliwy na różne wpływy genetyczne i środowiskowe. Dlatego też problemy zdrowotne matki w okresie prenatalnym mogą prowadzić do zwiększonego ryzyka wystąpienia zaburzeń neurorozwojowych (NDD) u potomstwa, jak na przykład zaburzeń ze spektrum autyzmu (ASD). Dotychczasowe badania na pacjentach wykazały związek pomiędzy infekcją prenatalną wywołującą aktywację układu immunologicznego matki i płodu (*ang. Maternal Immune Activation – MIA*), a wyższym ryzykiem wystąpienia zaburzeń neurorozwojowych u potomstwa.

Badania prowadzone do tej pory z wykorzystaniem gryzoni i ssaków z rzędu naczelnych, wykazały, że MIA ma znaczący wpływ na rozwój mózgu potomstwa, prowadząc do zmian w jego zachowaniu na kolejnych etapach rozwoju. Istniejące modele zwierzęce wykazały dotąd wysoką skuteczność w badaniu zaburzeń neurorozwojowych. Niestety mają one wiele ograniczeń, które zmniejszają ich wartość translacyjną w zakresie przyszłych zastosowań klinicznych. Prezentowana praca skupia się na badaniu wpływu MIA na zachowanie potomstwa wykorzystując niekonwencjonalny model zwierzęcy, a mianowicie owcę (*Ovis aries*), której wykorzystanie w badaniach wzbudza mniej wątpliwości na tle etycznym i jest mniej wymagające finansowo niż badania z wykorzystaniem ssaków z rzędu naczelnych.

Główna hipoteza badawcza prowadzonych badań zakłada, że iniekcja endotoksyny bakteryjnej (lipopolisacharyd - LPS) podawana ciężarnym owcom prowadzi do występowania u potomstwa zaburzeń behawioralnych charakterystycznych dla zaburzeń ze spektrum autyzmu (deficyty społeczne, zachowania perseweracyjne i zaburzenia komunikacji). Przedstawiona praca zakłada dwie szczegółowe hipotezy badawcze: (1) MIA w różnych okresach życia prenatalnego może mieć różny wpływ na późniejszy rozwój fenotypu behawioralnego u potomstwa; (2) Intensywność odpowiedzi immunologicznej matki na iniekcję LPS może być powiązana z nasileniem zaburzeń zachowania u potomstwa.

Szczegółowe cele przedstawianego badania to: (1) zbadanie wpływu aktywacji układu immunologicznego ciężarnej owcy na zachowanie jagniąt; (2) zbadanie odpowiedzi układu immunologicznego owiec na podanie endotoksyny bakteryjnej - LPS; (3) analiza związku pomiędzy natężeniem reakcji układu immunologicznego matki a poziomem zmian behawioralnych u jagniąt.

Doświadczenia przeprowadzone w ramach tej pracy były prowadzone przez trzy kolejne sezony hodowlane. Co roku trzydzieści owiec rasy świniarka było przydzielanych do dwóch grup. Każda owca kojarzona była z tym samym trykiem. W czasie ciąży, owcom z grupy MIA podawano dożylnie endotoksynę bakteryjną – LPS, natomiast zwierzętom z grupy kontrolnej wstrzykiwano roztwór soli fizjologicznej. Iniekcje LPS przeprowadzano w 125 dniu *post coitum* (eksperyment 1 – pojedyncza iniekcja) oraz 85 dniu *post coitum* (eksperyment 2 – pojedyncza iniekcja, eksperyment 3 – trzy iniekcje). Odpowiedź układu immunologicznego owiec na poziomie fizjologicznym i molekularnym była monitorowana w różnych punktach czasowych po zabiegu. Urodzone jagnięta poddawano szeregowi testów behawioralnych mających na celu ocenę konkretnych zaburzeń w zachowaniach typowych dla pacjentów z zaburzeniami neurorozowojowymi: deficyty społeczne, zaburzenia uczenia się, pamięci i elastyczności poznawczej, a także występowanie zachowań powtarzalnych. Bateria testów behawioralnych obejmowała: test izolacji od matki (ang. Isolation Test), test ominięcia przeszkody (ang. V-detour test) oraz test labiryntu w kształcie liery “T” (ang. T-maze Test). Do analizy uzyskanych materiałów wideo wykorzystano program Noldus EthoVision® XT 11.5, umożliwiający rejestrację i analizę behawioru zwierząt.

Uzyskane wyniki wskazują, że podanie LPS indukuje aktywację układu immunologicznego u ciężarnych owiec, prowadząc do wzrostu ekspresji cytokin zapalnych i reakcji fizjologicznych charakterystycznych dla stanu chorobowego. Jagnięta poddane aktywacji układu immunologicznego matki w okresie prenatalnym wykazywały subtelne zmiany w zachowaniu, wskazujące na zaburzenia procesu uczenia się, zapamiętywania oraz elastyczności poznawczej. Nie wykazano wpływu aktywacji układu immunologicznego matki na formowanie się więzi społecznych z matką, a także na reakcje na stres wywołany izolacją. Badania molekularne wykazały, że intensywność odpowiedzi układu immunologicznego matki na LPS, szczególnie w odniesieniu do poziomu IL-6 w osoczu, była powiązana z nasileniem zaburzeń

behawioralnych u potomstwa, obejmujących zaburzenia kontroli hamowania oraz występowanie zachowań perseweracyjnych.

Podsumowując, przeprowadzone doświadczenia pozwoliły nam na stworzenie solidnych podstaw do zdefiniowania owczego modelu MIA, w szczególności pod kątem występowania zaburzeń behawioralnych u potomstwa, typowych dla pacjentów z zaburzeniami neurorozwojowymi. Proponowany model może wykazywać potencjalnie wyższą wartość translacyjną niż gryzonie, a także wzbudzać mniej wątpliwości na tle etycznym niż wykorzystanie ssaków z rzędu naczelnych. W przyszłości może on się zatem stać użytecznym narzędziem do badania słabo dotąd poznanej etiologii i neuropatologii zaburzeń neurorozwojowych, a także pomóc w opracowywaniu nowych strategii terapeutycznych i profilaktycznych.

1. INTRODUCTION

Neurodevelopmental disorders (NDDs) are a heterogeneous group of psychiatric conditions caused by abnormalities in brain development and by behavioral impairments [1,2]. One of the most common NDDs is Autism Spectrum Disorder, the diagnosis of which is based uniquely on the presence of impairments in social communication and interaction, restricted interest, and repetitive patterns of behavior [2]. To better understand the basis of NDDs, well-tested animal models, mostly rodent ones, are used in preclinical studies. Although many controlled animal studies have been conducted up to date, focusing on the neuropathology of NDDs, the exact mechanisms leading to their occurrence are still not fully understood. Thus, there is a need to establish new methods and models, allowing to study the neuropathology of those disorders, as well as seeking possible therapies.

Growing evidence suggests that the fetal period is a vulnerable window for brain development, being sensitive to genetic and environmental perturbations. In this context, the immune system plays an important role in the pathophysiology of NDDs. Epidemiological studies indicate that maternal infection during pregnancy is a risk factor for NDDs, leading to brain developmental abnormalities and postnatal behavioral impairments. Well-validated animal models are used to study the effects of Maternal Immune Activation (MIA) elicited by prenatal infections on fetal brain development, as well as its consequences on the postnatal offspring behavior. Nevertheless, existing rodent and non-human primate models exhibit some limitations, which reduce their translational validity or pose ethical and cost-related concerns, respectively. Due to some of their similarities to humans, large animal models have already significantly contributed to understanding human disease etiologies, uncovering biological mechanisms, and establishing new therapeutic strategies [3]. Dogs, horses, pigs, and sheep have been previously used to model complex human neurological disorders like Parkinson's disease and epilepsy [4–7], among many others. Several similarities among humans and sheep in the hemodynamic and inflammatory responses to immune challenges [8,9], as well as their physiological resemblances, especially in the context of pregnancy [10], brain development [11,12] and its functioning [13–15], indicate the sheep as a valid candidate to model human prenatal infection, MIA and its association with NDDs. Establishing and validating an ovine model will be particularly useful to investigate the neuropathogenesis of NDDs and to develop

possible therapeutic and preventive strategies, which may help to significantly decrease the disease burden and the occurrence of NDDs in the future.

1.1. Neurodevelopmental disorders (NDDs)

NDDs are a heterogeneous group of mental illnesses that includes communication disorders, intellectual disability (ID), attention deficit hyperactivity disorders (ADHD), motor disorders, and autism spectrum disorders (ASD), among others [2,16]. Many lines of evidence suggest that NDDs are caused by abnormalities in the developing brain occurring during fetal stages [1], which leads to the manifestation of behavioral symptoms later in postnatal life, including impaired cognitive function, communication problems, motor, and affective disabilities, as well as social deficits [2].

NDDs typically have their onset before puberty, during childhood, or adolescence[17]. Early disturbances in the development of the brain involve crucial processes like neurogenesis, glial/neuronal proliferation, migration, synapse formation, and myelination [18–21], which may lead to lifelong changes in the behavior and physiology of the affected individuals [22–24]. NDDs often co-occur, and their symptoms overlap with other psychiatric illnesses. For instance, autism spectrum disorders often co-occur with intellectual disability, ADHD, and learning disabilities [25]. This makes it hard to define a disorder with a specific diagnosis, thus resulting in a wide spectrum of neuropsychiatric comorbidities [22–24], each characterized by alterations in various behavioral domains.

The early onset of NDDs and the overlapping symptoms among several disorders make diagnosis and treatment very challenging, requiring often specialists from many different fields (i.e. child psychiatrist, pediatrician, psychologist) [17]. Existing therapeutic strategies include behavioral therapy and rehabilitation (i.e. physical or speech therapy), as well as pharmacotherapy for the treatment of a subset of behavioral symptoms [16]. Due to a lack of understanding of the NDDs' etiology, biological mechanisms that could represent a target for developing new therapies are still far from being completely revealed. Moreover, the specific symptoms of the disorders are often detected after the optimal period for therapeutic intervention, thus, the development of effective and specific treatments for NDDs is highly challenging[26].

Autism Spectrum Disorder (ASD) is one of the most diagnosed neurodevelopmental disorders worldwide. The diagnosis of ASD is based on the sole evaluation of behavioral symptoms, which include: (1) persistent impairments in social

interaction, (2) impaired ability to communicate, as well as (3) restricted interest and repetitive patterns of behavior [2]. The behavioral impairments manifest in early childhood, usually before 3 years of age, and persist throughout life [2,27]. Recent studies show that the prevalence of ASD in the United States affects approximately 1 in 68 children, with a higher prevalence in boys, which is 1 in 42 [28]. In Europe, the prevalence of ASD differs depending on the country, with the highest occurrence in Sweden equal to 1/87 [29] and the lowest in Croatia – 1/5000 - 1/3333 children [30]. The data from Poland indicates that the occurrence of ASD in children between 0-16 years old, differs between two regions of the country: 1/313 in West Pomeranian and 1/263 in Pomeranian region [31]. Different studies performed across the world revealed that the prevalence of ASD worldwide has been increasing in recent decades [32,33]. For example, the study performed by Idring et al. [29] revealed the increase in ASD prevalence in Swedish children from 4.2/1000 in 2001 to 14.4/1000 in 2011. Another longitudinal study conducted in Australia estimated the prevalence of ASD among children as 14.1/1000 in 2005–2006 and 25.2/1000 in 2010–2011. Moreover, the most recent study reporting the prevalence of ASD in Australian children estimates it to be even 43.6/1000[34]. A study on Omani children reports the prevalence of ASD to be 15 times higher in 2018 than estimates in 2011[33]. Another study performed in Sweden over 10 years from 1993 to 2002 proved that the prevalence of autism symptoms remained stable during that period, while the official number of diagnoses increased substantially[35]. Summing up, such a major increase in the prevalence of ASD worldwide can be attributed to the improvement of diagnostic tools as well as a deeper awareness of the disorder.

Growing evidence indicates that NDDs result from a complex interaction between genetic and environmental factors [36]. The influences of genetics on ASD occurrence have been thoroughly studied and supported by twins studies, which demonstrated that monozygotic twins are co-diagnosed with ASD at a higher rate compared to dizygotic tweens [37]. In addition, several genetic variants have been associated with the development of ASD, including autosomal mutations (recessive and dominant), X-linked, copy number variations, as well as chromosomal aberrations. More recent studies based on whole-genome sequencing estimate that around 400-1000 genes are contributing to ASD susceptibility, such as *CHD8, GRIN2B, SCN2A* [38], although the vast majority of genes are yet to be discovered and require further studies based on larger cohorts of individuals.

On the other hand, recent evidence indicates that up to 40-50% of the variance in ASD liability is due to environmental factors. Twins' studies have yielded concordance rates of 47-96% in monozygotic twins and lower values (0-36%) for dizygotic twins. Such a range for monozygotic twins, rather than a 100% concordance, indicates that genetics is not the only etiological factor contributing to ASD occurrence and that environmental factors are also implicated. Considering the sensitivity of the developing brain to environmental influences, their contribution to the NDDs etiology is biologically plausible [39,40]. Environmental insults may lead to changes in the expression of key genes by inducing epigenetic marks, such as DNA methylation or histone post-translational modifications, which may disrupt brain developmental trajectories when occurring during crucial time points of embryo formation. Environmental factors may potentially affect offspring neurodevelopment during various time windows of development and can be distinguished into prenatal, perinatal, and postnatal risk factors. The prenatal risk factors occur during the gestational period and include maternal infection during pregnancy (bacterial or viral) [41], advanced parental age [42], as well as parental or embryonic exposure to specific drugs and chemicals [43,44]. Risk factors for ASD include also perturbations occurring around the time of delivery (perinatal risk factors), such as pre-term or post-term birth, fetal and maternal complications during parturition, as well as an abnormal fetal presentation or maternal hemorrhage, among others [45]. In the postpartum period, the vulnerability for ASD may be increased by early infant infection, as well as ischemia, encephalopathy, and physical trauma, among others [45].

The growing prevalence and its socio-economic burden, make NDDs a burning biomedical issue [46]. There is an urgent need to provide new, robust tools to allow further research in this area and investigate its still unclear etiological background. Considering the low availability of preventive and therapeutic strategies for NDDs, as well as the limited efficiency of the existing ones, examining the basis of these complex psychiatric disorders is especially important for reducing their impact on the society.

1.2. Animal models of NDDs

To better understand the biological mechanisms underlying the occurrence of neurodevelopmental disorders, well-validated animal models, mostly rodent ones, are used in experimental and preclinical studies. Developing valid animal models for complex disorders of the human brain is a challenging task. The validity of an animal model is determined by 3 criteria: (1) construct validity, which requires that the biological causes inducing brain and behavioral abnormalities in animals are the same as in human diseases, such as genetic mutations or environmental factors; (2) face validity, which requires that the specific behavioral alterations observed in the animal model, namely "endophenotype" [47], are comparable to those observed in human behavioral symptoms, such as social deficits, communication disorders, repetitive behaviors and restricted interest that defines ASD; (3) predictive validity requires that if a therapy is efficient in curing the specific human disorder, it will display similar efficacy in reversing the behavioral alterations observed in animal models, like reducing repetitive behaviors or improving the social deficits [48]. The optimal animal model possesses all three validity criteria - the experimental paradigm used to establish a new animal model may determine the level at which construct validity which focuses on the cause underlying the disorder, leads to face validity manifested by symptoms observed in these models, and provide predictive validity which may benefit as a therapeutic effect [49].

A growing number of mouse studies focus on the genetic risk underlying ASD occurrence. The number of different genetic mutations identified in ASD individuals indicates that each of these genetic defects may be worth examining in animal models carrying homologous mutations [50–52]. Most frequently, the mutations associated with ASD development in humans can be modeled in mice by deleting or mutating the homologous murine genes. Construct validity for that specific mutation is attained when the mutant mouse displays ASD-like behaviors [53–56]. In this context, rodents represent an optimal tool for studying the underlying genetics of ASD, especially in view of the recent development of genetic manipulation, such as CRISPR-Cas9 technologies. In addition, multiple animal models have been established to investigate the environmental insults on ASD occurrence. For instance, the literature evidence indicates that animals prenatally exposed to valproic acid (VPA, an anticonvulsant and mood stabilizer drug) display behavioral abnormalities resembling the core symptoms of ASD and additional symptoms observed in humans,

supporting the face validity of the model. Published data consistently indicate that prenatally VPA-exposed animals display behavioral abnormalities resembling both the core symptoms of ASD (social deficits, communication disorders, repetitive behavior, and restricted interest) and so-called "additional behaviors" (i.e. increased depression- and anxiety-like behaviors or circadian rhythm dysregulation) related to the human behavioral pathology, furtherly supporting face validity of the model [57].

Existing gaps in the knowledge about NDDs make it essential to provide additional tools for research focusing on determining the influence of genetic, neuroanatomical, and environmental components that contribute to ASD-like phenotypes. To this end, scientists started to develop alternative animal models using different species like knockout rats [58,59] and others with more developed social behavioral repertoires, such as voles [60] and non-human primates [61,62]. Rodent models of ASD also include inbred mouse strains that display robust ASD-like behaviors (i.e. social-communicative deficits and repetitive behaviors). Due to the lack of a specific connection between ASD-like behaviors and specific genetic or environmental perturbations, these inbred strains are considered to be models of idiopathic autism. Concerning the social domain, the inbred strains A/J, BALB/cByJ (BALB), BTBR T+Itpr3tf/J (BTBR), C58/J (C58), and 129S1/SvImJ display significant deficits in social interactions, in comparison with high social strains, such as C57BL/6J (C57) and FVB/NJ mice [63–66]. Moreover, some of those strains, including BTBR and C58 mice display motor stereotypies and other repetitive behaviors, like very high levels of self-grooming [64–68]. Among others, BTBR mice have been the most widely studied and well-validated mouse model of ASD-related behaviors. They exhibit not only social deficits and repetitive behaviors but also communication disorders, displaying altered patterns of ultrasonic vocalizations (USVs) during social interactions, as well as an unusual repertoire of USV calls [69,70].

Face validity of an animal model of NDDs is achieved when the abnormal behaviors observed in the model correspond to those observed in human patients. To this aim, studies on animal models utilize various behavioral assays, which allow for precise measurements of specific behavioral domains resembling NDDs symptoms (*Figure 1*). Rodents, like humans, are a social species, displaying a wide repertoire of social behaviors. The behavioral screening focusing on engagement in intraspecies reciprocal social interactions, parenting, and mating, as well as scent marking and aggressive behavior, may help to determine whether the model displays ASD-like social deficits

[71–73]. The most common paradigms include the 3-chambered social test [74], reciprocal social interactions test [66,71], the examination of social transmission of food preference [75], and social dominance [76]. Communication impairments are characteristic for ASD, as well as for other NDDs [2] (*Figure 1*). In humans, impairments in communication may manifest in different ways, depending on the degree of intellectual ability of the individual, and may include the absence of speech, language delay, and stereotyped speech, among many others [77]. Rodents communicate mostly through olfactory pheromones. However, during social interactions, as well as non-social situations, mice and rats emit vocalizations in the ultrasonic range (ultrasound vocalizations - USVs) [78]. USVs are analyzed in rodents under different experimental conditions with the use of dedicated equipment and software. Pups emit USVs when separated from the mother and the nest [79], while juvenile and adult mice emit USVs during social interaction with peers [69], as well as male-female courtship social interactions [80,81] or in response to female urine and other social scents [82,83]. The core diagnostic domain of NDDs symptoms includes also motor stereotypies, repetitive behaviors, restricted interest, and insistence on sameness [2]. In ASD patients, motor stereotypies include hand flapping and toe walking, while in mice they may include circling and jumping in frequencies higher than typical levels. The occurrence of behavioral stereotypies in rodents can be assessed in the home cage or novel empty cage by a trained investigator [84]. Repetitive behaviors in ASD patients may manifest as playing one video game repeatedly or placing the toys in the same order repeatedly. Also, ASD children display perseveration on specific habits and insistence on sameness, as well as a low degree of cognitive flexibility [85]. In mice, those behavioral disorders include repetitive self-grooming [86] and higher levels of marble-burying or digging [87]. Cognitive inflexibility in ASD has been modeled in many rodent models of autism, by dedicated behavioral assays including Morris water maze, spontaneous alteration on Y-maze, and rewarded T-maze [88]. In these assays, animals are habituated to act in a specific way to obtain the reward and are subsequently forced to change the behavior, which is called reversal learning. Another approach to evaluate the occurrence of cognitive inflexibility consists of the IntelliCage system (TSE Systems GmbH; Germany), allowing a home cage approach to test conditioned place preference in learning and reversal [89]. Behavioral methods described above have been extensively studied and reported in the literature and have been useful in evaluating the face validity of rodent models of ASD.

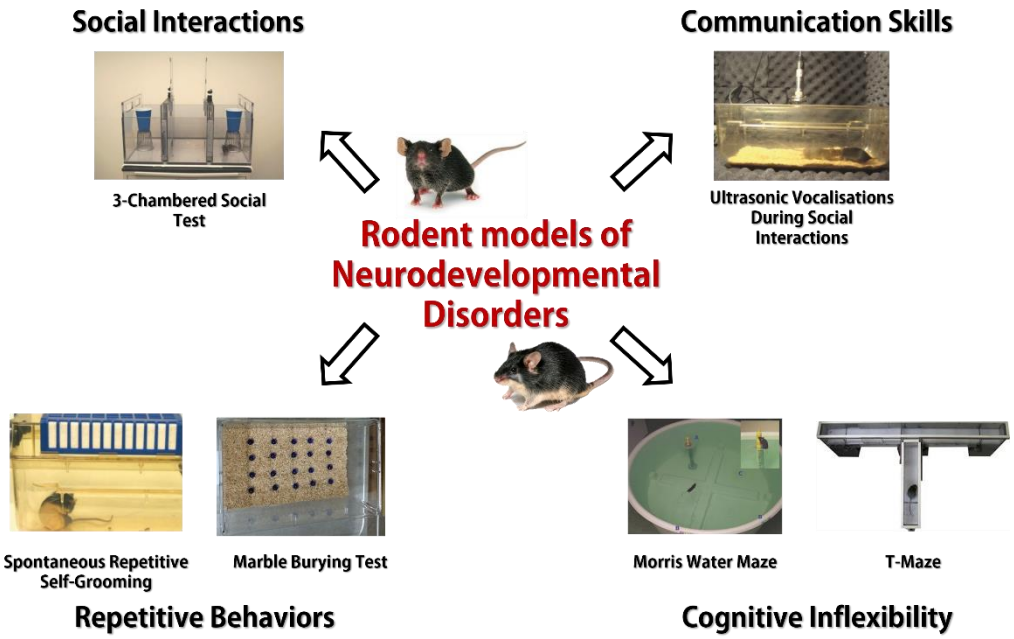

Figure 1. **Behavioral assays used to evaluate the face validity of rodent models of NDDs.** Four main behavioral domains corresponding to human ASD symptoms are evaluated in rodent studies. One of the core symptoms of NDDs includes social deficits. The most common approach to studying social interactions is the 3 chambered-social test and its modifications, although other paradigms like the reciprocal social interaction test and the social dominance test are also being employed for measuring mice sociability. The communication skills of rodents are examined by analyzing the ultrasound vocalizations emitted by animals during different social paradigms (i.e. male-female interactions, isolation-induced ultrasound vocalization, etc.) with the use of special software and microphones. The repetitive behaviors analyzed in rodents include observation of spontaneous repetitive self-grooming, marble burying, and digging, which have been widely used as a marker of autism-like repetitive behaviors in mice and rats. The cognitive flexibility of rodents is being examined with the use of different mazes, of which the most common are the Morris water-maze and T-maze tests, as well as Y-maze. Those assays are based on reversal learning, which may be defined as habituating the animal to some pattern of behavior, and subsequently changing the conditions to force it to change the learned behavior.

Other human neurodevelopmental disorders like ADHD or ID are also commonly modeled in mice and other species to investigate mechanisms and factors underlying the occurrence of these psychiatric conditions. Diagnostic criteria of ADHD, similarly to other NDDs, are based mostly on behavioral disturbances, including the inability to sustain attention, hyperactivity, and impulsivity, which can be mimicked in animal models [90,91]. Animal models of ADHD are either genetic (i.e. spontaneously hypertensive rat (SHR) [90], Wistar-Kyoto Hyperactive Rat [92]) or environmental, being evoked by an external insult to the central nervous system during development [91].

Despite the availability of a wide range of animal models of NDDs, as well as scientific tools allowing for their examination, the prevalence of NDDs is growing, and with that also its burden on the society and economy [46]. This creates an urgent need to establish new methods and models that would allow studying neuropathology and possible therapies, providing higher translational value in the context of human neuropsychiatric diseases. To better understand ASD and other NDDs further research involving multiple species needs to be undertaken to prove the occurrence of the same outcomes across different systems, displaying various similarities to humans.

1.3. NDDs and the immune system

Growing evidence suggests that alterations of the immune system may play an important role in the neuropathology of NDDs. Historically, the central nervous system (CNS) was considered to be independent of the immune system, with no or little interactions between those two [94–96]. At that time, the brain was assumed to be lacking the lymphatic system and major histocompatibility complex (MHC)[94–96]. However, recent discoveries suggesting that the dural sinuses are lined with the functional lymphatic vessels [97,98] and that neurons and glial cells express MHC molecules [99,100] gave new insight into the relation between the brain and the immune system. It has been also shown that monocytes, lymphocytes, and other immune cells are present in the CNS even under non-pathological conditions [101,102]. Moreover, the CNS possesses its own immune cells – microglia, which are implicated in mediating inflammation, directly combating infection, as well as clearing cellular debris through phagocytosis [103]. Microglia has been also revealed to be critical for complex neurodevelopmental processes. Signaling and phagocytic properties of these brain-resident immune cells have been shown to promote neurite formation and synaptogenesis, determine

neuronal fate by regulating programmed cell death, and strip excess synapses from developing neurons to permit the assembly of functional neuronal circuits [104].

A strong body of evidence indicates that alterations in the immune system occur in many neurodevelopmental and other neuropsychiatric disorders. Altered inflammatory functions have been linked with depression [105], schizophrenia [106], bipolar disorder [107], and post-traumatic stress disorder [108]. Several inflammatory mechanisms have been suggested to play a role in the development of neuropsychiatric conditions, including glial activation [109], neuronal damage and degeneration [110], increased oxidative stress [111], altered neurotransmitter metabolism [112], and blood-brain barrier disruption [113]. Genome-wide association studies (GWASs) indicate the involvement of immune-related genes in the development of autism and schizophrenia [114,115]. Patients suffering from ASD are also more prone to develop concurrent autoimmune disorders [116,117]. Moreover, allergic and atopic autoimmune disorders, as well as a previous occurrence of such disorders in the family, have been associated with ASD by several studies [118–120]. Epidemiological studies demonstrated a co-occurrence of ADHD with inflammatory and autoimmune disorders, suggesting a role of the immune system in the pathophysiology of this disorder [121,122].

Overall, evidence proves the crucial role of the immune system in the development and functioning of CNS and indicates its contribution to the pathogenesis of NDDs. A less tested hypothesis is whether maternal immune dysregulation during critical stages of gametes or embryo/fetus development may affect brain development and behavioral functions.

1.4. Maternal Immune Activation (MIA)

Maternal Immune Activation can be defined as a rise in the levels of pro-inflammatory markers above their normal range in the maternal body during pregnancy [123]. MIA occurs as a consequence of bacterial and viral infections. Moreover, common factors associated with inflammatory conditions are maternal psychopathology, psychosocial stress, prenatal infections, as well as high body mass index, which may induce a rise in the levels of pro-inflammatory molecules, thus leading to maternal inflammation during pregnancy [124]. Immune-related disruptions of early brain development may lead to a wide range of neurodevelopmental dysfunctions which may last until postnatal life, and in turn, influence the behavioral phenotype of the progeny over its lifespan. Among the most prevalent neuropsychiatric disorders,

two of them are especially vulnerable to immune-related disturbances of neurodevelopment: schizophrenia and autism. Epidemiological studies revealed a higher risk of schizophrenia in the offspring of mothers exposed during pregnancy to bacterial infections (sinusitis, tonsillitis, pneumonia, cystitis, pyelonephritis, bacterial venereal infection, and any other bacterial infection), viral infections (influenza, measles, herpes simplex virus type 2, rubella and polio) and parasites (*Toxoplasmosis gondii*) [125]. Moreover, the exposure to maternal infection in the first, as well as in the second trimester of pregnancy has been implicated in increasing the risk for schizophrenia [125]. Epidemiological evidence indicates also the link between MIA during pregnancy and a higher risk of developing ASD in offspring [126]. Viral infection in the first trimester, as well as a bacterial infection in the second trimester, have been linked with the higher occurrence of ASD in children [127]. Moreover, a retrospective study revealed increased levels of inflammatory cytokines at around midgestation in mothers bearing an ASD child [128]. Studies in rodents utilizing the human influenza virus for prenatal immunization have shown significant disturbances in offspring brain development, leading to long-lasting functional and structural changes in the brain areas associated with behavioral symptoms [129–133]. In further animal studies, it has been proved that behavioral changes were due to the immune response of the mother, rather than the pathogen itself [134–136].

Within a few hours after an infection is detected, the level of a broad range of pro-inflammatory cytokines increases in the fetal mice and rats' brains, including IL-1ß, IL-6, and TNF-α [137,138]. The work of Smith et al. [139] indicates the crucial role of IL-6 in the mediation of transcriptional and behavioral changes evoked by MIA in the offspring. In a rodent model of mid-pregnancy infection used in this study, eliminating the IL-6 from the immune response of the mother by blocking antibodies, prevents the occurrence not only of behavioral deficits but also of the transcriptional changes triggered by MIA in the offspring brain. Moreover, the single injection of IL-6 itself at mid-gestation evokes long-lasting changes in the behavior of the offspring, indicating the significant involvement of this inflammatory cytokine in MIA [139]. A more recent study by Choi et al. [140] indicates the importance of another cytokine in mediating the effect of MIA on the offspring – IL-17a. IL-17a is the predominant cytokine of TH17 lymphocytes, which are responsible for the immune response to pathogens. TH17 lymphocytes are also implicated in various inflammatory and autoimmune diseases, i.e. rheumatoid arthritis, or asthma. It has been shown that the pro-inflammatory

cytokine IL-6 is crucial for the differentiation of TH17 cells and the further production of IL-17a [141]. Lack of IL-6 in the dam exposed to MIA inhibits the strong increase of serum IL-17a, and in turn, prevents MIA effects on the fetal brain. A maternal IL-17a-dependent pathway was found to participate in the disruptions of cortical development in the offspring subjected to MIA during pregnancy. This may be due to direct exposure of the fetal brain to IL-17a. The presence of ASD-like behaviors in mice obtained following the direct injection of IL-17 into the fetal forebrain supports this latter hypothesis. Overall, there is a consensus concerning the importance of the IL-17a pathway as a key factor in mediating the behavioral changes evoked by MIA in the offspring [140].

Overall, the studies described above underlie the crucial role of the two cytokines – IL-6 and IL-17a in mediating the effect of prenatal infections on the developing fetal brain and future offspring behavior.

1.5. Current animal models of MIA

Studies in animal models examine the influence of MIA on offspring neurodevelopment and behavior by evoking an inflammatory response in pregnant animals (mice, rats, and non-human primates) and investigating whether a simulated infection may cause behavioral abnormalities in the offspring (*Figure 2*) [137,142]. The first pioneering study involved the exposure of pregnant mice to the human influenza virus to induce MIA [129,131,132]. Mice prenatally exposed to the human influenza virus displayed a global reduction in the reelin levels in the brain, as well as abnormal neuronal cell development and migration in the cortex [131]. Moreover, human influenza virus exposure during pregnancy was shown to alter the structure of the developing mouse brain by altering the cell density values of pyramidal and non-pyramidal cells, which leads to the development of abnormal behaviors in adult mice [132].

To study the specific effects of maternal inflammation on the developing offspring, experimenters can use infection-mimicking immunogenic substances to evoke MIA, rather than living pathogens. Nowadays, the majority of animal models of MIA are created with viral and bacterial mimics: synthetic double-stranded RNA analog polyinosinic:polycytidylic acid (Poly(I:C)) and bacterial endotoxin lipopolysaccharide (LPS), respectively. Double-stranded RNA is unique for viruses, thus Poly(I:C) induces the acute phase immune response resembling a viral infection,

while LPS mimics an innate immune response of the organism characteristic for bacterial infections. LPS and Poly(I:C) interact with toll-like receptors 4 and 3, respectively, and induce a pro-inflammatory signaling cascade leading to the production of proinflammatory cytokines such as IL-1ß, IL-6, and TNF-α, which mirror the course of an infection in the organism [143]. Both viral and bacterial mimics are well-documented in the literature and led to the establishment of animal models for several most common MIA-mediated neuropsychiatric disorders, including autism and schizophrenia [144].

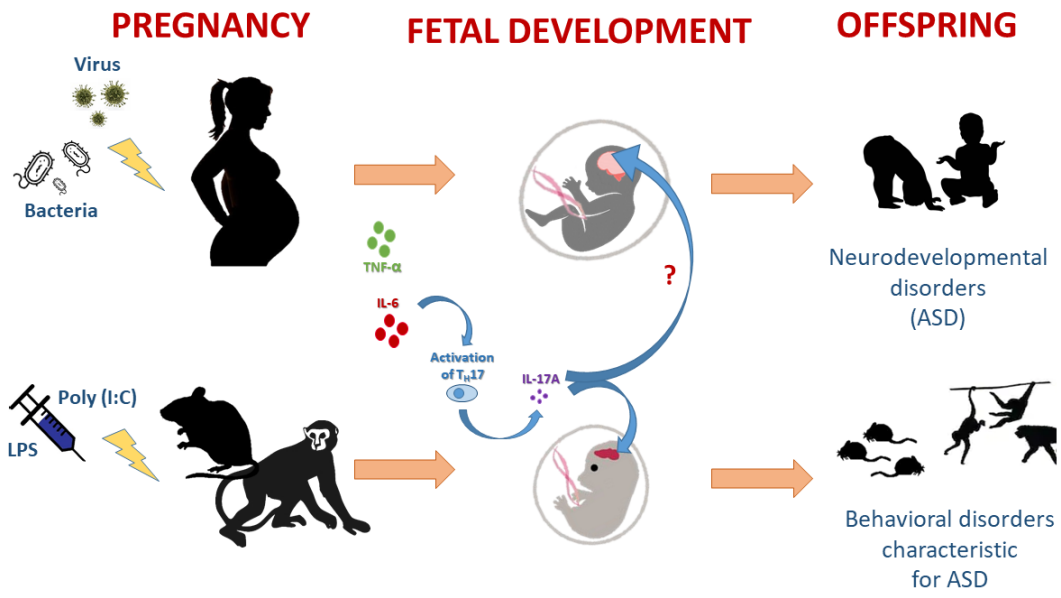

Figure 2. **Mechanism of MIA in the human and animal models.** Immune activation by a prenatal infection in humans is mimicked by immunogenic agents in animal models. This leads to the release of pro-inflammatory cytokines like TNF-α and IL-6, which activate T_H17 cells in the mother's bloodstream, in turn inducing the production of IL-17A. The cascade of pro-inflammatory cytokines influences the developing fetal brain and leads to behavioral changes in the offspring.

Evoking MIA with Poly(I:C) or LPS induces an immune response that is limited in time. Therefore, it is possible to identify specific developmental windows of vulnerability to immune challenges, which can be associated with different outcomes in the offspring [145,146]. We can distinguish certain windows during pregnancy, which display an increased susceptibility to the influence of MIA on brain development in rodents [144,147]. The vulnerability at a certain pregnancy time window may be associated with maternal, as well as fetal factors. Firstly, the immune response of the mother changes gradually during gestation, with a decrease in pro-inflammatory responses over time [148,149]. Moreover, specific maternal and fetal cytokines are activated at

different gestational windows, which may diversely influence the fetal brain [145]. Therefore, the long-term effects of the adverse maternal immune-related environment acting on the fetal brain substantially depend on the specific stage of maturation at which the developing fetus is exposed.

The offspring of mothers exposed to viral and bacterial mimics during gestation exhibit a wide variety of behavioral changes characteristic of ASD and schizophrenia. In rodents and non-human primates, gestational MIA has been associated with communication and social deficits, repetitive behaviors, elevated anxiety, reduction in sensorimotor gating, deficits in working memory and cognitive flexibility, as well as increased sensitivity to amphetamines, and many other behavioral alterations in the offspring [137,138,142,147,150]. For instance, mice exposed to poly(I:C) during fetal life, display an unusual pattern of vocalizations, decreased sociability, and increased levels of repetitive behaviors [151]. The offspring of mice injected with LPS at mid-pregnancy display anxiety- and depression-related behaviors [152]. In rhesus monkeys, poly(I:C) administration during pregnancy leads to abnormal offspring responses to separation from the mothers at weaning, as well as increased repetitive behavior and decreased affiliative vocalization [61]. Rats subjected to MIA administering poly(I:C) at gestational day 15 display impaired behavioral flexibility and occurrence of perseverative behaviors [153]. The variability of behavioral outcomes evoked by MIA reported up to date in rodent and rhesus monkey offspring, indicates that the utilized protocol of MIA (i.e. the type of immunogen, the timing of administration, as well as the timing of behavioral analyses) may lead to heterogenous consequences of MIA on the behavioral phenotype of the offspring [124,147].

Offspring of rodents subjected to MIA during prenatal life display many of the neuropathologies characteristics for neurodevelopmental disorders, including reduced cortical thickness and hippocampal volume, increased ventricular size, and cerebellar abnormalities [154]. Preclinical models of MIA exhibit also changes in the microglial cell number and activation, which is analogous to the findings from human ASD and schizophrenia postmortem studies [155,156]. Some studies describe behavioral and synaptic abnormalities not followed by an increase in the number of microglia or changes in their morphological activation [157–161]. This inconsistency may be the result of different factors: various animal models and MIA protocols, diverse timing of microglial activation analyses, as well as the protocol utilized to determine the activation of microglia. Moreover, current animal models of MIA exhibit imbalances

in the levels of dopamine, serotonin, and other neurotransmitters, which have been also shown to be involved in the neuropathology of ASD and schizophrenia [138,144].

Overall, existing animal models represent a robust tool to examine the effect of MIA during pregnancy on the offspring. Animals display impairments corresponding to those reported in children, thus supporting their validity for investigating human NDDs. Nevertheless, existing models carry many limitations that restrict their translational value in terms of useful clinical applications. This may be due to the lower degree of resemblance between rodent and human brains, indicating the need for new models with higher translational value than rodents, and being more cost-affordable and ethically acceptable than non-human primates.

1.6. Alternative animal models of MIA

As described above, rodent models are well-established and widely used to study the effects of MIA on offspring brain development and behavior. However, many of their physiological, anatomical, and behavioral characteristics are different from humans. Thus, there is a limited translation of the basic knowledge obtained by studying rodents to consistent clinical applications [162,163]. For instance, mice models of MIA have been criticized for not mimicking the important characteristics of the human immune response to microbial agents [164,165]. Moreover, the immunological phenotype, as well as the whole blood transcriptional responses to LPS in mice are markedly different from those found in humans [164,165]. The use of rodents to model complex human psychiatric disorders is contentious, since the regions of the brain responsible for social cognition, a crucial diagnostic aspect of ASD and schizophrenia, are not as well developed in rodents [166].

A possible alternative to rodents for modeling human disorders are non-human primate models [167]. Their phylogenetic proximity to humans and the large degree of conservation of gene maps, as well as many similarities between non-human primates and humans, including their genetics, physiology, but also behavior, make them the gold standard for modeling human diseases [168,169]. To date, non-human primate models have been extensively studied, especially concerning complex issues such as neurodevelopment and behavior. However, high costs related to their maintenance and specific facility requirements make it very challenging to provide sufficient welfare in a laboratory setting. Moreover, due to their emotional and personality features, the use

of non-human primates in experimental research raises no little ethical concerns among scientists and the society [170].

A new trend in neuroscience indicates the possible advantages of modeling NDDs in large livestock animals. Due to their genetic, phenotypic, anatomical and physiological similarities to humans, large animal models have significantly contributed to understanding human disorders and establishing new therapeutic strategies [3]. The use of large animals in studying human mental and behavioral disorders has already been documented in the literature. For instance, the dog has been proposed as a suitable model organism for epilepsy [4], Parkinson's disease [7], Anorexia Nervosa [171], as well as developmental disorders including Down Syndrome [172], ADHD [173], and many others. The horse has been indicated as a good candidate to model juvenile idiopathic epilepsy [174], environmentally acquired toxic parkinsonism [6], as well as depressive episode [175] and obsessive-compulsive disorder [176], among others. Moreover, the use of pigs in neuroscience is increasing, indicating the possible use of this species for experimental modeling of diverse human brain disorders including Parkinson's disease [177], mania [178], and postpartum psychosis [179]. Another species that has been recently taken into consideration to model complex neurological disorders is the sheep. In the last 4 decades, ovine models have been widely used to study many human diseases including respiratory conditions [180], fetal white matter injuries [181], more complex disorders like epilepsy [5], Creutzfeldt-Jakob disease [182] and Huntington's disease [183], as well as developmental disorders like fetal alcohol syndrome [184] and schizophrenia [185]. Importantly, the sheep have been useful to establish several biomedical innovations, such as somatic cell nuclear transfer in mammals [186], and an extra-uterine device supporting fetal development [187], among others.

1.7. Sheep as a model organism to study MIA

Several similarities between humans and sheep in the hemodynamic and inflammatory responses to endotoxin administration [8,9], as well as transcriptional response to LPS [188], support the use of the sheep as a good candidate to model MIA and its consequence on the offspring. For instance, LPS infusion in sheep has been proven to be accompanied by cardio-pulmonary responses that closely mimic those observed in humans [189–192]. Due to the similarities to humans concerning pregnancy, such as metabolic functioning and nutrient transport, it has been proposed as a model organism for human pregnancy[193]. Other pregnancy-related features show stronger

resemblances between humans and sheep, rather than rodents, such as parity, gestational length, and timing of fetal ontogeny [10]. Sheep display also similar brain development and architecture to those observed in humans, such as the timing of cortical layer formation, the subplate neuronal architecture, and a gyrified cortex [11,12]. Moreover, several behavioral aspects, especially in the context of social behaviors, make the sheep an optimal model for social deficits, characteristic of many NDDs. In terms of developing and sustaining mother-infant attachment, the sheep show strong resemblances to humans becoming a useful model for studying social attachment and bonding [13,194]. Unlike rodents that do not develop selective social interaction, sheep provide a better model to study the neurochemical and sensory control of selective attachment behaviors toward specific peers [13]. Other aspects of brain functioning, such as neuroendocrinology and neuromodulation of social behaviors in sheep have high degree of similarity to humans [14,15]. One important advantage of using sheep as a model organism for studying MIA during pregnancy is the timing of fetal immune system development, which in sheep, like in humans, starts already during pregnancy, while in rodents occurs only after birth [195]. Moreover, the immature ovine brain is similar to the preterm human brain at approximately mid-late pregnancy in terms of the completion of subcortical neurogenesis, the onset of cerebral sulcation, and the detection of several components of the cerebral cortex [196]. On the other hand, rodents have a lissencephalic (flat) brain cortex that does not resemble the gyrencephalic human brains, and several brain developmental milestones largely occur after birth in rodents [197].

The effects of prenatal inflammation on fetal brain development have been previously studied in sheep. For instance, it has been shown that exposure of the fetal lamb to LPS during pregnancy leads to substantial white and grey matter injuries in several brain areas, affects electroencephalogram measures, and is associated with loss of cortical volumes, as well as with a marked loss of subplate neurons in the fetal brain [198–201]. However, up to date, no previous studies have examined whether the abnormalities induced by LPS in the sheep fetal brain may turn into postnatal behavioral alterations resembling symptoms of NDDs.

To sum up, all the above-mentioned similarities between sheep and humans, make the sheep a potential model for MIA, being a possible robust alternative to rodents and non-human primates. In addition, being used for dairy and meat production, sheep have well-established zootechnical settings for breeding, welfare, and

management, thus representing a practical cost-affordable, and ethically acceptable alternative to non-human primate models by providing a new pre-clinical platform for NDDs studies.

2. HYPOTHESES

Existing evidence demonstrates that the administration of LPS during pregnancy elicits Maternal Immune Activation, impacting fetal neurodevelopment and being associated with behavioral alterations resembling the symptoms of Neurodevelopmental Disorders.

The main hypothesis underlying this research is that LPS administration during sheep pregnancy leads to Maternal Immune Activation (MIA) in pregnant ewes, and elicits the development of behavioral aberrations in the resulting lamb, which resembles symptoms of NDDs in humans.

Specific hypothesis 1. The administration of LPS during different gestational windows (early pregnancy and mid-pregnancy) may result in distinct behavioral outcomes in the offspring due to differences in fetal vulnerability to MIA.

Specific hypothesis 2. The intensity of maternal immune response consequent to LPS administration may be correlated with the severity of behavioral disorders in the offspring.

3. AIMS OF THE STUDY

The general objective of the present study was to explore the validity of the sheep (*Ovis aries*) as an experimental model to study the influences of MIA on offspring health and behavior.

The effects of immune challenges on the fetal brain have been extensively studied in developing lambs, which display specific brain injuries after administration of LPS in the fetal bloodstream [198–202]. However, it has not yet been examined whether brain alterations evoked by gestational immune challenges are followed by alterations in postnatal lamb behavior. Therefore, the ***specific aim 1*** of this study was to investigate the effects of LPS-induced MIA during sheep pregnancy on the lamb postnatal behavior.

Moreover, up to date, no study on sheep and only a few in rodents [158,203] focused on examining the relationship between the severity of the behavioral symptoms evoked by MIA in the offspring with the intensity of inflammatory responses of the mother. Thus, the ***specific aim 2*** was to examine the inflammatory responses of the ewe to endotoxin-LPS administration, and the ***specific aim 3*** of the present study was to investigate whether the intensity of the LPS-induced maternal inflammatory response during pregnancy may correlate with the severity of behavioral alterations in the lamb.

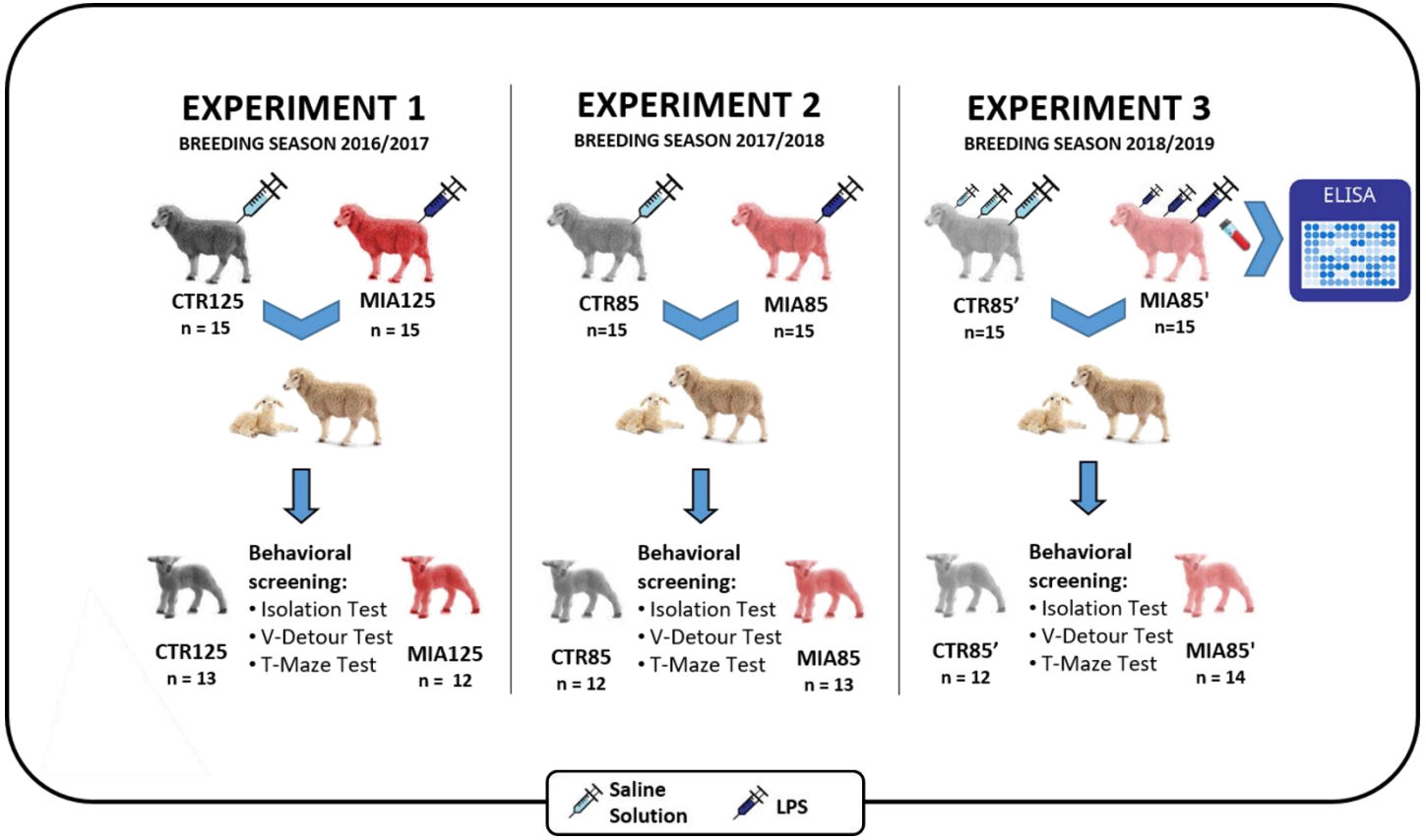

Figure 3. **The general scheme of experimental design applied in this study**. The figure represents an overview of each of the three experiments conducted through three consecutive breeding seasons. The red color indicates animals from the groups exposed to MIA during pregnancy, while the grey indicates controls. The shades of both colors indicate the experiment, with the most saturated color in experiment 1, and least saturated in experiment 3. The differences between the three experiments reside mainly in the protocol used for MIA: EXP 1. – a single injection of 1,2 µg/kg b.w.m. on 125th *dpc;* EXP 2.- a single injection of 1,2 µg/kg b.w.m. on 85th *dpc; EXP 3.*- triple injections with increasing dose of LPS 0,4 - 0,8 - 1,2 µg/kg b.w.m. on 85th,86th and 87th *dpc*

The work described in this thesis consisted of three experiments, each performed in one of the three consecutive breeding seasons. The differences between the three experiments reside mainly in the protocol used for MIA, as presented in Table 1.

Table 1. Doses and schedules of LPS administration.

	Time points of the LPS administration [a]	**Dosage of LPS [µg/kg b.w.m.]** [b]
EXPERIMENT 1	125 *dpc*	1.2
EXPERIMENT 2	85 *dpc*	1.2
EXPERIMENT 3	85 - 86 - 87 *dpc*	0.4 - 0.8 – 1.2

[a] indicates the specific time point of the pregnancy at which the injections were administered; dpc - days *post coitum*.

[b] indicates the dosage of LPS in µg per 1 kg of body weight mass.

A general overview of the experimental design is presented in *Figure 3*. 30 ewes of the świniarka breed were mated in each season, always with the same ram throughout the whole project. Pregnant ewes were then assigned to two experimental groups: MIA animals (MIA125, MIA85, and MIA85') intravenously injected with 2 ml saline solution containing the endotoxin LPS, and control animals (CTR125, CTR85, and CTR 85') injected with 2 ml of saline solution (SAL). Administration of SAL and LPS doses was performed with the timing described in Table 1. Assignment to either experimental group was pseudo-random, considering the balance between experimental and control lambs in each pen's group. Ewe-lamb dyads were video-monitored and supervised during the entire course of pregnancy, as well as at delivery, by an experienced veterinarian. Subsequently, lambs were subjected to a battery of behavioral assays consisting of three different tests: Isolation Test, V-detour Test, and T-maze Test. Moreover, blood samples were collected from the ewes at different time points before and after LPS/SAL administration to monitor their immune response by measuring the level of selected pro-inflammatory cytokines (namely, IL-6 and TNF-alpha) in the plasma. Furthermore, the levels of cortisol in response to stress induced by social isolation were evaluated in the lambs of experiment 3.

4.2. Animals

Ninety primiparous female sheep (*Ovis aries*) of the świniarka breed were recruited for this study. Świniarka is a Polish, primitive ovine breed (*Figure 4*). Adult females reach no more than 35 kg, which makes them easy to handle during manipulations required for behavioral tests. Moreover, świniarka ewes usually bear one single lamb, making them ideal for modeling human singleton pregnancy.

In our study, animals were kept in wooden pens (3x3 m), in a group of 5 ewes before lambing, and subsequently in 4-5 ewe-lamb dyads per pen. Animals participating in the study were fed hay once a day (between 7 and 8 AM) and their diet was supplemented with fresh oat grain (250-300 g/ewe/day). Adult ewes weighed on average 30.5 kg in the middle pregnancy and 37.6 kg in the late pregnancy. All animals had free and constant access to water and salt stones.

Figure 4. **Pictures of the świniarka breed. A.** Adult ewes of the świniarka breed marked with different colors for identification purposes. **B.** 80-days old male lamb of świniarka breed in its home-pen.

Mating was performed at the turn of November and December. All ewes were mated with a single ram to standardize the paternal genetic contribution. The mating was performed naturally under supervision, by allowing the ram to mate the ewes that were under spontaneous estrus, within their housing pens. Pregnancy was detected by ultrasonography (USG) at 40–70 days *post-coitum* (dpc). The summary of the lambing outcomes of each breeding season is reported in table 2.

The experiments were conducted in the facilities of the Department of the Institute of Genetics and Animal Biotechnology of the Polish Academy of Sciences (Jastrzębiec, Poland; coordinates: 52.032, 20.829). Experiments have been approved by the Second Warsaw Local Ethics Committee for Animal Experimentation (approval no. WAW2/35/2017).

Table 2. Number of animals used in the study.

	Pregnant ewes/ Total no of ewes [a]	Lambs excluded from the study [b]	Lambs enrolled in the study		
			♂	♀	Total
EXPERIMENT 1 **Breeding season 2016/2017**	28/30	3 (♄ ⌀ ↕)	13	12	25
EXPERIMENT 2 **Breeding season 2017/2018**	28/30	3 (x x ⁂)	17	8	25
EXPERIMENT 3 **Breeding season 2018/2019**	29/30	4 (⌀ x ↕ ⁂)	14	12	26

[a] indicates the number of pregnant sheep as detected by USG analyses, compared to the entire number of sheep subjected to breeding;
[b] indicates the number of ewes/lambs excluded from the study due to the following reasons:
♄ - illness symptoms in the ewe during pregnancy (not connected to the LPS administration);
⌀ - miscarriage (not connected to the LPS administration);
↕ - post-partum rejection of the lamb;
x - lamb stillborn or died soon after the delivery;
⁂ - twins.

During the first breeding season (2016/2017), the USG analyses indicated pregnancy in 28 out of 30 ewes. Of these, one of the ewes displayed symptoms of illness during late pregnancy, thus it was not subjected to the treatment, and it has been excluded from the study. Another ewe has been excluded because of mid-pregnancy miscarriage. During the lambing, 26 lambs were born. One of the lambs was rejected by its dam just after the delivery, thus it was excluded from the study. At last, 25 healthy lambs, 12 females and 13 males were enrolled in experiment 1 and subjected to the behavioral screening.

During the second breeding season (2017/2018), the USG analyses showed the occurrence of pregnancy in 28 out of 30 sheep. One lamb was born dead and the other died just after delivery. Moreover, one ewe delivered twins. In this case, the second sibling born was immediately removed from the ewe and excluded from the study, leaving only one lamb for each ewe. At last, 25 lambs, 8 females and 17 males were subjected to the behavioral battery of tests.

During the last breeding season (2018/2019), USG analysis revealed pregnancy in 29 out of 30 ewes. One sheep miscarried during the mid-gestational period. During the lambing, 29 lambs were born. One lamb was born dead and one lamb was rejected by its mother after delivery. Moreover, one sheep delivered twins, one of which (the one born later) was separated from the ewe just after parturition and excluded from the study. Then, 26 healthy lambs, 14 females and 12 males entered the behavioral analyses in experiment 3.

4.3. Maternal Immune Activation protocol

The endotoxin LPS was administered to pregnant ewes to evoke MIA, whereas saline solution was administered to the control group, to standardize the stress derived from handling the animal during LPS/SAL administration.

Before starting the procedure, animals were weighed on a standard scale for small ruminants and the concentration of LPS solution was calculated based on the body weight to obtain a 2ml bolus. Just before the injection, the animal was restrained by the caregiver by embracing its head with one hand and the groin with the other, while the physiological parameters of the animal, such as rectal temperature, respiratory rate, and heartbeat, were monitored by an experienced veterinarian. Animals from MIA groups received the specific dosage (according to Table 1) of Escherichia coli O111:B4 LPS (Sigma, St. Louis, MO) diluted in 2 ml of saline solution, while the control animals received a 2 ml bolus of SAL. Injections were performed intravenously through the *vena jugularis externa* by an experienced veterinarian. Before performing the procedure, the injection site was disinfected with 70% alcohol. After each administration, the general health condition, and physiological parameters of the animals (temperature, respiratory rate, and heartbeat) were monitored at 2, 4, and 24 hours after the injection (in experiments 1 and 2) and analyzed. The analyses revealed that the physiological parameters of the ewe 2 hours after the injection doesn't display any significant changes, thus in experiment 3 the general health conditions were examined at 4 and 24 hours after each injection.

4.4. Behavioral analyses

To examine whether LPS administration during pregnancy affects postnatal lamb behaviors, the lambs enrolled in the study were subjected to a battery of behavioral tests, which were designed to examine specific behavioral domains characteristic of neurodevelopmental disorders. Behavioral tests were designed to measure the ewe-lamb attachment and separation-induced stress responses (in the Isolation Test), the lamb's inhibitory control (in the V-detour test), as well as spatial learning, memory, and cognitive flexibility (in the V-detour and T-maze tests). All the performed procedures were video-recorded and registered with a CCTV monitoring system, which allowed us to monitor the performance of animals during the tests, as well as to review and analyze them afterward.

The behavioral testing arenas were manufactured using wood panels within the same facility where the sheep were housed during the whole experiment. The specific display and the photos of the experimental facility are shown in *Figure 5*.

Two operators were involved in performing each test. All animals were previously habituated to the handling procedures and the operators to avoid novelty-induced stress. The person responsible for carrying the lamb (namely, Main Operator) was the same for all experiments and tests, to eliminate any possible influence of the operator on the subject. The other person (namely, Helper Operator), responsible for handling the ewe during the test and for time measurements, was the caregiver that daily fed and took care of the animals. Through all behavioral analyses, operators were blind concerning the experimental groups the ewes and lambs belong to.

Each procedure was preceded by 10 minutes pre-test phase. At first, the lamb-ewe-dyad was separated from the flock and transferred to the waiting room (2 m x 2.1 m) for 5 minutes initial phase (pre-test phase I). This phase was the same for all three tests performed. Subsequently, the ewe was taken by the helper operator into the specific Target Zone, depending on the performed test. All tests were based on a separation-reunion paradigm, in which the lamb seeks to reunite with its ewe after social isolation. Then, the lamb was subjected to 5 minutes of social isolation (pre-test phase II), which was performed to increase its motivation for the social reunion in the subsequent phases of each test. Subsequently, the behavioral tests were performed as described below.

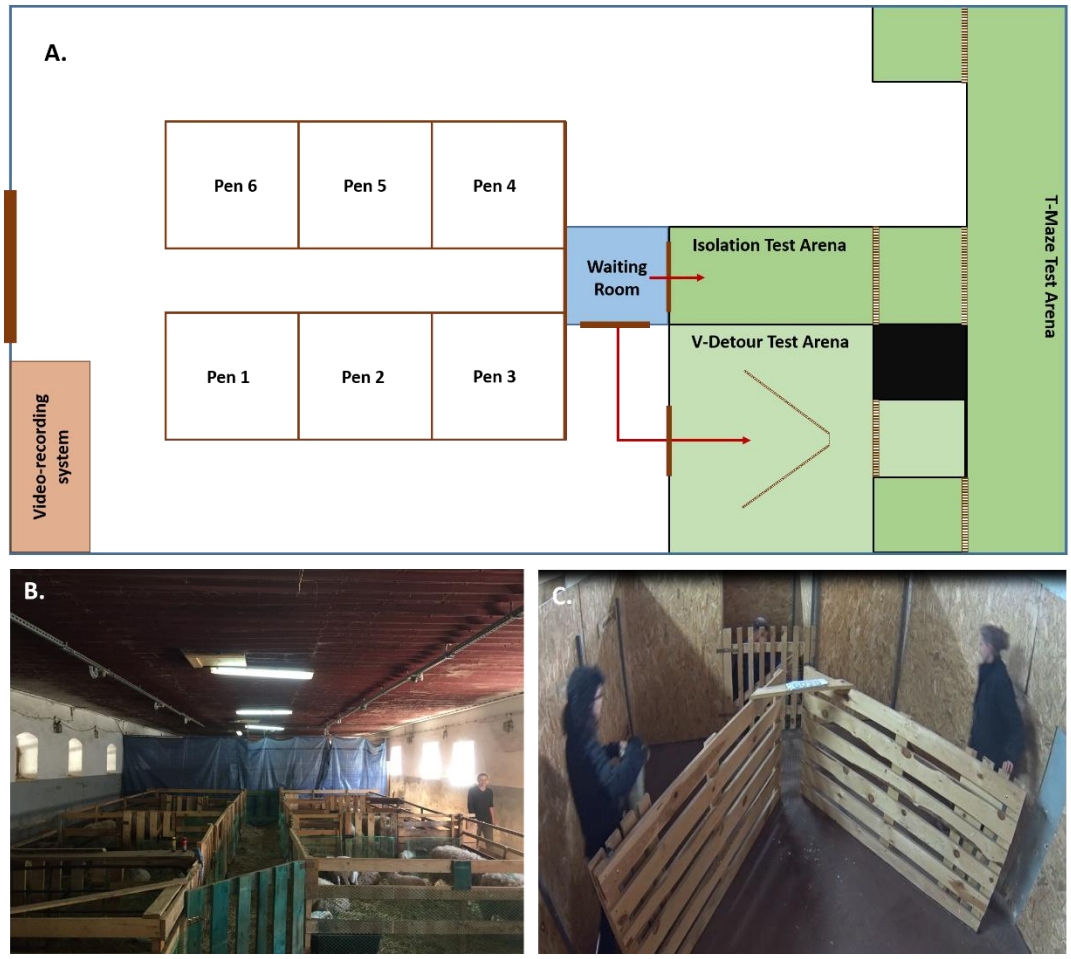

Figure 5. **Scheme and the photos of the experimental facility. A.** The detailed scheme of the facility. Six wooden pens were located on one side of the facility and housed the animals during experiments. The behavioral arenas were settled in the back part of the facility. During behavioral analyses, animals were transferred to the waiting room directly from their home pen. Subsequently, depending on the test performed, they were transferred to a specific arena (as indicated by red arrows). The arena for isolation and T-maze tests shared the same central corridor. Depending on the test performed two movable gates were added to the setup, creating a target zone for the Isolation Test. **B.** Photo presenting the front part of the facility containing six wooden pens, in which animals were kept daily. The blue curtain visible on the back divides the pens from testing arenas. **C.** The photo of the V-detour test arena during the test depicts the two operators approaching the target zone by two opposite sides of the maze.

4.4.1. Isolation Test

At the age of 20 days, lambs were subjected to the Isolation Test to evaluate their social attachment to the mother, the stress response to physical separation, as well as the effects of visual cues on the lambs' behavioral responses. The scheme of the testing arena is presented in *Figure 6*. The arena consisted of a rectangular room where the lamb was tested (1,1 m x 4,5 m), and an adjacent target zone (1,1 m x 1,06 m), in which the ewe was placed as a social stimulus during the test.

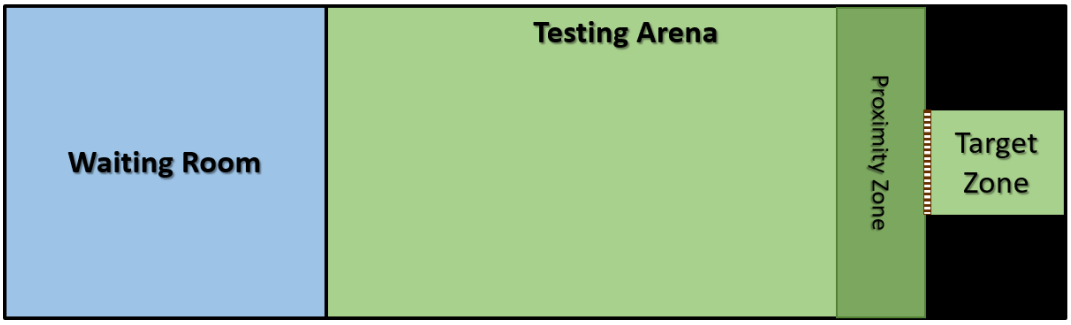

Figure 6. The detailed scheme of the Testing Arena used in the Isolation Test.

After the pre-test phase, the main operator opened the gate of the waiting room and the lamb was let to freely explore the Testing Arena for 5 min (*Figure 7*). Subsequently, the lamb was rewarded with 5 minutes of re-union with the dam within the target zone. Then, the lamb was again transferred by the main operator into the waiting room and animals were subjected to another 5 min isolation phase correspondingly to the pre-test phase II. Subsequently, the lamb was subjected to a second trial according to the protocol utilized in the first trial. The difference between trials consisted of an additional black panel located in front of the target zone, preventing visual contact between the lamb and the ewe.

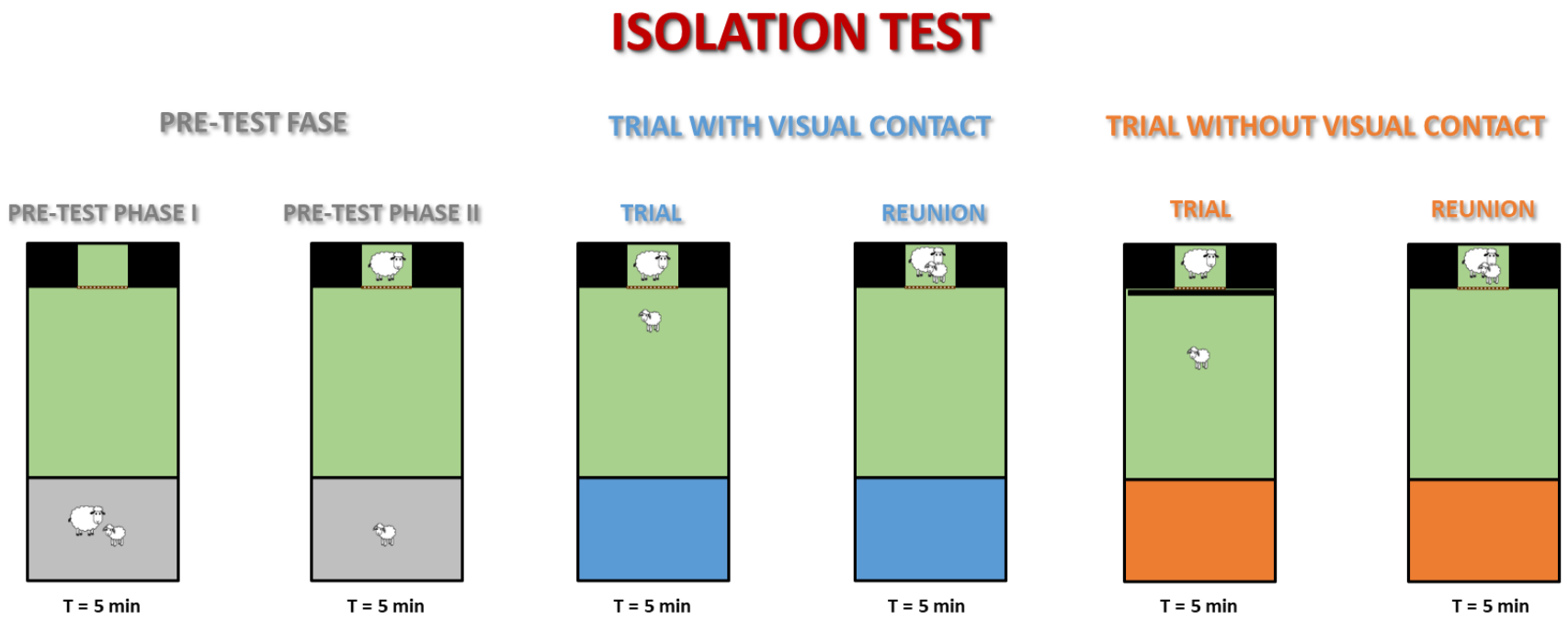

Figure 7. Schematic representation of the Isolation Test. The colors on the scheme represent specific phases of the test: Pre-Test Phase – grey, trials with visual contact with the ewe – blue and trials without visual contact – orange. The test is performed in one day and preceded by the pre-test phase divided into two parts. Then, lambs are subjected to one trial in which they have visual contact with their mothers. Subsequently, the target zone where the dam is placed is covered with an opaque black panel, thus preventing visual contact between animals.

4.4.2. V-detour Test

At the age of 40-43 days, lambs were subjected to the V-detour Test to evaluate inhibitory control toward a visual stimulus, as well as to measure spatial learning, memory, and different aspects of cognition. The test was performed in two days. The second day of the test was 3 days (72 h) after the first one. The detailed scheme of the testing arena is shown in *Figure 8*. The arena consisted of a rectangular room (3.8 m x 3.5 m) containing a V-shaped, semi-transparent, wooden obstacle in the center (arms: 2 m, connected in the center by a 0.2 m wide wire net) facing a target zone (1.25 m x 1.1 m).

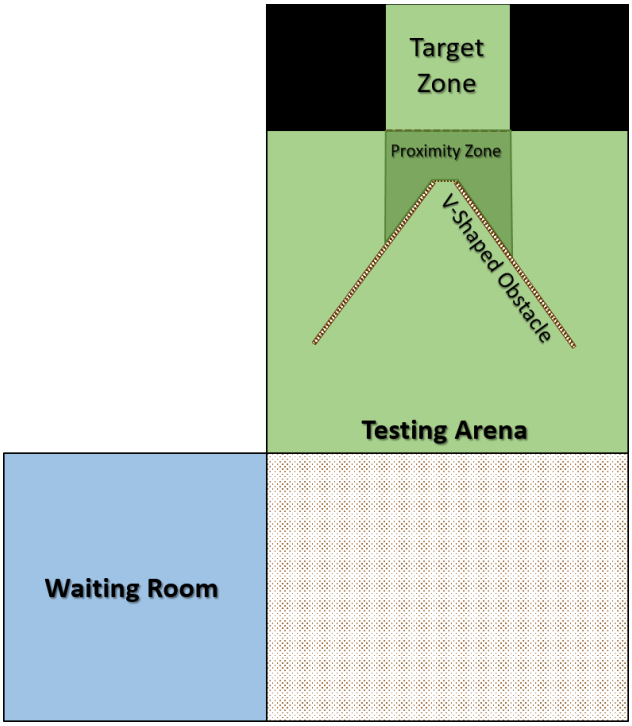

Figure 8. Representative scheme of the Testing Arena used in the V-detour Test.

At the beginning of the test (*Figure 9*), the lamb-ewe dyad was subjected to the pre-test phase, which was conducted as described above for the Isolation Test. After the ten-minute pre-test phases, the lamb was transferred by the main operator into the center of the V-shaped maze and left free to reach the target zone by detouring the obstacle from the left or the right side (habit acquisition trials). Then, after reaching the area facing the target zone (proximity zone: 1 m x 1.5 m, excluding the front part of the V-shaped obstacle) with at least two legs, the lamb was rewarded with 2 min of reunion with the dam within the target zone.

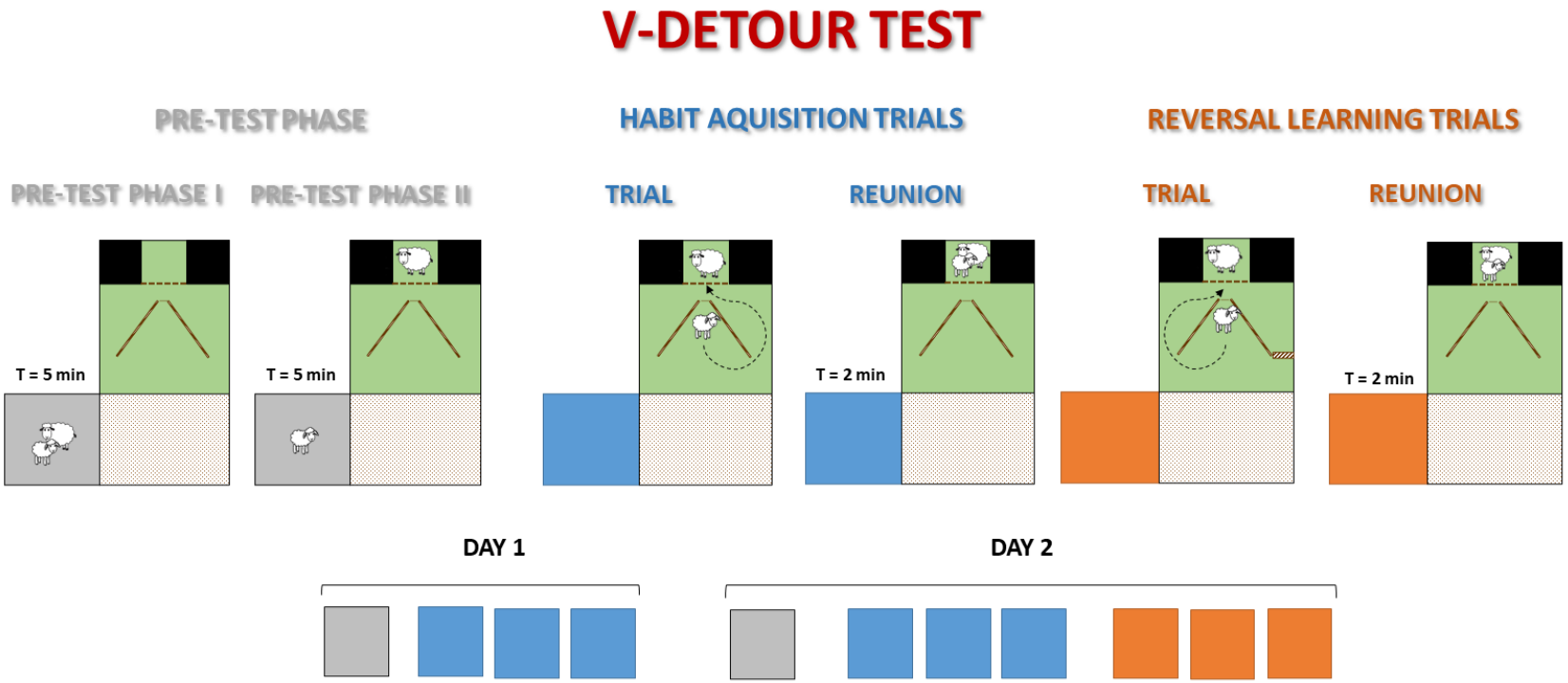

Figure 9. **The schematic presentation of the V-detour test.** The colors on the scheme represent specific phases of the test: Pre-Test Phase – grey, Habit Acquisition Trials – blue and Reversal Learning Trials – orange. The test is performed in two days, and each day of the test is preceded by the pre-test phase, divided into two 5-minute-long phases. During the 1st day of the test, lambs are subjected to habit acquisition trials in which they have to detour the V-shaped obstacle from one of the two sides (on the picture as an example is shown on the right side). During the 2nd day of the test, three further habit acquisition trials are performed as on day 1 and followed by three reversal learning trials, in which the chosen side is closed, and the lamb has to reach the dam from the opposite side of the obstacle.

On the 1st day of the test, the task was considered accomplished when the lamb reached the proximity zone moving along the same side of the "V" for three consecutive trials. On the 2nd day of the test (3 days later), lambs were subjected to three further habit acquisition trials using the same paradigm as on day 1. Then, the animal was subjected to 3 reversal learning trials, in which the side previously chosen by the lamb was closed with a semitransparent gate, thus making the acquired spatial navigation habit unsuitable anymore. The task was considered successfully completed when the lamb reached the proximity zone within 20 min for the first reversal trial, and within 10 min for the next two. After 3 unsuccessful trials, the test was considered failed.

4.4.3. T-maze Test

At the age of 60 days, lambs were subjected to the T-maze test to evaluate spatial learning and cognitive flexibility under a different experimental paradigm than in the V-detour test. The test was performed on two consecutive days.

The test was conducted in the T-shaped arena (depicted in *Figure 10*), including a rectangular entrance corridor (5.85 m x 1.1 m), and two side arms (3.8 m x 1.5 m). Two target zones – left and right, consisting of small enclosures (1.3 m x 1.25 m) located at the two distant ends of each arm, housed the dam during testing.

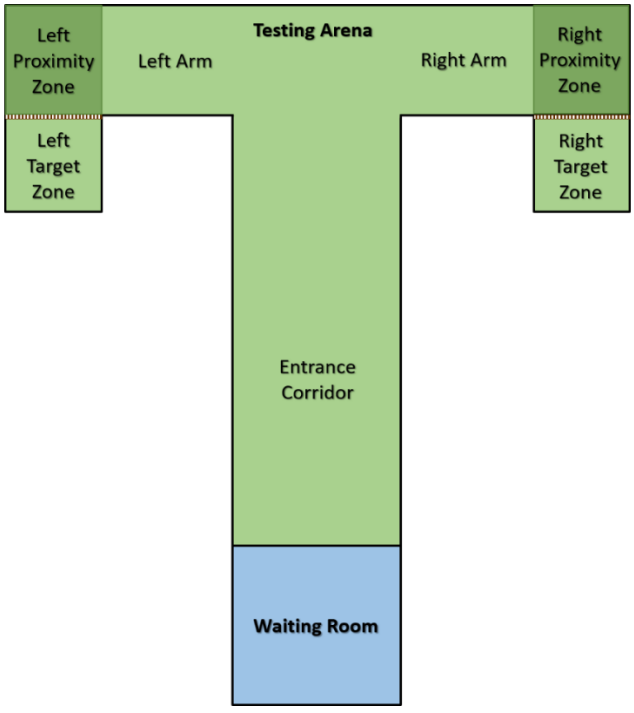

Figure 10. **The detailed scheme of the testing arena used in the T-maze test.**

At the beginning of the test (*Figure 11*), the lamb-ewe dyad was subjected to the pre-test phase, as described above. The target zones (left or right), into which the ewe was transferred, were selected according to the side chosen by each lamb during habit acquisition trials in the V-detour test. After the pre-test phase, the gate of the waiting room was opened, and the lamb was let free to enter the entrance corridor and navigate the maze to reach the ewe. The trial was considered successful when the lamb entered with two front legs in the proximity zone (1.5 m x 1.3 m) adjacent to the target zone in which the ewe was placed. After each trial, the lamb was rewarded with 1 minute of reunion with the ewe within the target zone. On the first day of the test, the lamb was subjected to four habit acquisition trials. On the second day of the test, lambs were again subjected to the pre-test phases, and then to 4 further habit acquisition trials as on the 1st day of the test. When the lamb had successfully completed the first four trials, four additional trials were performed (reversal learning trials), in which the position of the ewe was on the opposite target zone. The time limit for a successful acquisition of reversal learning was settled at 20 min for the first trial, and at 10 min for the next 2 trials. After 3 unsuccessful trials, the test was considered failed.

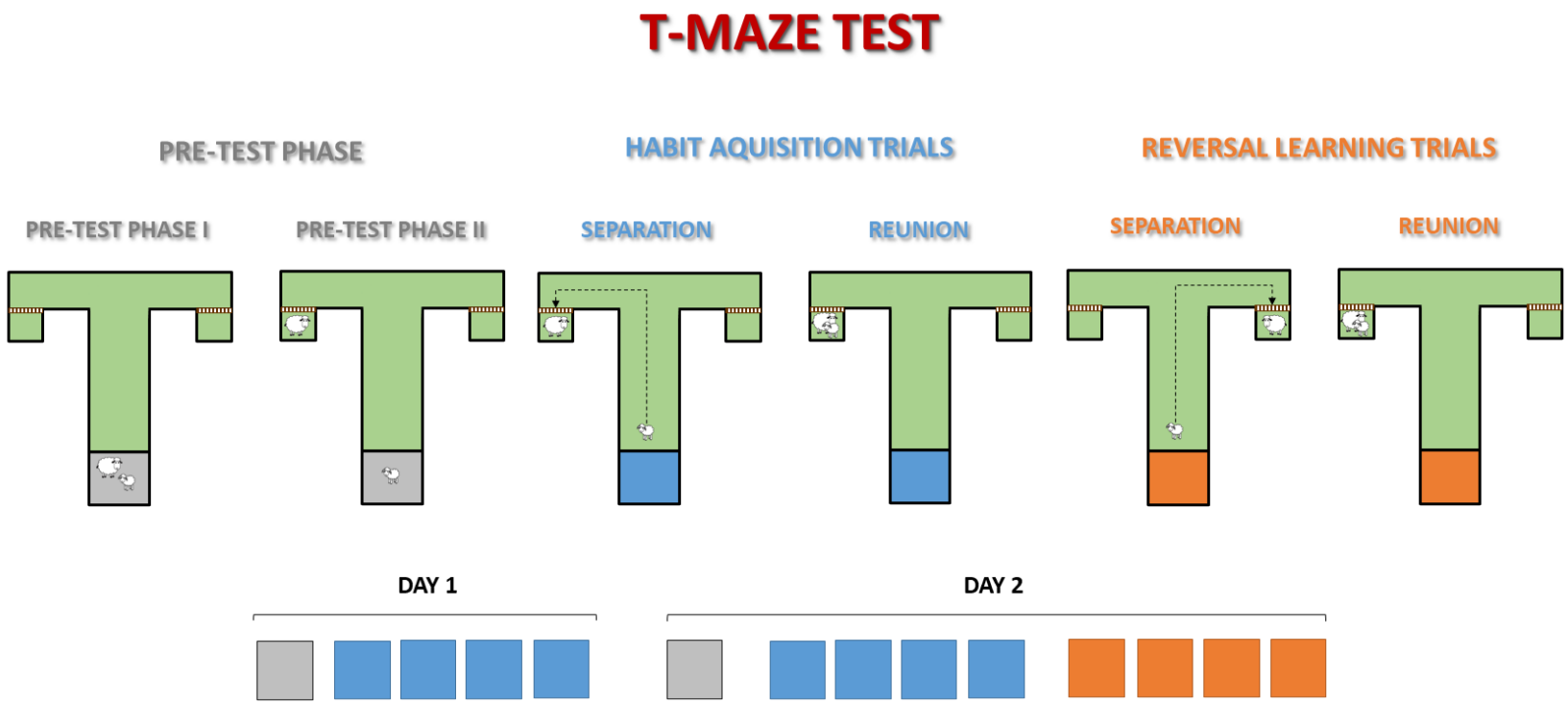

Figure 11. **Schematic representation of the T-maze test.** The colors on the scheme represent specific phases of the test: the pre-test phase – grey, habit acquisition trials – blue and reversal learning trials – orange. The test is performed in two days, and each day of the test is preceded by the pre-test phase consisting of two 5-minute phases. Subsequently, during the first day of the test, lambs are subjected to habit acquisition trials in which they have to reach the dam placed at the end of one of the arms of the maze (on the picture as an example is used left side). During the second day of the test, Habit Acquisition Trails corresponding to those from day 1 are followed by reversal learning trials, in which the ewe is transferred at the end of the opposite arm of the maze and the lamb has to reach her, changing the learned path.

4.4.4. Video-tracking analysis

All behavioral tests were video recorded and subsequently analyzed with the use of the video-tracking software EthoVision® XT 11.5 (Noldus Information Technology, Netherlands). This software tracks and analyzes the body activity as well as animals' movement coordinates within the area, by tracking the center of its gravity. For efficient tracking, it is critical to precisely identify the animal and distinguish it from the background, which in our setup was the floor of the different arenas. Different experimental set-ups might be more prone to light and background changes, thus it's essential to adjust the detection method specifically for each experiment. The software allows fine-tuning the different parameters of the detection, such as gray scaling, static subtraction, dynamic subtraction, and differencing, or automatically set up all the parameters, based on the sample video.

The approach used in this study consisted of applying an automated detection setting, which, based on the contrast between the animal and the background, as well as light conditions assigns an optimal set-up for all light and contrast parameters. Subsequently, the detection settings were adjusted manually to the specific conditions of each assay for each test, what allowed us to obtain the optimal tracking of the moving lamb under various light conditions. Using automated detection was more reliable, compared to the manual set-up, and helped to minimize the effect of environmental changes (mainly light conditions).

To detect the animal in the different arenas, all settings were adjusted to the contrast and brightness of each specific video. In the first experiment, the floor of the arenas was light grey, thus the contrast between the background and the moving lamb was not very clear and the detection setting required substantial manual arrangements. To overcome this problem, in experiments 2 and 3, the floor of the arenas was painted dark brown using non-toxic paint. This made the automatic video-tracking much more efficient and significantly decreased the number of corrections needed.

Photos of the arena settings from the EthoVision® XT software are displayed in *Figure 12* (Isolation Test), *Figure 13* (V-detour test), and *Figure 14* (T-maze test). Five different zones were defined for the Isolation Test: proximity zone, center front, central, and back, as well as the back of the arena. The analyzed parameters included the time spent and the frequency of entries in each zone. Moreover, an experienced operator was manually scoring the number of jumps performed by the lambs, defined as when

the lamb detaches all four hooves from the ground, eventually hitting one of the walls of the maze.

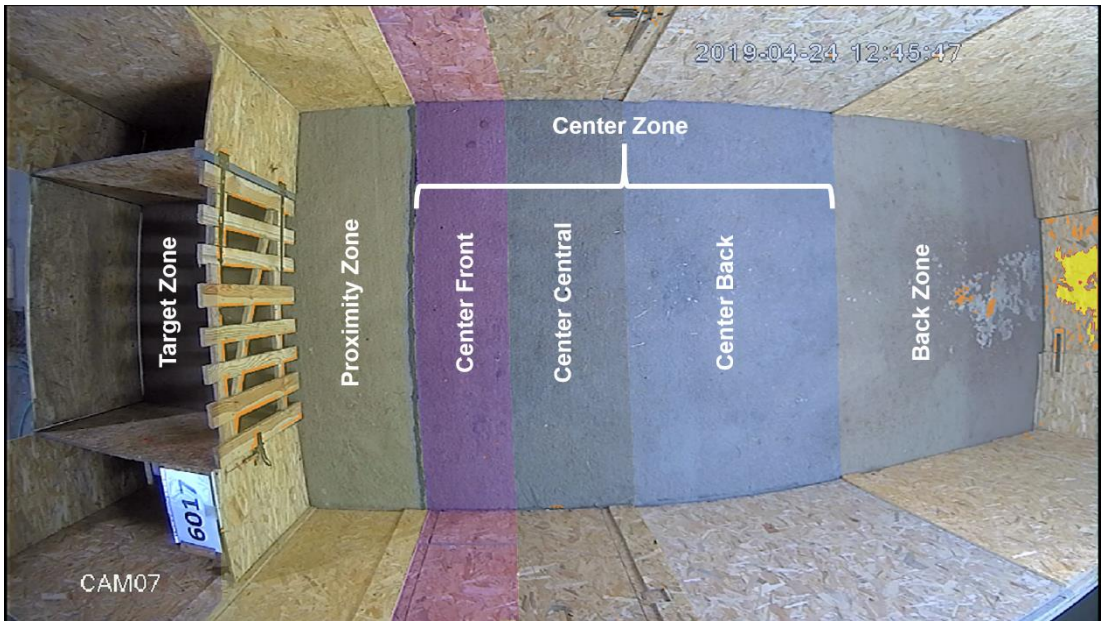

Figure 12. **Representation of the Isolation Test arena and the zones setting for EthoVision® XT analysis.** Five different zones were defined for the Isolation Test: proximity zone, center front, central and back, as well as the back of the arena. Three middle zones were considered as one in the further analyses and called center zones.

In the set-up for the V-detour Test, four zones were defined: central zone, left and right zones, and proximity zone. In each trial, the analyzed parameters included the total time spent by the lamb to reach the proximity zone, as well as the frequency of entrances into either the central, the left, or the right zones.

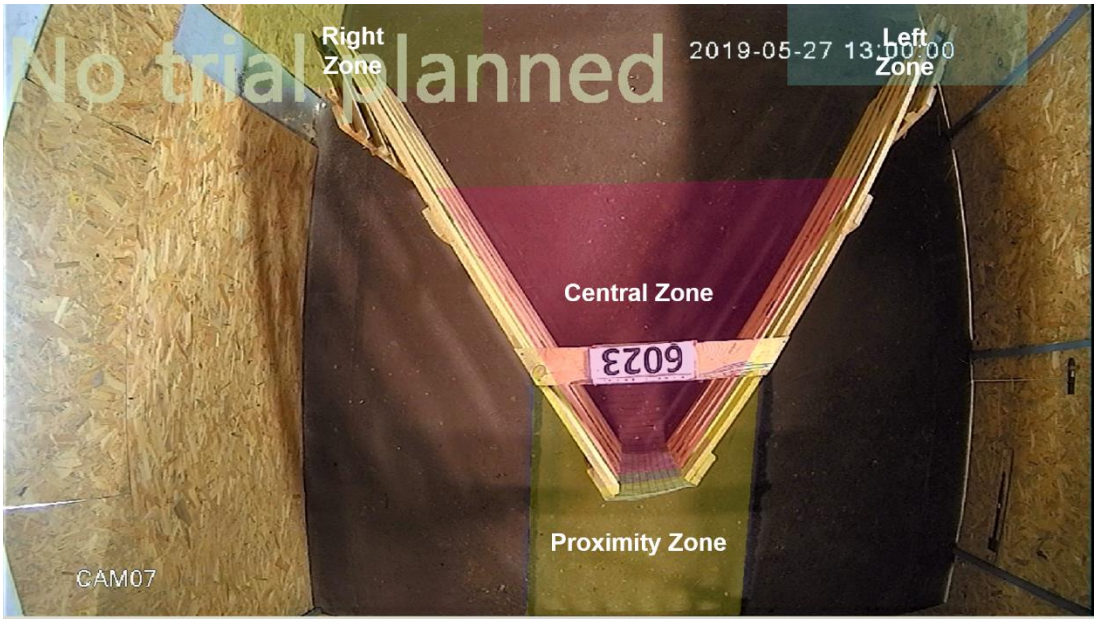

Figure 13. **Representation of the V-detour test arena and the zones setting for EthoVision ® XT analysis.** Four different zones were defined. In each trial, the software calculates the total time spent by the lamb to reach the proximity zone, as well as the frequency of entries into either the central, left or right zones.

For the T-maze, six different zones were distinguished: start arm, center, left and right arms, as well as left and right proximity zones. In each trial, the total time spent by the lamb to reach the left or right proximity zone in which the dam was located, as well as the frequency of entries into either the start, left or right arm, and the center, were calculated.

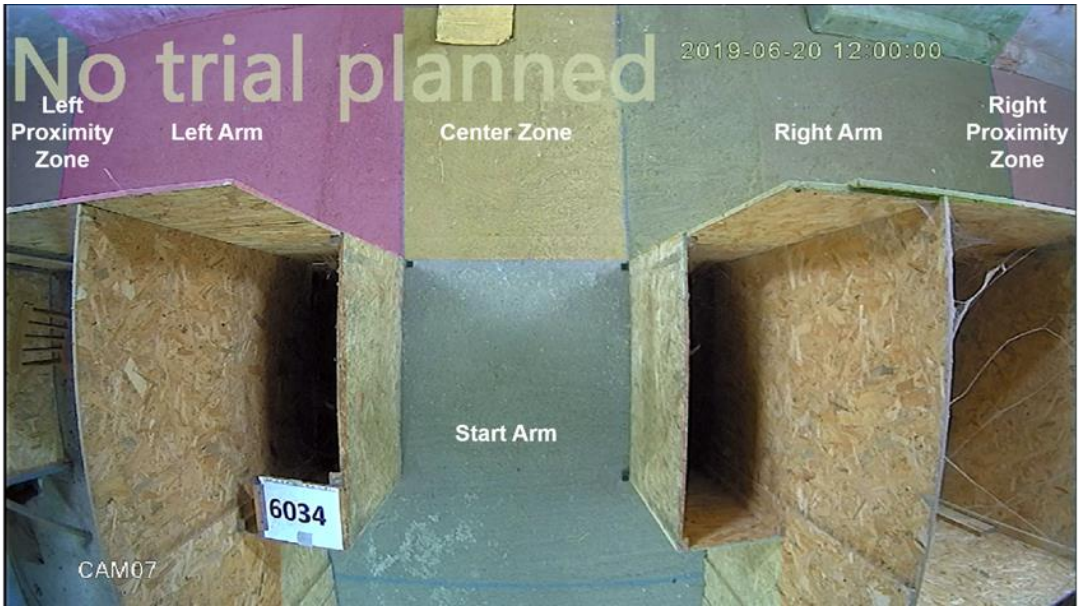

Figure 14. **Representation of the T-maze test arenas and the zones setting for EthoVision ® XT analysis.** Five different zones were defined. In each trial, the software calculates the total time spent by the lamb to reach the left/right zones, as well as the frequency of entries into either the start, left or right arm.

In EthoVision® XT the tracking of each trial is conducted according to specific rules, which define the conditions for starting and finishing the animal tracking. In the Isolation Test, the end of the trial was considered 5 minutes after the first detection of the lamb in the arena. In the V-detour test, the trial finishes when the lamb enters the proximity zone adjacent to the target zone with at least the two front legs. The latency of this event was considered in further analyses as the time spent to complete the trial. In the T-maze test, the trial finishes when the lamb enters the proximity zone adjacent to the target zone with at least two front legs. When the lamb does not reach the proximity zone the tracking is stopped manually. All the video tracks were inspected by an experienced operator, *a posteriori.* In case the tracked coordinates did not correspond to the real position of the lamb visible on the video, the tracked trajectories were corrected manually.

4.5. Inflammatory cytokines profiling in ewes

Inflammatory cytokines profiles were examined to monitor the inflammatory response of the pregnant ewes after the LPS-induced immune challenge. These analyses were conducted only for experiment 3, by measuring the levels of the pro-inflammatory cytokines IL-6 and TNF-alpha by ELISA.

4.5.1. Sample collection

Blood samples were collected from pregnant ewes just before, and at specific time points after LPS/SAL injections, as summarized in *Figure 15*.

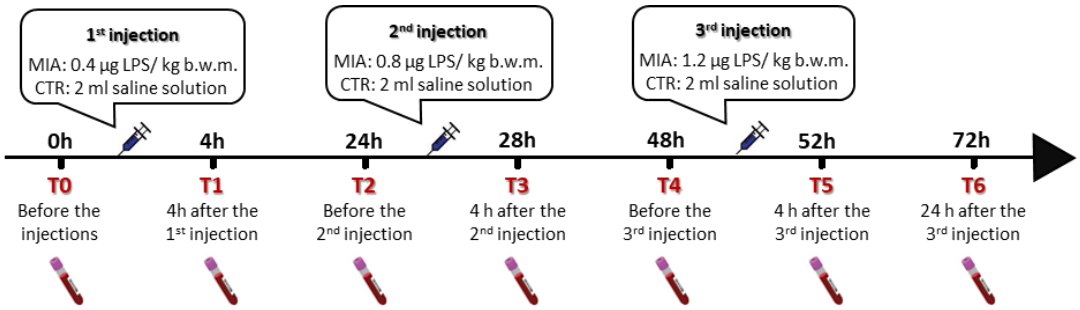

Figure 15. Scheme presenting the blood collection schedule in experiment 3. The syringes indicate the time points in which the LPS was administered, while the vials under the axis indicate the time points of blood collection.

Animals were restrained by the operator as described above for the LPS/SAL administration procedure. Blood was collected from the *vena jugularis externa* with the use of vacuum probes (BD Diagnostics, USA) coated with a K2EDTA

anticoagulant. Afterward, samples were centrifuged for 20 min at 4200 rpm to separate plasma from other blood components. The supernatant was carefully collected with the use of a Pasteur pipette, transferred to a clean 1.5 ml tube, and finally stored at -80°C until further analyses.

4.5.2. ELISA for IL-6 and TNF-alpha measurements

The analyses of the levels of pro-inflammatory cytokines – IL-6 and TNF-alpha were performed with the use of sandwich enzyme-linked immune-sorbent assay technology. The kits were purchased from FineTest® (Wuhan Fine Biotech Co., Ltd., China). The assays were performed on 96-well plates pre-coated with anti-IL-6 or anti-TNF-alpha antibodies, according to the manufacturer's instructions. The samples destined for TNF-alpha analyses were pre-processed according to the manufacturer's instructions, obtaining the final dilution of 1:3. Plasma samples used for IL-6 ELISA were instead not diluted. The standards of note concentrations provided by the manufacturer and plasma samples were transferred to the wells and left for 90 minutes of incubation at 37°C. The plates were then washed with the wash buffer. Subsequently, the samples were incubated for 60 min at 37°C with biotin-conjugated detection antibodies. After further washing, streptavidin conjugated with horseradish peroxidase (HRP) was added and left for an additional, 30 minutes incubation at 37°C. Unbound conjugates were then washed away with wash buffer. Subsequently, the TMB substrate was added, and plates were again incubated for 30 min at 37°C in the dark. HRP catalyzes the TMB substrate to produce a blue product. To stop the reaction, an acidic solution was added, leading to the change of color into yellow, which allowed visualizing the HRP enzymatic reaction that just occurred. The absorbance was measured spectrophotometrically at 450 nm in a microplate reader within 5 minutes after HRP reaction.

The final concentrations of IL-6 or TNF-alpha in the samples was calculated by comparing the optical density (OD) measured for each sample to the standard curves created with the use of the OD standard curve values calculated for standards provided by the manufacturer.

4.6. Stress response profiling in lambs

Isolation-induced stress response was analyzed in weaning lambs (66-98 days old), by measuring the levels of cortisol, the concentrations of which rise in response

to physical or emotional stress [25]. The analyses were performed after finishing all the above-mentioned behavioral tests, to exclude the influence of stress caused by other manipulations. This analysis was conducted only in experiment 3.

4.6.1. Social isolation procedure and sample collection

This procedure was performed just before the weaning, after at least 10 days following the last T-maze trial. Each lamb was transferred by the main operator into the rectangular room (1.25 x 1.2 m), without visual contact with the rest of the flock mates. After 5 minutes, the lamb was returned to the home pen. Two lambs were subjected to the isolation procedure simultaneously, on the two distant sides of the facility. Blood samples were collected just before and 25 minutes after the isolation procedure. Existing evidence indicates that the level of cortisol in sheep rises around 30 minutes after stressful events like isolation and restraining [205,206]. Thus, in the presented experiment, samples were collected 25 minutes after the isolation to record the peak of the cortisol level. Sample collection as well as their further processing and storage was performed as described above. Briefly, the lambs were restrained by the caregiver embracing with one hand their head and holding the groin with the other, and an experienced veterinarian collected the 5 ml of blood from the *vena jugularis externa*.

4.6.2. ELISA for cortisol (social-solation-induced cortisol surge)

The plasma levels of cortisol were examined using a Competitive-ELISA kit provided by Elabscience® (Elabscience Biotechnology Co. Ltd., China). The procedure was performed according to the protocol provided by the manufacturer. The 96-well plates provided with the kits were pre-coated with cortisol. The standards and examined samples were placed in the wells. Subsequently, the biotinylated detection antibody specific to cortisol was added to all wells and left for 45 minutes of incubation at 37°C. During the reaction, cortisol present in the standards or samples competes with a fixed amount of the cortisol on the plate for sites on the biotinylated detection antibody. The surplus conjugate, as well as unbound standards or samples, were washed from the plate with the wash buffer. Then, the avidin conjugated to HRP was added to each well and left for 30 minutes incubation at 37°C. After washing, a TMB substrate solution was added to each well and left for 15 minutes of incubation at 37°C in the dark. The enzyme-substrate reaction catalyzed by HRP was terminated by adding the stop solution which led to the color change from blue to yellow.

Immediately after, the optical density (OD) was measured spectrophotometrically with the use of a microplate reader at a wavelength of 450 nm. The final concentration of cortisol was calculated by comparing the OD of the samples to the standard curve, created with the OD values of the standards.

4.7. Statistical analyses

The statistical analyses were performed using GraphPad Prism Software (GraphPad Software, USA) with α set of 0.05. The statistical analyses were performed on the data from all the lambs of one experiment, without distinction of sex. All the obtained data were checked for normal distribution with the use of a D'Agostino-Pearson omnibus normality test. The distribution was considered normal for a p-value>0.05. Subsequently, all the data regarding physiological parameters (temperature, heartbeat, respiratory rate), as well as the body weight of the lambs and the data obtained from molecular analyses were checked for significant outliers with the Grubbs' test, which was conducted using the online tool provided by GraphPad (available at: https://www.graphpad.com/quickcalcs/Grubbs1.cfm), with α of 0.05. All data with normal distribution were subjected to a One-Way analysis of variance to analyze the differences in outcome measurements between the time points after the injection. Differences among the groups were further investigated with the Tukey post-hoc test. When data were not normally distributed, a Kruskal-Wallis Test followed by Dunn's multiple comparisons was performed. Further comparisons between the experimental groups were performed with Unpaired T-Test for normal distribution of the data, or Mann Whitney Test in other cases. Two-way ANOVA with Bonferroni post-test was used to investigate the effect of treatment, time points, and their interaction, on the physiological parameters of the ewes and lambs, as well as the cytokine levels of the ewes.

The correlation between the ewes' body temperature and the levels of cytokines was studied using STATISTICA 13 software (StatSoft Polska Sp. z o.o., Poland), utilizing Pearson's correlation test. The correlation coefficient was considered significant for p-values<0.05, while the strength of the correlation was considered high for r=0.7-0.9, moderate for r=0.5-0.7, low for r=0.3-0.5, and negligible for values lower than 0.3.

To investigate the association between specific behavioral outcomes observed in the V-detour test and the LPS treatment, linear regression models were created for each

of the experiments. The sex of the lamb and the ewe body temperature at 4 h post-treatment were introduced into the model as confounding factors and effect modifiers. The same parameters were used in the models for all investigated trials and behavioral outcomes.

For experiment 3, a linear regression model included the trial results as dependent variables and tested against IL-6 and TNF alpha concentrations at time points in 4 hours after each injection. Each model was corrected by cytokine concentrations before the 1st injection, sex of the lamb, and the temperature of the ewe 4 hours post-treatment. The associations were considered significant for $p<0.05$.

5. RESULTS

5.1. Effects of a single LPS dose at 85 and 125 *dpc* on the ewes' physiological outcomes

To evaluate the response of the pregnant ewes to the single LPS immune challenge, physiological parameters, including rectal temperature, heartbeat, and respiratory rate, were evaluated just before, and at different time points after LPS/SAL administration.

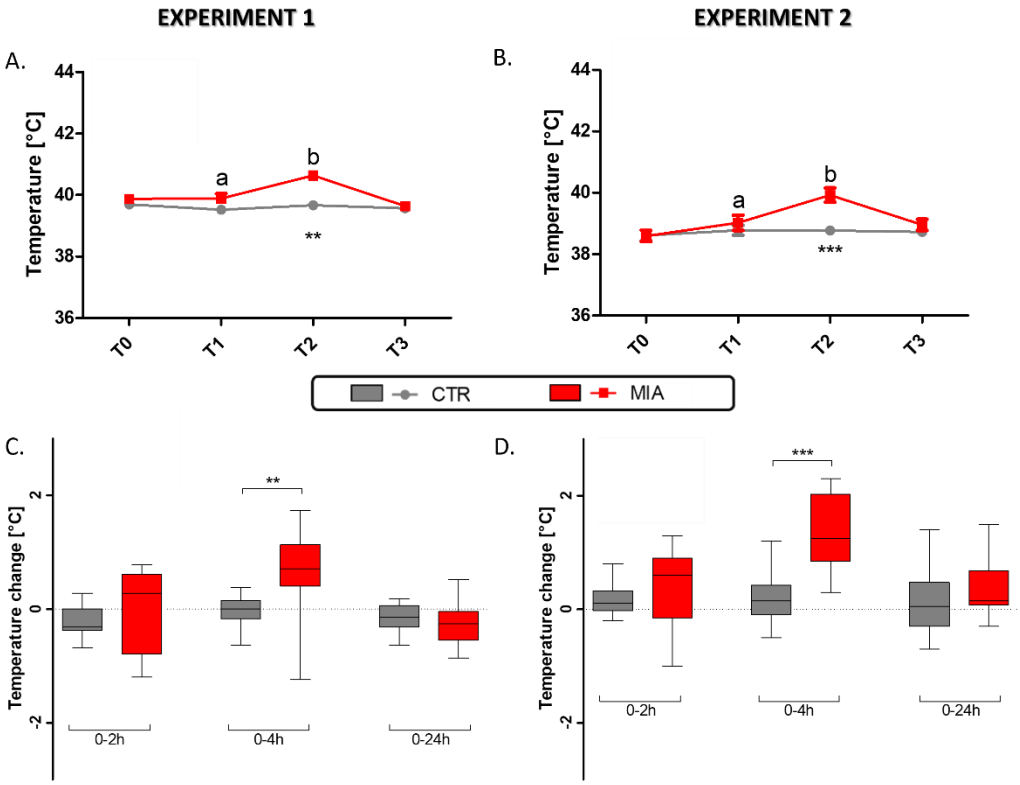

Figure 16. Ewes' temperature at different time points after LPS/SAL administration: MIA animals were (MIA125, MIA85, and MIA85') intravenously injected with 2 ml saline solution containing the endotoxin LPS, and Control Groups animals (CTR125, CTR85, and CTR 85') injected with 2 ml of saline solution (SAL). **A.** Absolute temperature of the ewes in experiment 1 just before (T0), 2 h after (T1), 4 h after (T2) and 24 h after (T3) the treatment. "a" indicates the difference between T0 vs T2 in MIA lambs, $p<0.001$; "b" indicates the difference between T1 vs T2 in MIA lambs, $p<0.001$; "c" indicates the difference between T2 and T3 in MIA lambs, $p<0.001$; $^{**}p<0.01$, $^{***}p<0.001$ and indicates the difference between CTR and MIA groups; **B.** Absolute temperature of the ewes in experiment 2 just before (T0), 2 h after (T1), 4 h after (T2) and 24 h after (T3) the treatment. "a" indicates the difference between T0 vs T2 in MIA lambs, $p<0.001$; "b" indicates the difference between T1 vs T2 in MIA lambs, $p<0.05$; "c" indicates the difference between T2 and T3 in MIA lambs, $p<0.01$; $^{***}p<0.001$ and indicates the difference between CTR and MIA groups; **C**. Ewes' temperature change after the treatment in experiment 1. 0-2h indicates the differences of the temperature between T0 and T1, 0-4 h – between T0 and T2, and 0-24h – between T0 and T3. $^{**}p<0.01$ and indicates the difference between CTR and MIA lambs. **D.** Ewes' temperature changes after the treatment in experiment 2. 0-2 h indicates the differences of the temperature between T0 and T1, 0-4 h – between T0 and T2, and 0-24h – between T0 and T3.$^{***}p<0.001$ and indicates the difference between CTR and MIA lambs The differences between the time points were measured with One-Way ANOVA, followed by Tukey post-hoc test, while the differences between the groups were evaluated with unpaired t-test (A, B, and D) or Mann-Whitney test (C).

Figure 16 displays the temperature changes in different time points post-injection of the ewes belonging to MIA and CTR groups of experiments 1 and 2. Ewes

injected with LPS displayed a significantly higher temperature at 4 h (*Figure 16 A-B*) after administration, compared with SAL-injected controls in both experiments. No differences were found comparing T0 vs. T3, indicating that the temperature dropped back to its basic level after a few hours. The change in body temperature (*Figure 16 C-D*) tends to be higher at 2 and 4 hours after the injections of LPS compared to controls in both experiments although the differences were significant only when measured 4 h after the injection.

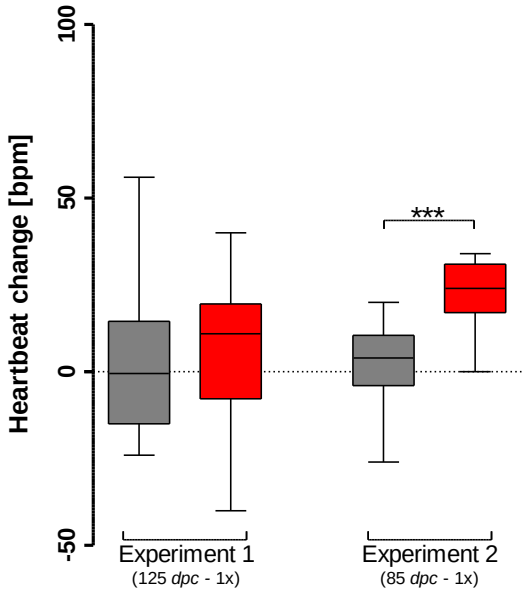

Figure 17. **Ewes' heartbeat changes 4h post-LPS/SAL administration in experiments 1 and 2.** MIA animals (MIA125, MIA85 and MIA85') were intravenously injected with 2 ml of saline solution containing the endotoxin LPS, and Control Groups animals (CTR125, CTR85 and CTR 85') were injected with 2 ml of saline solution (SAL). Differences between groups were examined by the Unpaired T-test. ***$p<0.001$.

Heartbeat rises 4 h post-LPS/SAL administration tends to be more consistent in LPS-injected ewes compared to controls (*Figure 17*). These differences were significant only in experiment two ($p<0.001$).

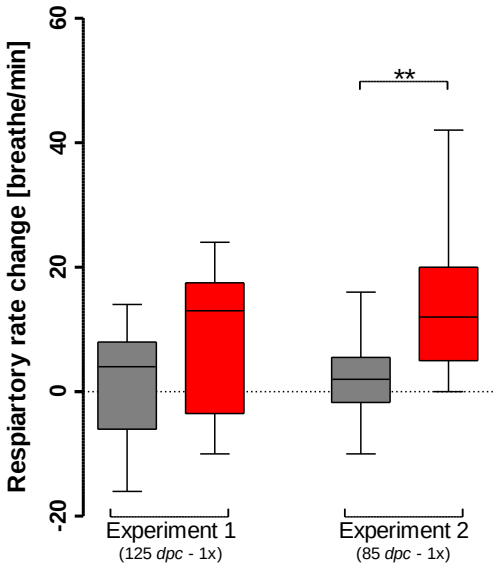

Figure 18. **Ewes' respiratory rate changes 4h post LPS/SAL administration in experiments 1 and 2.** MIA animals (MIA125, MIA85 and MIA85') were intravenously injected with 2 ml of saline solution containing the endotoxin LPS, and Control Groups animals (CTR125, CTR85 and CTR 85') were injected with 2 ml of saline solution (SAL). Differences between groups were investigated by Unpaired T-Test. $^{**}p<0.01$.

Pregnant ewes subjected to the injection of LPS displayed a tendency towards a greater increase of respiratory rate 4h after LPS injection in both experiments (*Figure 18*), although the observed differences were statistically significant only for experiment 2 ($p<0.01$).

5.2. Ewes' physiological responses to chronic LPS challenge

The results obtained from experiments 1 and 2 indicate that the biggest changes in the temperature response to LPS challenge occur after 4 h. Thus, in experiment 3 the physiological parameters, including rectal temperature, heartbeat and respiratory rate of the ewes were examined at 4 and 24 h after each of the 3 injections.

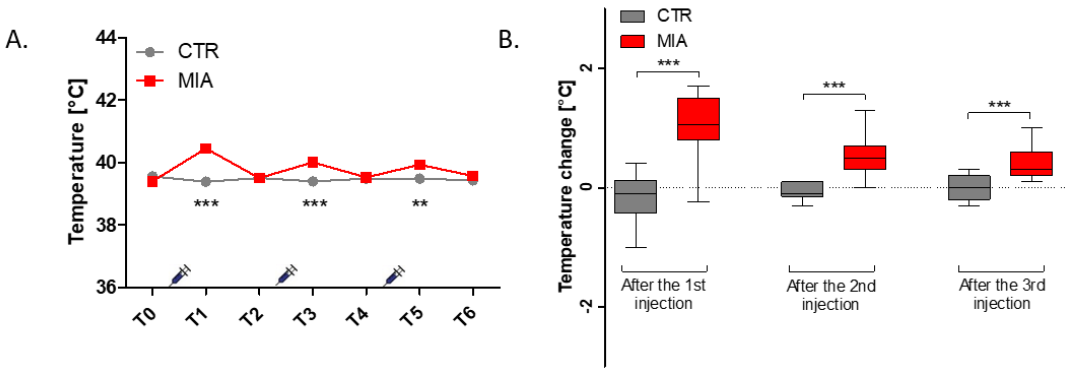

Figure 19. **Ewes' temperature in different time points after LPS/SAL administration in experiment 3.** MIA animals were administered three intravenous injections with a growing dose of saline solution containing the endotoxin LPS, while Control Groups animals (CTR85') were injected three times with 2 ml of saline solution (SAL). **A.** The absolute temperature of the ewes after injections. T0, T2 and T4 indicate the time before and 24 hours after each injection (starting from T2), while T1, T3 and T5 indicate time points 4 h after each administration. The syringes indicate the time of the injections; ***p<0.001 and **p<0.01 indicate differences between CTR and MIA groups. **B.** The change of the ewes' temperature in 4h after each injection. ***p<0.001 and indicates differences between CTR and MIA groups. The differences between groups were evaluated with the Two-Way ANOVA followed by Bonferroni post-test (A) or unpaired t-test (B).

Just before the injection, the average temperature of the ewes (*Figure 19 A*) was similar in both experimental groups (MIA: 39.40±0.07, CTR: 39.56±0.08). A significant effect of treatment was found in ewes' body temperature ($F(1,27)=12.30$; $p<0.0016$), with post-hoc analysis showing significant differences between MIA vs CTR ewes at all time points 4h after administration - T1 ($p<0.001$), T3 ($p<0.001$) and T5 ($p<0.01$), although the differences between groups were not significant at time point zero, before LPS/SAL shots were administered. Moreover, while in the MIA group, the temperature raised post-LPS (*Figure 19 B*), in the CTR group, no differences were observed among time points. The analysis of the temperature change indicates that MIA ewes had significantly higher temperature rises after each of the LPS injections, compared to controls ($p<0.001$).

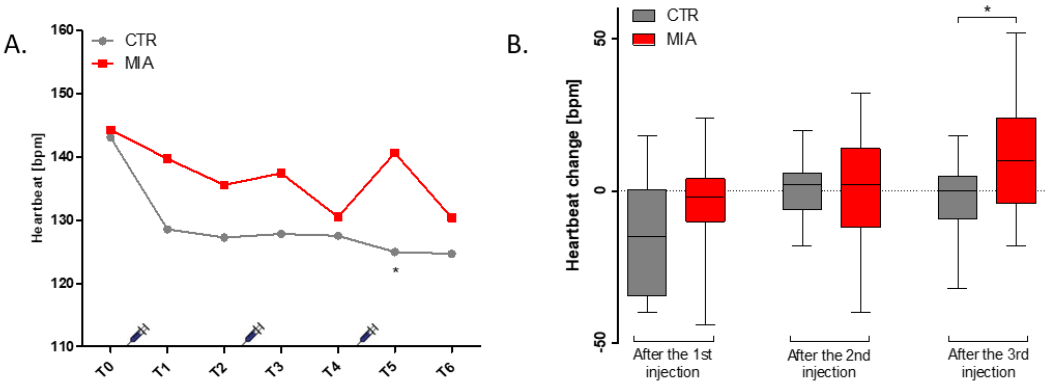

Figure 20. **The heartbeat of the ewes after LPS or saline administrations in experiment 3.** MIA animals were administered three intravenous injections with a growing dose of saline solution containing the endotoxin LPS, while Control Groups animals (CTR85') were injected three times with 2 ml of saline solution (SAL). **A.** The absolute heartbeat of the ewes. T0, T2, and T4 indicate the time before and 24 hours after each injection (starting from T2), while T1, T3, and T5 indicate time points 4 h after each administration. The syringes indicate the time of the injections. *p<0.05 and indicates the difference between MIA and CTR animals. **B.** The change of the ewes' heartbeat in 4h after each injection. *p<0.05 and indicates differences between CTR and MIA groups. The differences between groups were evaluated with the Two-Way ANOVA followed by Bonferroni post-test (A) or unpaired t-test (B).

Before the first injections, the average heartbeat (*Figure 20 A*) was at similarly high levels in all experimental groups (MIA: 144.3±3.99, CTR: 143.1±3.88). A significant effect of treatment was found in the ewes' heartbeat ($F(1,27)=5.353$; $p=0.0285$) with post-hoc analysis showing significant differences only comparing MIA vs CTR ewes at 4 h after the last injection ($p<0.05$). The change in the heartbeat rate indicates that MIA ewes display a significantly higher rise after the 3rd injection (*Figure 20 B*).

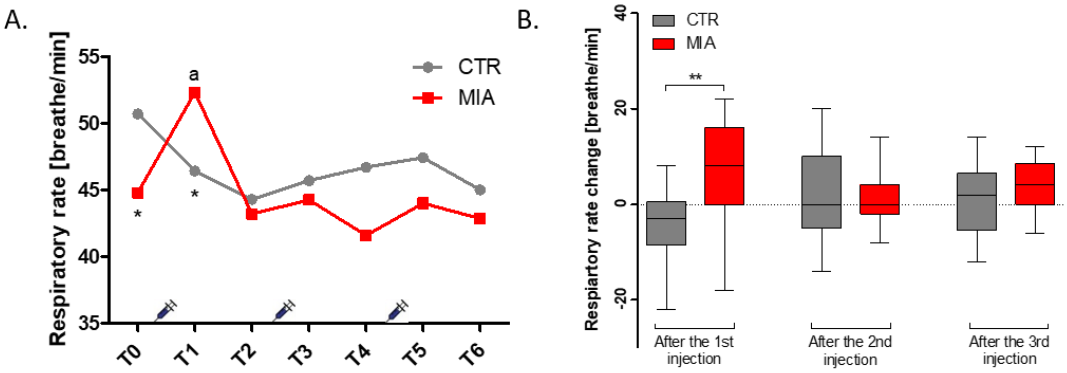

Figure 21. **Respiratory rate of the ewes after LPS or saline administrations in experiment 3.** MIA animals were administered three intravenous injections with a growing dose of saline solution containing the endotoxin LPS, while Control Groups animals (CTR85') were injected three times with 2 ml of saline solution (SAL). **A.** The absolute respiratory rate at different time points after LP/SAL injections. T0, T2, and T4 indicate the time before and 24 hours after each injection (starting from T2), while T1, T3, and T5 indicate time points 4 h after each administration. The syringes indicate the time of the injections. "a" indicates the difference between T1 vs T4 and T1 vs T6 in the MIA group, $p<0.01$; *$p<0.01$ and indicates differences between CTR and MIA groups. **B.** The change of the ewes' respiratory rate in 4h after each injection. **$p<0.01$ and indicates differences between CTR and MIA groups. Differences between groups were measured with an Unpaired T-Test, while the effect of time was checked with Two-Way ANOVA test.

Two-way ANOVA showed no effect of the treatment on the respiratory rate (*Figure 21 A*) of the ewes, although a significant interaction between time points and the treatment was revealed ($F(6,27)=3.262$; $p=0.0047$). Further analysis showed that just before the 1st injection the respiratory rate was significantly lower in the MIA group, compared to controls ($p<0.05$), whereas the lambs from the MIA group displayed a higher respiratory rate than lambs from the CTR group ($p<0.05$) on 4 h after the first injection (T1). Accordingly, the respiratory rate of the ewes subjected to MIA during pregnancy displayed a significant rise 4 h after the first injection ($p<0.01$) and tended to rise after each of the next two injections, although the differences were not significant. The respiratory rate change displays significant differences only after 1st injection, showing a higher rise in MIA lambs, compared to controls ($p<0.01$) (*Figure 21 B*).

5.3. Effects of chronic LPS challenge on the ewes' cytokines inflammatory response

To evaluate the inflammatory response of the pregnant ewes to chronic LPS administration, blood levels of pro-inflammatory cytokines TNF-alpha and IL-6 were examined in experiment 3.

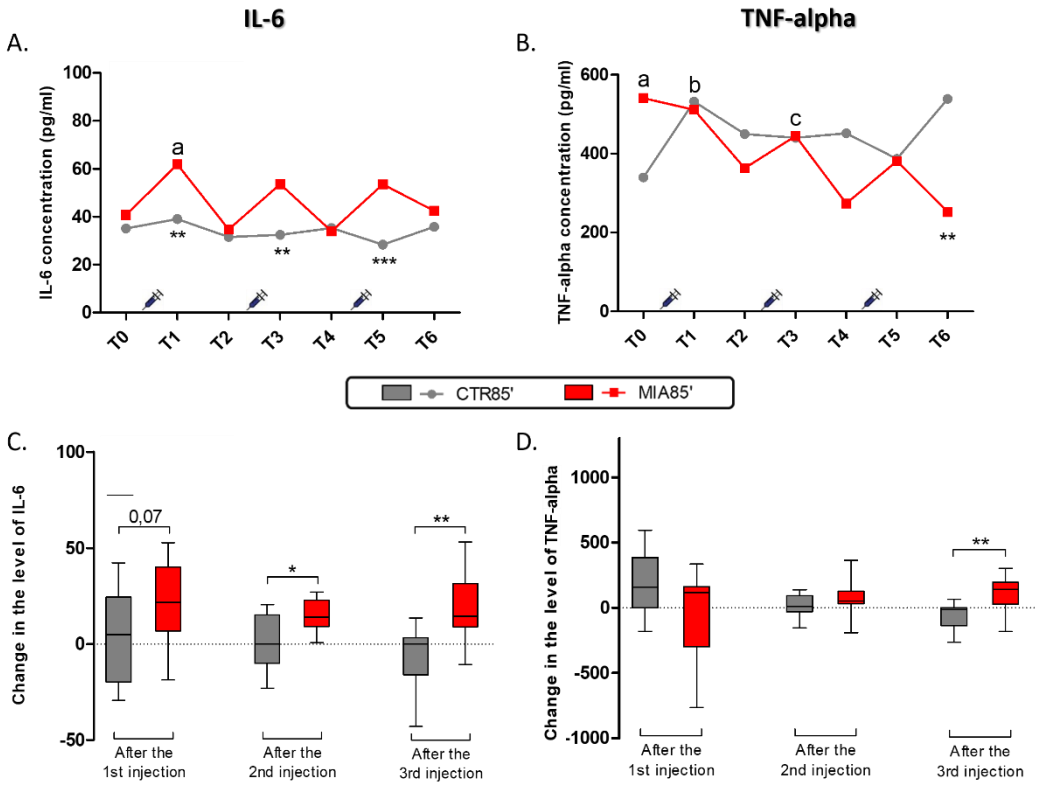

Figure 22. **Protein levels of pro-inflammatory cytokines TNF-alpha and IL-6 in the plasma of the pregnant ewes injected with saline or LPS in experiment 3, before and 4 hours after each injection.** MIA animals were administered three intravenous injections with a growing dose of saline solution containing the endotoxin LPS, while Control Groups animals (CTR85') were injected three times with 2 ml of saline solution (SAL). **A.** The absolute concentration of IL-6 in the plasma of the ewes. The syringes indicate the time of injection. T0, T2 and T4 indicate the time before and 24 hours after each injection (starting from T2), while T1, T3 and T5 indicate time points 4 h after each administration.; "a" indicates the differences between T1 vs T2 and T1 vs T4 in MIA85', $p<0.01$; **$p<0.01$, ***$p<0.001$ and indicates the differences between CTR85' and MIA 85'. **B.** The absolute concentration of TNF-alpha in the plasma of the ewes. The syringes indicate the time of injections. T0, T2 and T4 indicate the time before each injection, while T1, T3 and T5 indicate time points 4 h after. "a" indicates the differences between T0 vs T6 in MIA85', $p<0.05$; "b" indicates the differences between T1 vs T4 and T1 vs T6 in MIA85', p<0.05; "c" indicates the differences between T3 vs T6 in the MIA 85', p<0.05. **p<0.01 and indicates the differences between CTR85' and MIA 85'. **C.** The changes in the plasma levels of IL-6 4 h after each injection. *$p<0.05$, **$p<0.01$. **D.** The changes in the plasma levels of TNF-alpha 4 h after each injection. **$p<0.01$. The differences between the groups were evaluated by Two-Way ANOVA followed by Bonferroni post-test (A-B), Mann-Whitney test (C), or unpaired t-test (D), while differences between the trials (A-B) were evaluated with One-Way ANOVA

Just before the injection, lambs from both experimental groups displayed comparable levels of IL-6 in plasma (MIA: 40.68±4.991 pg/ml; CTR: 35.09±3.637 pg/ml). As shown in *Figure 22 A*, MIA lambs display significantly higher level of plasma IL-6 level in 4 h after the 1st injection compared to 24 after the 1st and 2nd injection ($p<0.01$). Moreover, a significant effect of the treatment on the plasma levels of IL-6 was found ($F(1,23))=8.099$, $p=0.0091$), with post-hoc analyses showing significant differences in the plasma level of IL-6 between MIA vs CTR dams at all time points 4 h after the injections - T1($p<0.01$), T2($p<0.01$) and T5($p<0.001$). The change in the plasma levels of IL-6 (*Figure 22 C*) was significantly higher in the MIA

group after the 2nd (p<0.05) and the 3rd injections (p<0.01), compared to controls. Moreover, the same tendency was observed after the first injection, although the differences were not significant (p=0.07).

The plasma level of TNF-alpha (*Figure 22 B*) in pregnant MIA ewes displays significant differences between the time points just before the 1st injection and 24 h after the last injection (p<0.05). Significant differences were observed also in the MIA group comparing the cytokine levels at 4 h after the first injection with 24 h after the second injection (p<0.05) and 24 h after the last injection (p<0.05), as well as time point 4 h after the 2nd injection with the time point 24 h after the 3rd injection. This suggests the conclusion that sheep display a reduced sensitivity and homeostatic adaptation to subsequent LPS administration, even despite the increasing dose. Two-way ANOVA showed no effect of the treatment on the TNF-alpha levels in the plasma, although a significant interaction between the time point and the treatment was observed ($F(6,23)=4.677$, p=0.0002), with post-hoc analyses showing a significant difference between CTR and MIA in the last time point, 24 h after the last injection (p<0.01). The level of the TNF-alpha displays a significantly higher rise (*Figure 22 D*) in MIA ewes after the 3rd injection, compared to controls (p<0.01).

Correlation analyses (*Figure 23*) showed a moderate positive correlation between the changes in the body temperature and changes in the plasma level of IL-6 after each injection. The values of the correlation coefficient were the lowest after the first injection (r=0.5001, p-value=0.011), slightly higher after the 2nd injection (r=0.5168, p-value=0.008), and the highest relationship between variables after the 3rd administration (r=0.6473, p-value=0.000). Moreover, the higher values for temperature rise, as well as IL-6 level, are displayed by MIA lambs, while CTR lambs are concentrated at the bottom-left part of the correlation graph, thus generating two distinct treatment-specific clusters.

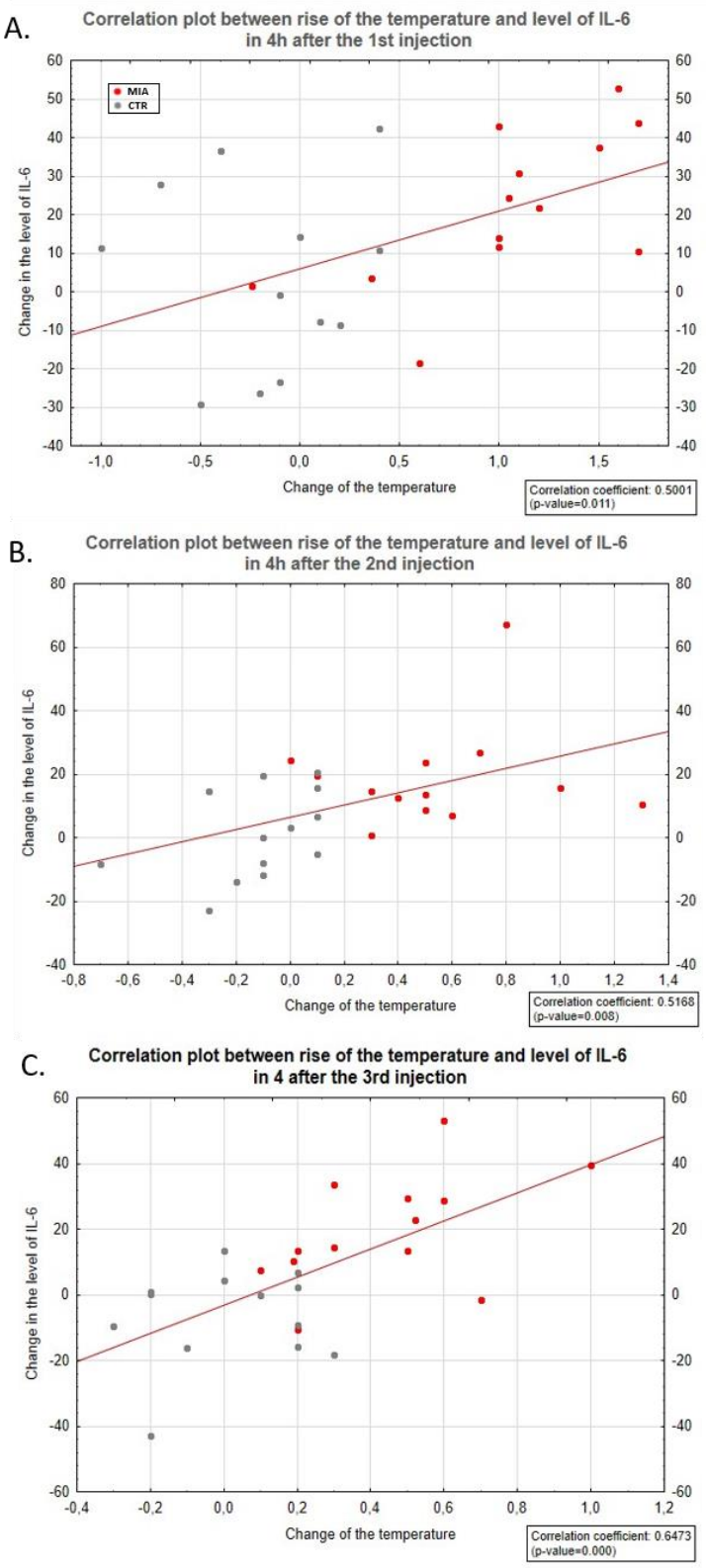

Figure 23. **Correlation plots between the changes in the plasma level of IL-6 and the rise of the temperature in 4 h after each injection. A.** Correlation plot concerning the 1st injection. The analysis revealed a positive correlation between the rise of IL-6 and temperature change in 4 h post-injection; correlation coefficient: 0.5001 (p-value=0.011). **B.** Correlation plot concerning the 2nd injection. The data display a positive correlation between the rise of IL-6 levels and temperature change in 4 h post-treatment; correlation coefficient: 0.5168 (p-value=0.008). **C.** Correlation plot concerning the 3rd injection. The positive correlation occurs between the rise of IL-6 and temperature change in 4 h post-injection; correlation coefficient: 0.6473 (p-value=0.000). The data were analyzed with Pearson's correlation.

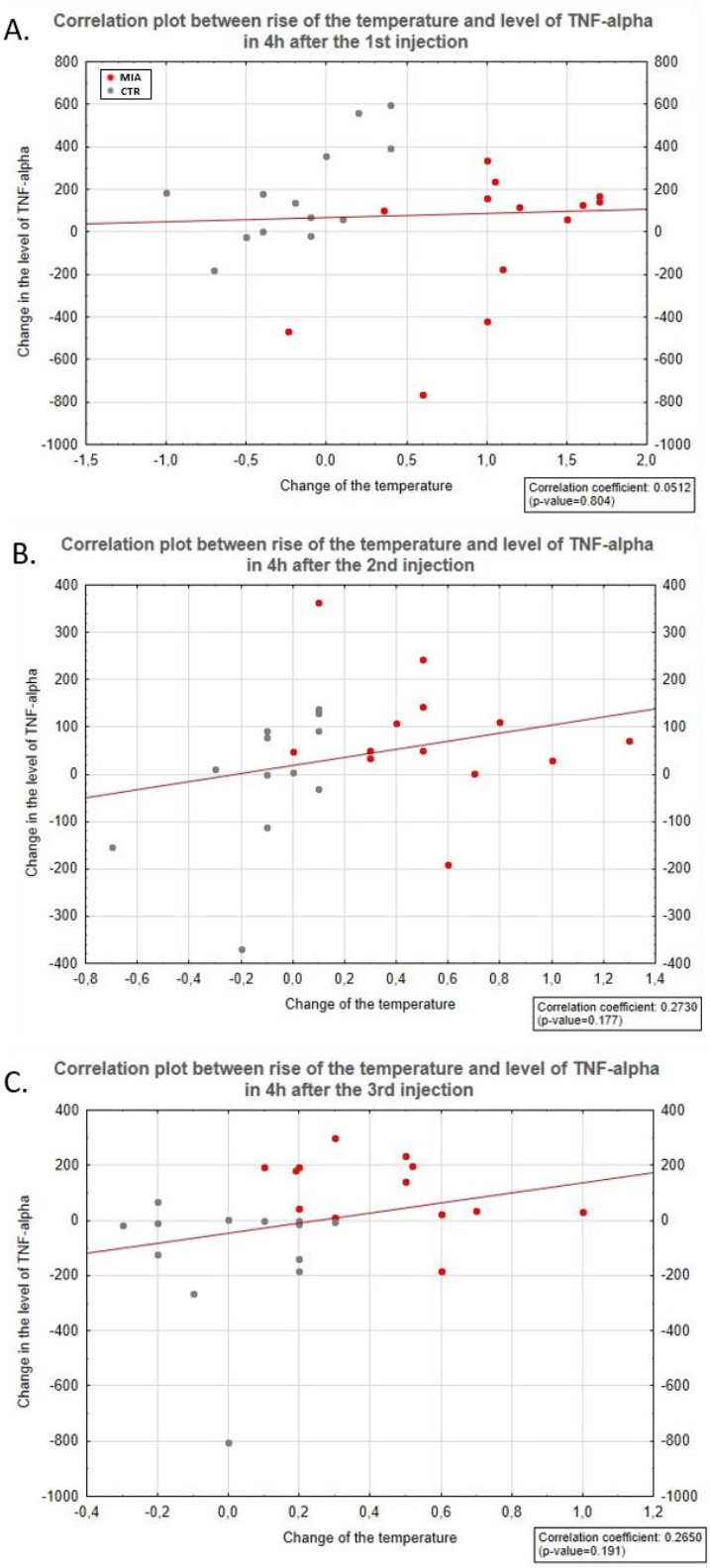

Figure 24. **Correlation plots between the changes in the plasma level of TNF-alpha and the rise of the temperature in 4 h after each injection. A.** Correlation plot concerning the 1st injection. The analysis revealed no correlation between the rise of TNF-alpha and temperature change in 4 h post-injection; correlation coefficient: 0.0512 (p-value=0.804). **B.** Correlation plot concerning the 2nd injection. The data displays no correlation between the rise of TNF-alpha levels and temperature change in 4h post-treatment; correlation coefficient: 0.2730 (p-value=0.177). **C.** Correlation plot concerning the 3rd injection. A positive correlation occurs between the rise of TNF-alpha and temperature change in 4 h post-injection; correlation coefficient: 0.2650 (p-value=0.191). The data were analyzed with Pearson's correlation.

Correlation analyses (*Figure 24*) revealed no relationship between the change of TNF-alpha and the change of the temperature in 4 h post-injection after any of the administrations (1st: r=0.0512, *p*-value=0.804; 2nd: r=0.2730, *p*-value=0,177; 3rd: r=0.2650, *p*-value=0.191).

5.4. General health and body weight of the lambs

All lambs used in this study displayed proper and constant growth, as well as good general health. After delivery, all lambs stand up on their 4 legs within 30 minutes post-partum. No differences between groups were observed in the latency to stand up after delivery (data not shown). No differences in infectious disease vulnerability or visible gross malformations were observed comparing MIA with CTR. However, data concerning the body weight showed some important differences among experiments (breeding seasons).

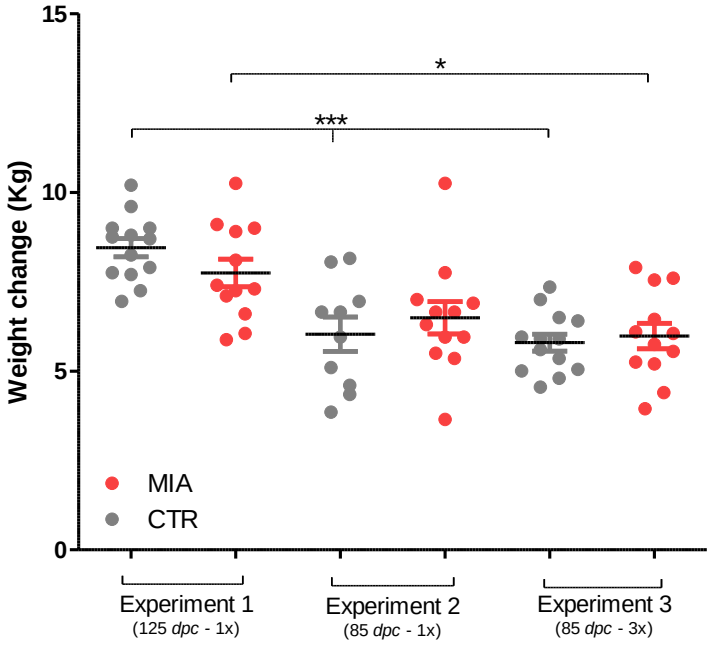

Figure 25. Lambs' body weight change between postnatal day 20 and postnatal day 60. Differences between groups were measured by One-Way ANOVA. ***$p<0.001$, *$p<0.05$.

As indicated in *Figure 25*, lambs from the CTR group of experiment 1 showed significantly higher weight gain between PND 20 and PND 60, compared with the lambs from the corresponding groups enrolled in the other experiments ($p<0.001$). Moreover, we observed a higher weight change in the lambs from the MIA group of experiment one, compared to lambs from the MIA group of the other two experiments, although these differences were significant only for experiment 3 ($p<0.05$).

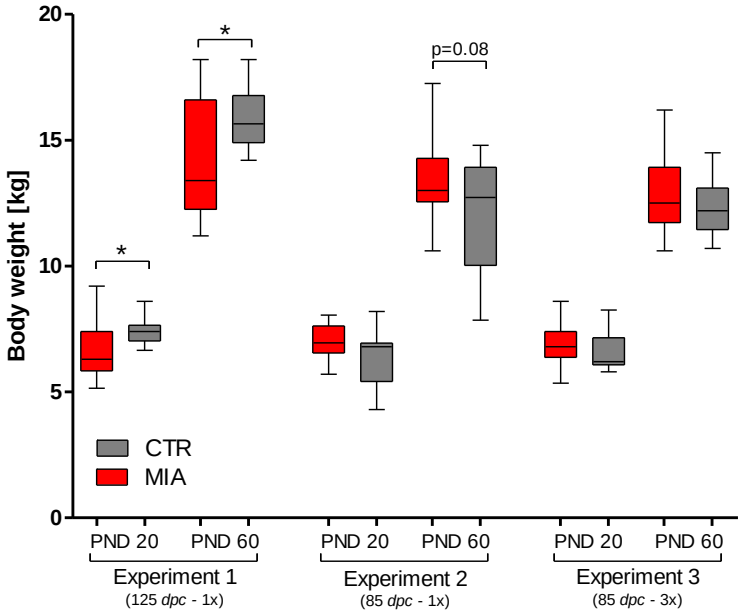

Figure 26. **The body weight of the lambs on postnatal day 20 (PND 20) and postnatal day 60 (PND 60).** Differences between groups were measured by Unpaired T-Test. *p<0.05.

As shown in *Figure 26*, the MIA lambs of experiment 1 displayed significantly lower body weight than CTR lambs in PND 20, as well as in PND 60 (p<0.05). In experiment 2 and experiment 3, no significant differences in body weight were found, although MIA lambs from experiment 2 tend to be heavier compared to CTR animals (p=0.08 with Unpaired T-Test), thus showing an opposite trend with respect to the first experiment.

5.5. Isolation Test – Behavioral response to maternal separation

The Isolation Test was performed on 20-day-old lambs to evaluate the social attachment to the mother and the behavioral response to social separation with (T1) and without (T2) having visual contact with the ewe.

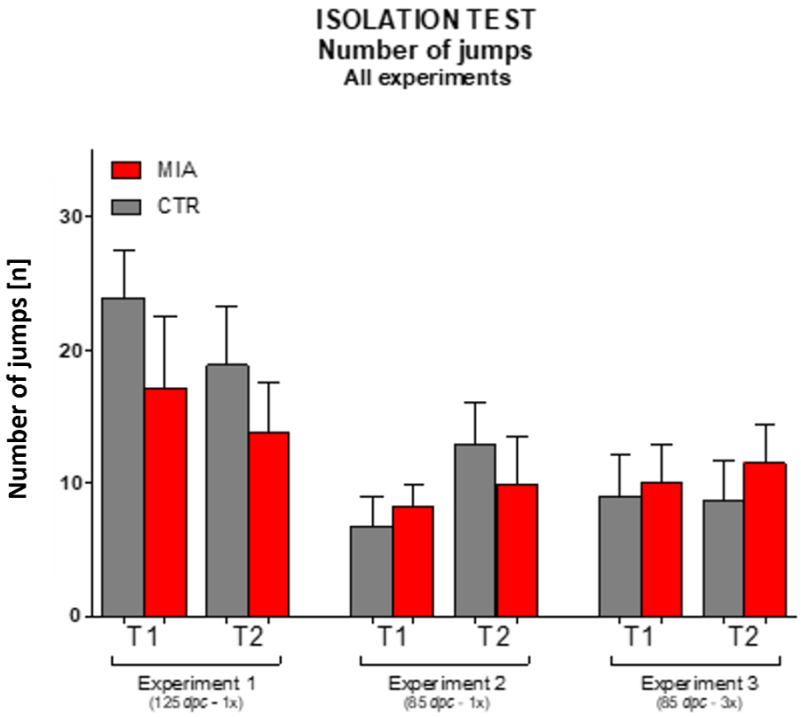

Figure 27. **The number of jumps during the Isolation Test.** T1 indicates the first trial of the Isolation Test, with visual contact, while T2 indicates the trial without visual contact with the dam. No differences between groups and between trials were observed. The differences between groups were evaluated by Mann –Whitney test, while differences between the trials were analyzed by the Kruskal-Wallis test, with Dunn's multiple comparisons.

The number of jumps during maternal separation in the Isolation Test was examined as a measure of social separation stress (*Figure 27*). No differences between the groups were found in this parameter in any of the experiments and trials analyzed.

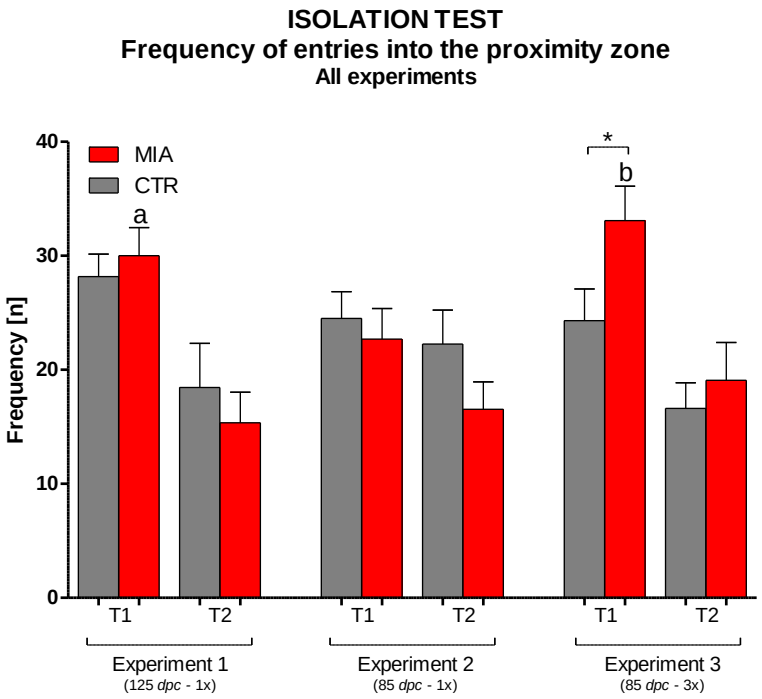

Figure 28. **The frequency of entries into the proximity zone in the Isolation Test.** T1 indicates the first trial of the Isolation Test, with visual contact, while T2 indicates the trial without visual contact with the dam. "a" indicates the difference between T1 vs T2 in MIA lambs, $p<0.01$; "b" indicates the difference between T1 vs T2 in MIA lambs, $p<0.01$; *$p<0.05$ and indicates the difference between CTR and MIA animals. The differences between groups were evaluated by unpaired t-test, while differences between the trials were analyzed with One-Way ANOVA followed by Tukey's post-hoc test.

As depicted in *Figure 28*, the frequency of entries into the proximity zone tends to decrease in the trial without visual contact with the dam (T2), compared to the trial in which lamb maintains the visual contact with the mother (T1) in all experimental groups and among all experiments. The differences between T1 and T2 in the frequency of entrance into the proximity zone were statistically significant for the MIA lambs in experiment 1 ($p<0.01$) and experiment 3 ($p<0.01$), while CTR lambs did not show such a significant tendency. Moreover, MIA lambs from experiment 3 displayed significantly more entries into the proximity zone on the 1st trial of the Isolation Test without having visual contact with the mother, compared to CTR ($p<0.05$).

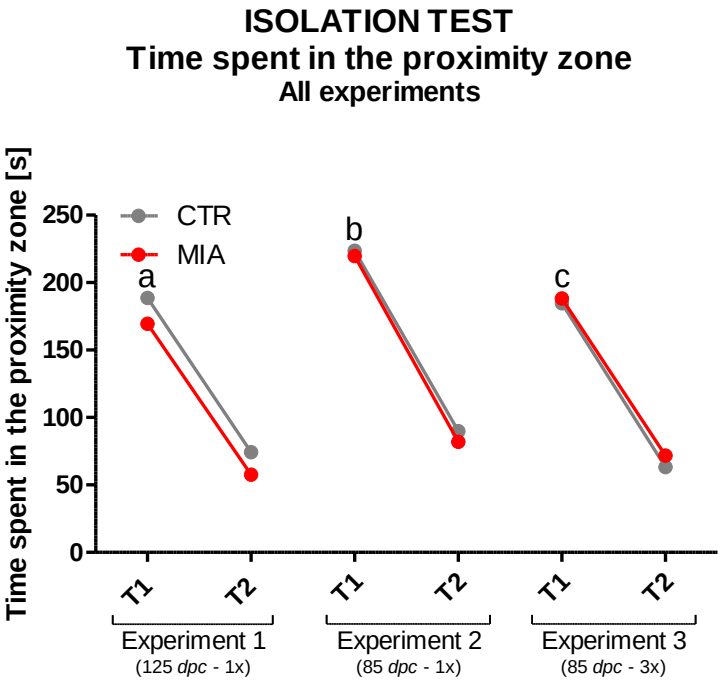

Figure 29. **The time spent by the lamb in the proximity zone in all three experiments.** T1 indicates the first trial of the Isolation Test, with visual contact, while T2 indicates the trial without visual contact with the dam. "a" indicates the difference between T1 vs T2 in MIA and CTR lambs, $p<0.001$; "b" indicates the difference between T1 vs T2 in MIA and CTR lambs, $p<0.001$; "c" indicates the difference between T1 vs T2 in MIA and CTR lambs, $p<0.001$. No differences between experimental groups were observed. The differences between the trials were evaluated with the One-Way ANOVA followed by Tukey's post-test, while differences between the groups were checked with an unpaired t-test.

Lambs from all experimental groups in all three experiments spent significantly more time in the proximity zone (*Figure 29*) in the trial when they had visual contact with the ewe, compared with trials when the view was prevented by the opaque panel ($p<0.001$).

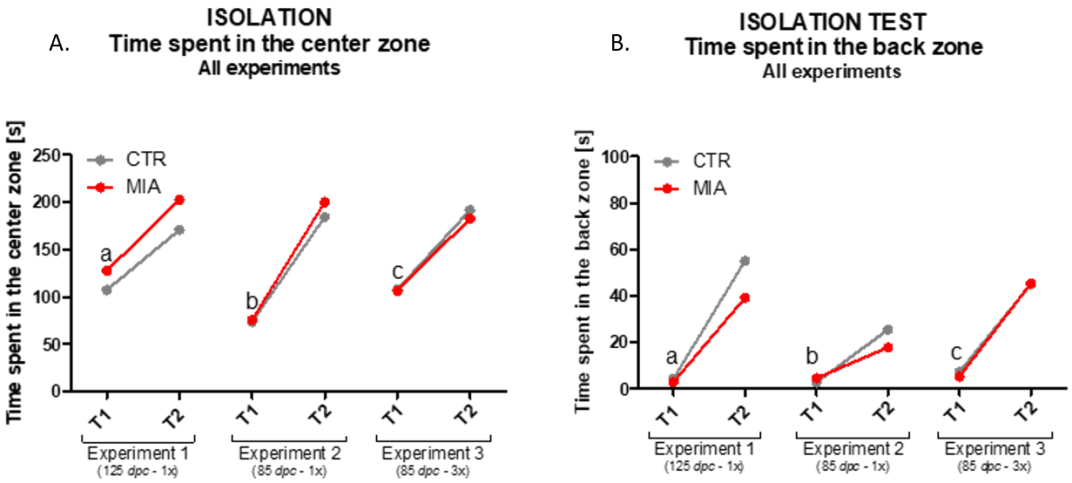

Figure 30. **The time spent by the lamb in the center and the back zone during the Isolation Test.** T1 indicates the first trial of the Isolation Test, with visual contact, while T2 indicates the trial without visual contact with the dam. **A.** Time spent in the center zone by lambs from MIA and CTR groups. The letter "a" indicates a significant difference between T1 vs T2 in MIA and CTR lambs, $p<0.05$; "b" indicates the difference between T1 vs T2 in MIA and CTR lambs, $p<0.001$; "c" indicates the difference between T1 vs T2 in MIA and CTR lambs, $p<0.001$. No differences between experimental groups were observed. The differences between the trials were evaluated with the One-Way ANOVA followed by Tukey's post-test, while differences between the groups were checked with an unpaired t-test. **B.** Time spent in the back zone by lambs from MIA and CTR groups. "a" indicates the difference between T1 vs T2 in CTR lambs, $p<0.01$; "b" indicates the difference between T1 vs T2 in CTR lambs, $p<0.05$; "c" indicates the difference between T1 vs T2 in MIA and CTR lambs, $p<0.05$. No differences between experimental groups were observed. The differences between the trials were evaluated with the One-Way ANOVA followed by Tukey's post-test, while differences between the groups were checked with an unpaired t-test.

Consequently, all tested lambs spent significantly more time in the center zone (*Figure 30 A*) in the 2nd trial of the test, when visual contact with the ewe was interdicted, compared to trials in which they could see the mother ($p<0.05$). Seemingly, the time spent in the back zone of the arena (*Figure 30 B*) on the trial in which lambs could maintain visual contact with the mother (T1) is close to 0 in all experimental groups. In all experiments, CTR lambs displayed significantly more time in the back zone in the trials without visual contact (T2), compared to T1 ($p<0.05$), while MIA lambs displayed such significant change only in experiment 3 ($p<0.05$). No statistically significant differences between treatment groups were observed concerning time spent and frequencies of entrance in the back and central zones.

5.6. Spatial learning, memory, and inhibitory control of the lambs

Learning difficulties and problems with memory are common symptoms of many psychiatric diseases, including neurodevelopmental disorders. To evaluate spatial learning and memory skills, as well as inhibitory control of visual stimuli in the

lambs, the time spent to complete the tasks in the V-detour and the T-maze tests were measured.

In the habit acquisition trials of the 1st V-detour test day (*Figure 31 A*), all experimental groups displayed a tendency toward a decreased time spent to solve the task already on the second trial (T2). The differences in the time to complete the learning task between T1 vs T3 are significant in all the experiments, for both CTR and MIA lambs ($p<0.01$). However, while controls from experiment 2 solved the maze significantly faster already in the second trial ($p<0.05$), for MIA lambs this improvement was not significant. Conversely, in the 3rd experiment, MIA lambs solved the task significantly faster on T2 compared to T1 ($p<0.01$), while CTR lambs did not show such a significant difference. Concerning the first three trials of the first day, there were no significant differences between groups in the time spent to solve the V-detour test, in experiments 1 and 2, whereas, in experiment 3, MIA lambs spent significantly less time solving the task in T2, compared to controls ($p<0.05$). In the habit acquisition trials of the 2nd day of the test (*Figure 31 B*), when lambs were again subjected to the V-detour to test memory skills, no differences between trials were observed. Interestingly, in experiment 1, CTR lambs spent significantly less time solving the task in all trials (T4, T5, and T6), compared to MIA ($p<0.05$). The same trend was observed in experiment 2, but not in experiment 3.

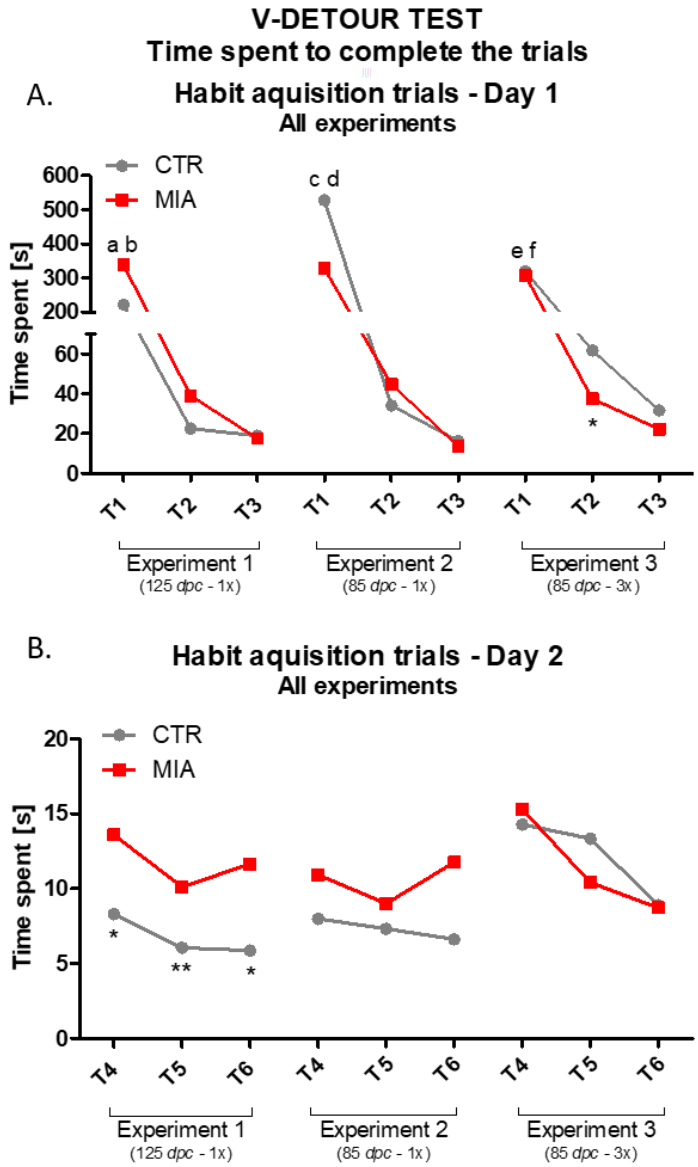

Figure 31. **Time spent to complete the task in the V-detour Test.** T1 – T6 indicates the consecutive trials on the 1st and 2nd day of the V-detour Test. **A.** The latency to complete the task in the habit acquisition trials on the 1st day of the test. "a" indicates the difference between T1 vs T3 in MIA group, $p<0.001$; "b" indicates the difference between the T1 vs T3 in CTR lambs, $p<0.01$; "c" indicates the difference between T1 vs T3 in MIA group, $p<0.001$; "d" indicates the difference between T1 vs T2 and T1 vs T3 in the CTR group, $p<0.05$; "e" indicates the difference between T1 vs T2 and T1 vs T3 in MIA lambs, $p<0.01$; "f" indicates the difference between T1 vs T3 in the CTR lambs, $p<0.001$. $p<0.05$ and indicates the difference between CTR and MIA lambs. **B.** The latency to complete the task in the habit acquisition trial on the 2nd day of the test. No differences were found in comparing trials. $^{**}p<0.01$, $^{*}p<0.05$ and indicates the differences between CTR and MIA lambs. The differences between the trials were evaluated with Kruskal-Wallis Test, followed by Dunn's post-test, while differences between groups were evaluated with the Mann-Whitney test.

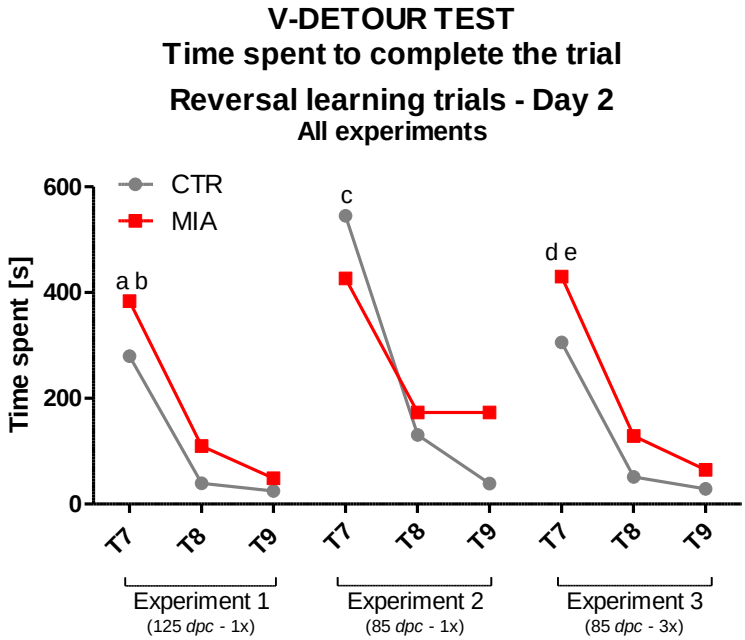

Figure 32. **The latency to complete the reversal learning task on the 2nd day of the V-detour test.** T7 – T9 indicate the consecutive trials on the 2nd day of the V-detour Test. "a" indicates the difference between T7 vs T9 in MIA lambs, $p<0.05$; "b" indicates the differences between T7 vs T8 and T7 vs T9 in CTR, $p<0.01$; "c" indicates the difference between T7 vs T8 and T7 vs T9 in CTR group, $p<0.05$; "d" indicates the difference between the T7 vs T9 in MIA group, $p<0.001$; "e" indicates the difference between T7 vs T8 and T7 vs T9 in CTR, $p<0.01$.

In the reversal learning trials (*Figure 32*), all lambs spend significantly more time solving the task on the 1st trial after blocking the previously learned path (T7), compared to the consecutive ones. In experiment 1 and experiment 3, MIA lambs display significantly lower times in the last T9 trial compared to T7 ($p<0.05$), indicating successful reversal learning in MIA lambs. On the other hand, lambs from the CTR group from all the experiments displayed such improvement already in the second reversal learning trial (T8) ($p<0.05$), indicating that they can reverse the learned habit faster than MIA lambs.

In experiment 1 all lambs managed to accomplish the V-detour test and change the acquired habit to reach the dam. In the second experiment, 3 male lambs of the MIA group didn't manage to accomplish the reversal learning ($p=0.07$, comparing only CTR vs. MIA male lambs with the Chi-Square test). In experiment 3 only 1 female lamb didn't successfully pass the test and no significant differences between groups were observed.

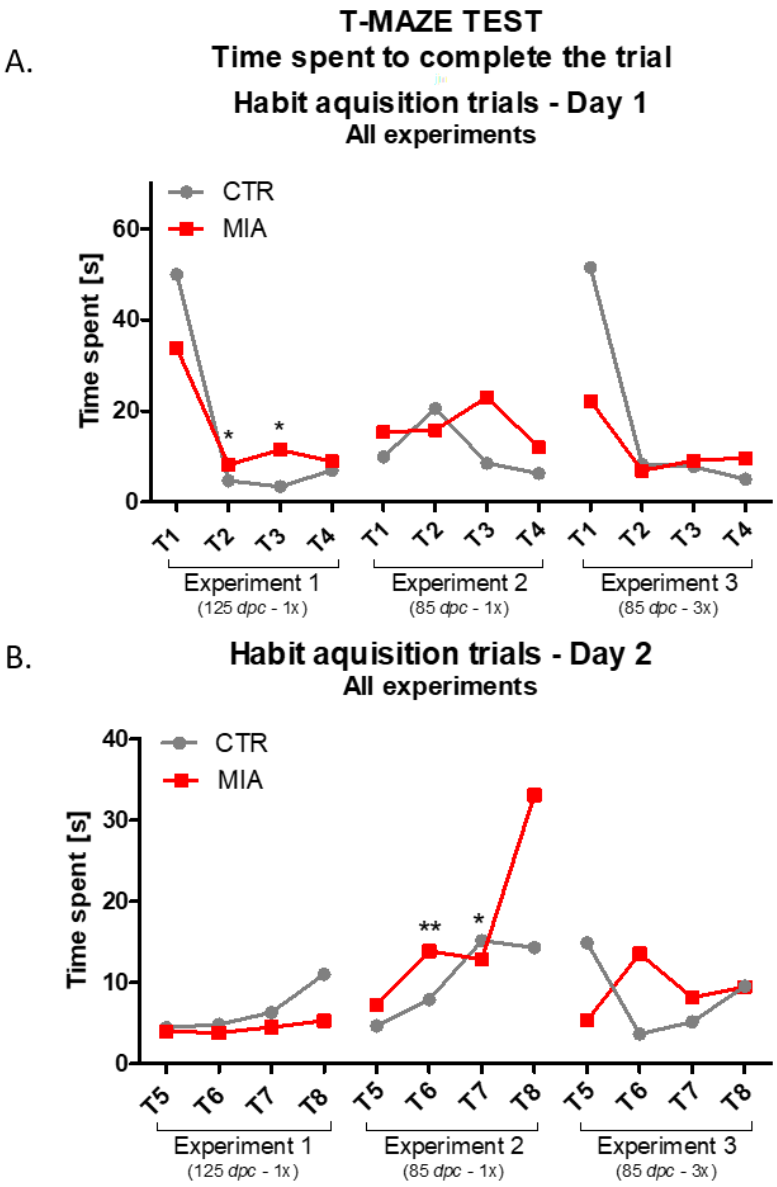

Figure 33. **Time spent to complete the task in the habit acquisition trial in the T-maze test.** T1 – T8 indicates the consecutive trials on the 1st and the 2nd day of the T-maze Test. **A.** The latency to complete the task in the habit acquisition trials on the 1st day of the test. **p*<0.05 and indicates the differences between CTR and MIA lambs. **B.** The latency to complete the task in the habit acquisition trial on the 2nd day of the test. ***p*<0.01 indicates the difference between CTR T6 and MIA T6; **p*<0.05 indicates the difference between CTR T7 and MIA T7. The differences between the groups were evaluated with the Mann-Whitney Test, while the differences between the trials were analysed with Kruskal-Wallis Test, followed by Dunns' post-test.

In the habit acquisition trials on T-maze test day 1 (*Figure 33 A*), no unique trend can be distinguished among the three experiments, as compared with the clear learning curve observed in the V-detour test. In the T-maze's T2 and T3 of experiment 1, lambs from the MIA group spent significantly more time completing the task than lambs from the CTR group. This result indicates a slower learning acquisition of MIA lambs towards the target zone in which they can reunite with their mother. On the 2nd day of

the test, in the habit acquisition trials (*Figure 33 B*), lambs from the CTR group from experiment 2 show a significantly lower time in solving the maze at T6 ($p<0.01$) and significantly higher time to complete the T7 ($p<0.05$) compared to MIA lambs.

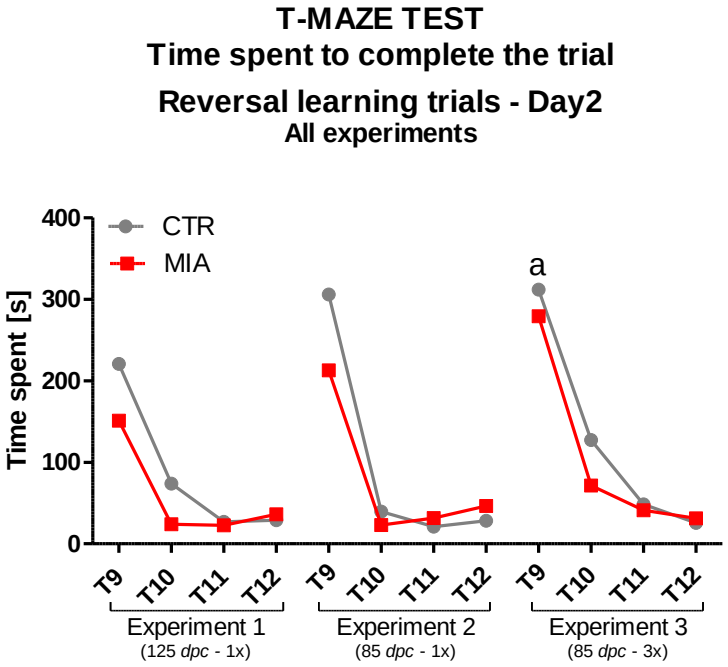

Figure 34. **Time spent to complete the reversal learning task on the 2nd day of the T-maze test.** "a" indicates the difference between T9 vs T11 and T9 vs T12 in MIA lambs, $p<0.05$; No differences between the groups were found. The differences between the groups were evaluated with the Mann-Whitney Test, while the differences between the trials were analysed with Kruskal-Wallis Test, with Dunns' posttest.

In reversal learning (*Figure 34*) lambs need more time to solve the task in the 1st trial (T9), as compared to previous habit acquisition and consecutive trials in all experiments. Moreover, in experiment 3, MIA lambs show a significantly lower time to accomplish the task on T11 and T12, compared to the first reversal trial ($p<0.05$). No differences in the latency to finish the task between the groups were found.

The results obtained from V-detour and T-maze tests indicate that the V-detour is a more robust tool to examine the learning skills and inhibitory control of the lambs. Thus, to have better insight into the relationship between maternal immune activation and offspring behavior, we conducted a linear regression analysis focusing on the measurements collected in the V-detour test. The behavioral outcomes examined included the improvement rate, calculated as a percentage of improvement in the consecutive trials, and time spent to solve the maze on the second day of the test, trials 4-9.

Table 3. Linear regression analyses of the relationship between LPS treatment and the behavioral outcomes of the V-detour test.

"Behavioral outcome"~Treatment[SAL] + Sex[M] + Temperature[continuous]			
Behavioral outcome	**Estimate [a]**	**Standard error**	**P-value [b]**
Improvement rate T1-T2-T3	37.47	29.09	0.202
Improvement rate T3-T4	28.05	13.46	0.040 *
Improvement rate T4-T6	57.61	21.18	0.008 **
Improvement rate T7-T8	11.93	10.08	0.240
Improvement rate T8-T9	2.15	23.75	0.929
Time spent to complete the trial on T4	-1.91	1.81	0.295
Time spent to complete the trial on T5	-0.48	1.74	0.783
Time spent to complete the trial on T6	-5.88	1.97	0.003 **
Time spent to complete the trial on T7	-141.93	81.58	0.086 •
Time spent to complete the trial on T8	-96.83	55.04	0.082 •
Time spent to complete the trial on T9	-110.77	38.62	0.005 **

[a] indicates the estimated value of the coefficient.
[b] indicates the p-value of the obtained results; the results were considered significant for p-value<0.05. *p<0.05, **p<0.01, • p<0.1.

The results obtained from linear regression analyses (*Table 3*) indicate a significant effect of maternal LPS treatment on the improvement rate between the last trial of habit acquisition on 1st day of the V-detour test (T3) and the first habit acquisition trial on the 2nd day of the test (T4), as well as between T4 and the last trial of habit acquisition trial on the 2nd day of the test (T6). This effect is still significant after considering the lamb sex and the maternal body temperature change as

confounding factors/effect modifiers. No effect of the treatment was found on the improvement rate during reversal learning trials. Overall, the linear model analyses indicate that MIA influences the ability of lambs to recall a learned behavior in the context of both short- and long-term memory. No effect of sex was observed in any of the analyzed models.

5.7. Perseveration and insistence on the sameness of the lambs

Perseveration is defined as an uncontrolled repetition of behavior that occurs despite the absence or cessation of the stimulus that evoked it. Perseveration, the insistence on sameness, and inflexible adherence to routines are key diagnostic symptoms of some neurodevelopmental disorders, including autism spectrum disorder. To evaluate the occurrence of such repetitive behavior and perseveration, the error rate was measured as the frequency of entrances into correct/wrong zones of the V-detour and T-maze Tests arenas. In the V-detour, the center of the V-shaped obstacle and the closed side of the obstacle in reversal learning trials were considered as "perseveration" zones.

As shown in *Figure 35 A*, all lambs in all three experiments tend to return fewer times to the center of the V-shaped obstacle on the 2nd (T2) and the 3rd (T3) trial of habit acquisition on V-detour test day 1, compared to the 1st trial (T1), although the differences are not significant. On the habit acquisition trials on the 2nd day of the test (*Figure 35 B*), there were no significant changes in the frequency of return into the center of the "V" between trials, nor between groups.

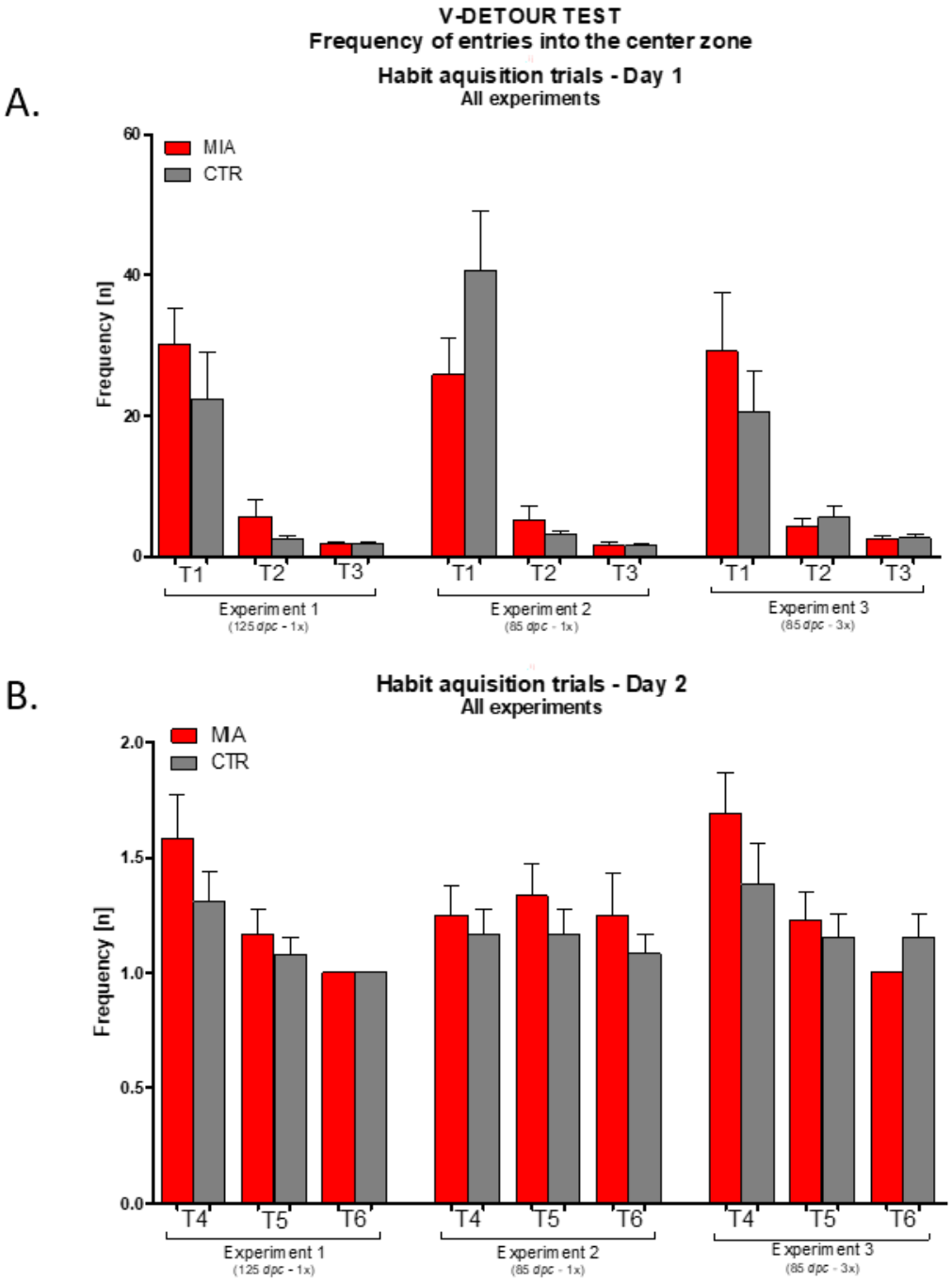

Figure 35. **Frequency of the entries into the center of the V-shaped obstacle in the V-detour Test.** T1 – T6 indicates the consecutive trials on the 1st and 2nd day of the V-detour Test. **A.** Frequency of entries into the center zone of the V-shaped obstacle during the trials based on habit acquisition during the 1st day of the test. **B.** The frequency of entries into the center of the V-shaped obstacle during habit acquisition trials on the 2nd day of the test.

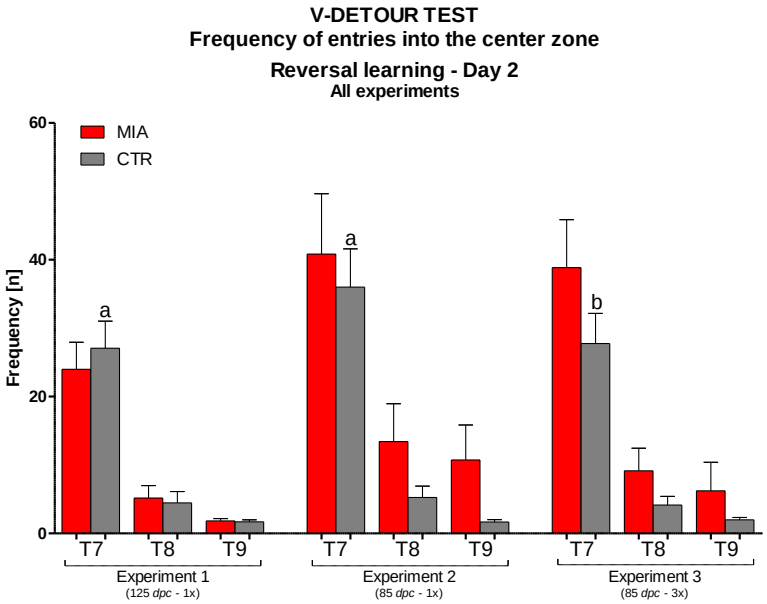

Figure 36. **Frequency of the entries into the center of the V-shaped obstacle in the reversal learning trials during the V-detour Test.** T7 – T9 indicate the consecutive trials on the 2nd day of the V-detour Test. The differences were evaluated by the Kruskal-Wallis test. "a" indicates the difference between T7 vs T9 in CTR lambs (p<0.01); "b" indicates the difference between T7 vs T9 in CTR lambs (p<0.05).

On the reversal learning trials on the V-detour test, day 2 (*Figure 36*), lambs tend to display a higher frequency of entries into the center of the V-shaped obstacle on the first reversal learning trial T7, compared to previous and next trials. However, while CTR lambs showed a significant drop in this perseverative behavior after two trials, comparing T7 with T9 (p<0.05), MIA lambs did not display such significant improvement and maintained higher frequencies of perseveration until the last trial of the test.

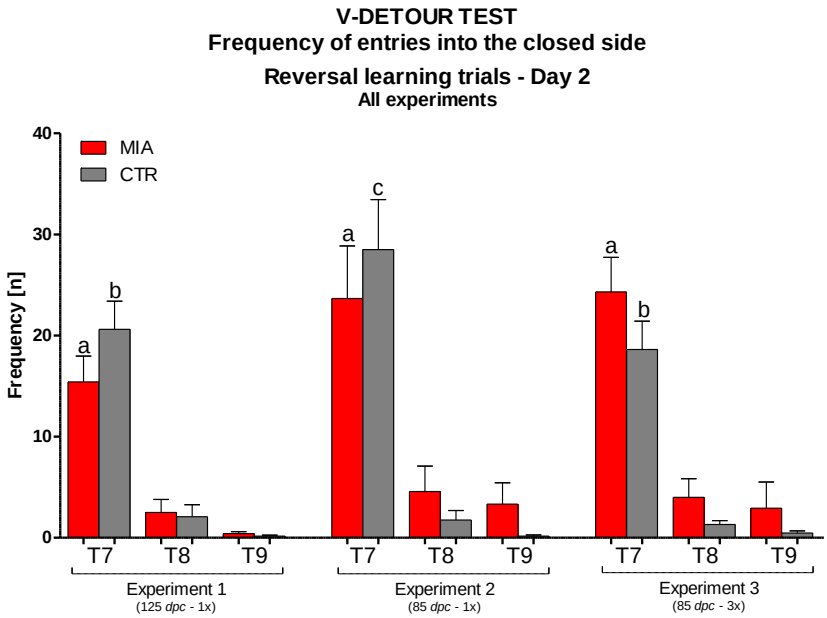

Figure 37. **Frequency of the entries into the closed side of the V-shaped obstacle in the reversal learning of the V-detour Test.** T7 – T9 indicate the consecutive trials on the 2nd day of the V-detour Test. The statistical significance of the differences was evaluated with Kruskal-Wallis Test. **"**a" indicates the difference between T7 vs T9 in MIA lambs (p<0.05); "b" indicates the difference comparing T7 vs T9 in CTR lambs (p<0.001); "c" indicates the difference between T7 vs T8 and T7 vs T9 in CTR lambs (p<0.001).

As depicted in *Figure 37*, lambs from the MIA group showed a significant decrease in the frequency of entries into the closed side between the first (T7) and the last (T9) trials, in all three experiments (p<0.05). The same trend was observed in the lambs of the CTR group (p<0.001). Moreover, CTR lambs from experiment 2, showed significantly fewer entries into the closed side already in the T8, compared to T7 (p<0.01), while MIA lambs did not show such fast improvement and kept perseverating until the last trial.

In the T-maze test, lambs were forced to change the learned path to find their mothers in the reversal learning trials, and the number of returns to the previously learned path was calculated as a measure of perseverance. All lambs displayed a higher frequency of entries into the proximity zone that did not contain the ewe (*Figure 38*).

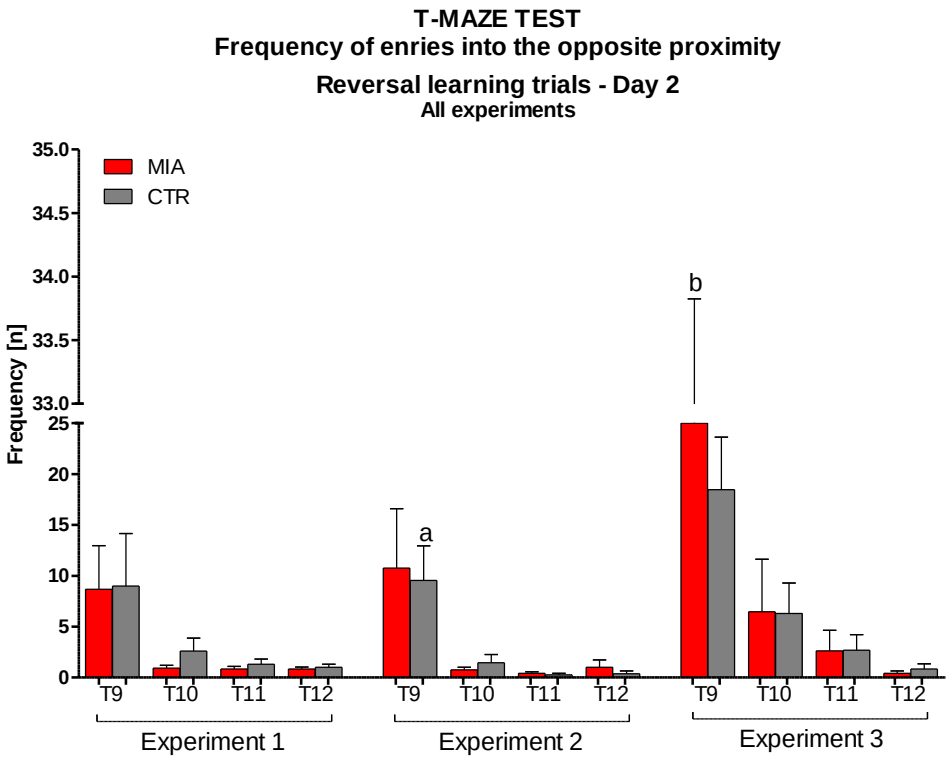

Figure 38. **Frequency of the entries into the proximity zone opposite to the one adjacent to the position of the dam in the reversal learning trials of the T-maze test.** T9 – T12 indicate the consecutive trials on the 2nd day of the T-maze Test. **"**a" indicates the difference between T9 vs T11 and T9 vs T12 in CTR lambs (p<0.05); "b" indicates the difference between T9 vs T11 and T9 vs T12 in MIA lambs (p<0.05). No differences between the groups were found. The differences between the trials were evaluated by the Kruskal-Wallis test followed by Dunns' post-test, while the differences between groups were calculated with the Mann-Whitney test.

Performed analyses revealed that the V-detour test allowed us to obtain more robust results concerning the perseverative behaviors and insistence on sameness of the lambs, as compared to the T-maze test. Consequently, the relationship between the treatment and the specific behavioral parameters analyzed in the V-detour test was further examined by linear regression analyses considering only selected parameters of the V-detour test. In particular, the models examined the association between the treatment and the frequency of entries into the closed side displayed by the lamb during reversal learning trials (T7-T9).

The obtained results (*Table 4*) showed significantly lower frequency in CTR lambs on the last two trials of reversal learning – T8 (p=0.042) and T9 (p=0.03). No effect of the treatment was found on the frequency of entries into the closed side on the 1st trial after closing one side of the obstacle (T7).

Table 4. Linear regression analyses of the relationship between the treatment and frequency of entries into the closed side in reversal learning trials of the V-detour test.

"Behavioral outcome"~Treatment [SAL] + Sex [M] + Temperature [continuous]			
Behavioral outcome	**Estimate [a]**	**Standard error**	**P-value [b]**
Frequency of entries into the closed side on T7	-5.50	3.98	0.171
Frequency of entries into the closed side on T8	-3.23	1.56	0.042 *
Frequency of entries into the closed side on T9	-3.25	1.47	0.030 *

[a] indicates the estimated value of the coefficient.
[b] indicates the p-value of the obtained results; the results were considered significant for p-value<0.05. *p<0.05.

5.8. Response to isolation-induced stress

To evaluate the possible influence of MIA on the stress response, lambs were subjected to short 5 minutes of social isolation when aged 60-90 days. The rectal temperature and the cortisol plasma levels were evaluated as measures of the stress response.

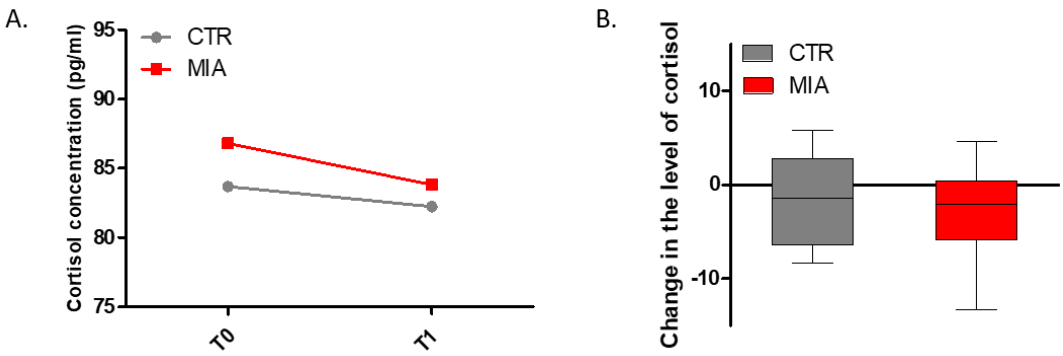

Figure 39. Protein levels of cortisol in the plasma of the lambs just before and 25 minutes after short isolation. A. The absolute concentration of cortisol in the plasma of the lambs. T0 indicates the time before the isolation, while T1 indicates the time point 25 min after. No significant differences between the groups and time points were observed. **B.** The changes in the level of cortisol in the plasma of the lambs after isolation. No significant differences between the groups were observed.

As indicated in *Figure 39* A. the absolute concentration of cortisol in the plasma of the lambs doesn't show significant changes 25 minutes after the short isolation. The changes in the cortisol level after the Isolation Test didn't display any differences between MIA and CTR lambs.

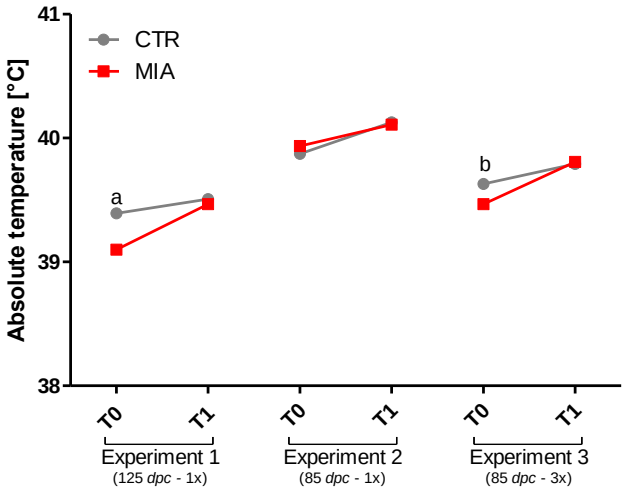

Figure 40. **The absolute temperature of the lambs just before and 25 minutes after short isolation.** T0 indicates the time before the isolation, while T1 indicates the time point 25 min after. The differences were evaluated with Unpaired T-Test. "a" indicates the difference between T0 vs T1 in MIA lambs, $p<0.05$; "b" indicates the difference between T0 vs T1 in the MIA group, $p<0.01$.

The absolute temperature of the lambs (*Figure 40*) showed a significant increase after isolation in MIA lambs of experiment 1 ($p<0.05$) and experiment 3 ($p<0.01$), while in experiment 2 there were no significant differences between time points. On the other hand, lambs of the CTR group did not display such a rise in body temperature after isolation, in any of the experiments.

5.9. Association of maternal cytokine levels after LPS administration with the behavior of the lamb in the V-detour test

To evaluate the relationship between the maternal immune response to LPS/SAL administration during pregnancy and the behavioral outcome of the lambs during V-detour test, the linear regression analyses were performed. In these models, we analyzed the association between the levels of maternal proinflammatory cytokines (IL-6 or TNF-alpha) after LPS administrations with the lamb V-detour improvement rate (calculated as a percentage of improvement in the time spent to completely detour the maze) and the frequency of lambs' entries into the center of the V-shaped obstacle. The results (Table 5) indicate that the level of IL-6 in the ewes exposed to LPS is significantly associated with the offspring behavior. Post-LPS maternal IL-6 levels were negatively associated with the improvement rate in the habit acquisition trials between the 1st (T4) and the 2nd (T5) habit acquisition trials on the 2nd day of the V-detour test ($p=0.004$ and $p=0.024$, respectively). No relationship was found comparing

the level of IL-6 in any of the time points with the improvement rate between other trials than T4 and T5. In the present analyses, the level of IL-6 at 4h after the 1st injection was linked to the frequency of entries into the center of the V-shaped obstacle on T1. Moreover, the level of IL-6 after the 2nd injection was shown to be associated with the frequency of entries into the center of the "V" on T2 (p=0.026). Intriguingly, a similar trend was shown for IL-6 levels post 1st injection, although the results remain only suggestive (p=0.092). The level of TNF-alpha in 4h after the 2nd LPS injection was associated with the improvement rate between T7 vs T8 (p=0.017) and the frequency of entries into the center of the "V" on T8 (p=0.015). No other behavioral outcome measured in the V-detour test displayed an association with the levels of TNF-alpha of the ewes after the immune challenge during pregnancy.

Table 5. Linear regression analyses of the relationship between the behavioral outcome of the lambs in V-detour test and the immune response of the ewe to LPS/SAL administration.

"Behavioral outcome"~ Concentration of IL-6/TNF-alpha at T0 + Concentration of IL-6/TNF-alpha at T1/T3/T5 + Temperature																		
Behavioral outcome	**IL-6**									**TNF-alpha**								
	4h after the 1st injection			**4h after the 2nd injection**			**4h after the 3rd injection**			**4h after the 1st injection**			**4h after the 2nd injection**			**4h after the 3rd injection**		
	Estimate[a]	**Standard error**	**p-value[b]**	**Estimate[a]**	**Standard error**	**p-value[b]**	**Estimate[a]**	**Standard error**	**p-value[b]**	**Estimate[a]**	**Standard error**	**p-value[b]**	**Estimate[a]**	**Standard error**	**p-value[b]**	**Estimate[a]**	**Standard error**	**p-value[b]**
Improvement rate T1-T2-T3	0.028	0.339	0.935	0.471	0.428	0.284	-0.581	0.515	0.271	-0.007	0.035	0.832	-0.044	0.041	0.300	-0.060	0.059	0.315
Improvement rate T3-T4	-0.282	0.401	0.490	-0.415	0.518	0.432	0.510	0.624	0.423	-0.037	0.043	0.397	0.003	0.052	0.955	-0.115	0.069	0.112
Improvement rate T4-T5	0.923	0.514	0.087•	1.891	0.582	0.004 **	1.761	0.724	0.024 *	0.097	0.059	0.117	-0.009	0.075	0.906	0.125	0.101	0.230
Improvement rate T5-T6	-0.060	0.266	0.822	-0.269	0.339	0.436	-0.531	0.378	0.175	0.030	0.028	0.293	0.024	0.034	0.484	-0.023	0.047	0.628
Improvement rate T7-T8	0.262	0.281	0.361	0.343	0.363	0.355	0.095	0.425	0.824	0.021	0.032	0.518	-0.088	0.034	0.017 *	-0.052	0.054	0.343
Improvement rate T8-T9	0.398	0.904	0.664	1.573	1.123	0.176	1.232	1.322	0.362	-0.071	0.102	0.492	0.136	0.118	0.265	0.086	0.173	0.622
Frequency of entries into the center of the „V" on T1	-0.571	0.233	0.023 *	-0.281	0.336	0.411	-0.105	0.391	0.790	-0.110	0.029	0.712	-0.020	0.034	0.570	-0.059	0.047	0.228
Frequency of entries into the center of the „V" on T2	0.086	0.049	0.092•	0.145	0.060	0.026 *	0.062	0.077	0.425	0.004	0.006	0.546	-0.004	0.007	0.564	-0.001	0.010	0.951
Frequency of entries into the center of the „V" on T7	-0.099	0.193	0.613	0.388	0.237	0.116	0.219	0.285	0.450	-0.007	0.022	0.756	0.011	0.025	0.665	-0.011	0.035	0.757
Frequency of entries into the center of the „V" on T8	-0.105	0.092	0.268	0.134	0.120	0.276	0.001	0.142	0.993	0.009	0.010	0.349	0.028	0.010	0.015 *	0.001	0.017	0.944

[a] indicates the estimated value of the coefficient;
[b] indicates the p-value of the obtained results; the results were considered significant for p-value<0.05. *p<0.05, **p<0.01, • p<0.1.

6. DISCUSSION

In recent decades, the prevalence of NDDs has been drastically growing, and with that, also their socio-economic impact [46]. A number of preventive and therapeutic strategies for NDDs have been proposed, although their action is mostly limited to reducing the severity of symptoms and there is not a definitive cure for these conditions. Therefore, there is a pressing necessity to develop new, robust tools which will help to investigate the still unclear etiology and pathogenesis of NDDs.

Existing epidemiological evidence indicates that maternal infections during pregnancy increase the risk of bearing children affected by NDDs [41,125]. The results presented in this thesis support this association, lining up with other animal studies conducted in other model species [167] and suggesting that the activation of the ewes' immune system during pregnancy prompts fetal reprogramming, which leads to long-term subtle effects on the lamb behavioral phenotype. Some of those differences in the postnatal lambs' behavior resemble the symptoms of human neurodevelopmental disorders. Therefore, this study adds important observations to the current knowledge about MIA in the ovine model.

The physiological and inflammatory response of ewes to LPS administration during pregnancy

Physiologically, an infection leads to numerous changes in the organisms of all mammals. Similar responses have been confirmed in the present ovine model of prenatal infection induced by acute or chronic LPS-endotoxin administration. Based on the results obtained in experiments 1 and 2, we concluded that the time point at 4 h after the injection is the most sensitive to the LPS challenge in terms of temperature rise. This observation led to some changes in the experiment 3 - health monitoring and blood sampling were conducted only at two time points, 4 h and 24 h after LPS/SAL administration. That allowed to reduce the number of manipulations and decrease the stress for the animal. In experiment 3, when LPS was administered chronically with increasing doses for 3 consecutive days, the body temperature of the ewes was significantly higher at 4 h after each injection in MIA compared to controls. Despite the low dose of LPS the rise in the temperature was higher in the first injection than in consecutive two increasing doses. This may be due to the habituation of the organism and increasing tolerance to LPS [207]. LPS administration also leads to a significant rise in the heartbeat and respiratory rate when injected as a single dose in mid-pregnancy

(85 DPC). In the ewes from experiment 3, the heartbeat of all animals just before the 1st injection was at similarly high levels when comparing MIA with CTR. The high level of the heartbeat at time zero could be due to the stress elicited by novelty related to the first restrain of the animals, which get later habituated to the manipulations. Moreover, the significant rise of the heartbeat in MIA ewes of experiment 3 was observed only after the 3rd injection. This may indicate that during previous injections, the dose of LPS (0.4 and 0.8 μg/kg body weight mass) was not high enough to elicit the changes in the heartbeat, while in the 3rd injection, the dose (1.2 μg/kg body weight mass) led to a significant rise of the heartbeat. This is consistent with the significant rise of the heartbeat in MIA animals injected with a single dose of LPS during mid-pregnancy (experiment 2) and indicates that the rise of the heartbeat may be dose dependent. Conversely, the respiratory rate displays a significantly higher rise in MIA ewes from experiment 3 only after the first injection. In experiment 2 the same tendency occurs in MIA ewes compared to CTR, indicating that the respiratory rate rises due to the first contact with the immunogen, and further injection doesn't elicit any changes in this parameter. The raise in the heartbeat and respiratory rate in response to LPS administration has been previously reported in other species [208]. In the study conducted by scientists from Comenius University in Bratislava on rats, LPS-induced endotoxemia elicited a significant continuous increase in the heart rate. Moreover, animals subjected to LPS injection displayed a significant increase in respiratory rate and elevated body temperature.

While the above-mentioned results indicate that MIA elicits measurable changes in physiological parameters (body temperature, respiratory rate and heartbeat), the analyses of the level of the pro-inflammatory cytokines delivered a deeper insight into the molecular immune response of the pregnant ewe to LPS. To evaluate the inflammatory response of the pregnant ewes, the plasma levels of pro-inflammatory cytokines IL-6 and TNF-alpha were measured after chronic LPS administration. LPS has been well-documented as evoking changes in the levels of pro-inflammatory cytokines in sheep, such as IL-6 and TNF-alpha [209,210]. The study performed by Kabaroff et al. [209] shows that the level of IL-6 in pregnant ewes rises significantly in response to LPS in each stage of gestation. Under our experimental paradigm, IL-6 was at a significantly higher level 4 hours after each LPS administration, compared to controls. This result is in alignment also with the study performed by McLure et al.

where the peak of the IL-6 level in pregnant sheep was 4 hours after the LPS administration.[210].

The rise in the level of IL-6 was significantly higher in MIA ewes after the 2nd and 3rd LPS administration, compared to controls. Moreover, a strong positive correlation between the changes in the rectal temperature of the ewe and the level of IL-6 was observed, indicating this cytokine as a good marker of immune activation in sheep. The level of TNF-alpha in MIA in pregnant sheep shows a significant drop after all 3 injections. Concerning that such trend doesn't occur in the CTR group, we can conclude that the LPS challenge leads to changes in the physiological level of TNF-alpha in the blood, although its trend along different time points didn't display any significant variation.

Overall, these results show that LPS administration causes an illness-like status in the pregnant ewes, manifested by the change of physiological parameters (body temperature, heartbeat, and respiratory rate), and shows that LPS induces immune activation in ewes both at mid and late pregnancy. Considering the molecular inflammatory response, the observed rise in IL-6 levels is of particular interest in the understanding of the mechanisms underlying MIA. Based on rodent studies, IL-6 is an important biomarker of MIA-induced fetal brain damage [211] and it is a key intermediary in behavioral and transcriptional alterations in offspring [139]. Another inflammatory marker worth considering in future studies is IL-17, which has been already linked to MIA effects on developing fetal mouse brain [140], as well as therapeutic targeting of T_H17 helper cells has been proposed to reduce the likelihood of ASD in the offspring[140]. The presented study also covered the attempts to evaluate the plasma levels of IL-17 of the ewes by immunoassays. However, there are no ELISA kits having high sensitivity for ovine IL-17 yet, thus we used a kit specific for the bovine cytokine. Recent reports validated the use of such kits for measuring IL-17 in sheep [212], showing that it is possible to detect native ovine IL-17A using and ELISA for bovine IL-17. Nevertheless, under our experimental conditions, we did not obtain sufficient reliability and repeatability, thus the results have not been presented in this thesis. Further work should consider better adjustments of the protocol. As indicated by the data, the level of TNF-alpha doesn't display such a clear trend as IL-6. No differences were observed in the levels of TNF-alpha between the time points in the CTR group. Some significant changes were displayed by MIA animals, nevertheless,

the influence of the treatment is not so strong as in the case of IL-6, thus we may conclude that TNF-alpha is not an optimal marker of inflammatory response in sheep.

The effect of LPS administration during sheep pregnancy on the general health and body weight of the lamb

The literature indicates that LPS challenges during pregnancy may lead to fetal loss [213], preterm birth [213,214] or respiratory problems in offspring [215]. Under our experimental paradigm, we did not observe any effect of LPS administration on these pregnancy outcomes. Moreover, MIA didn't influence the general health of the lambs in any of the experiments. The negative effect of the LPS on pregnancy outcomes and offspring postnatal health depends on the dose of endotoxin, thus the doses administered in our experiment were at the lowest, safe level, preventing severe pregnancy and neonatal complications. Nevertheless, we observed subtle effects of MIA on the lamb body weight. In the first experiment, when LPS was administered in a single dose (1.2 μg/kg b.w.m.) during late pregnancy, the MIA group displayed significantly lower postnatal body weight than CTR animals. The opposite trend was observed in experiment 2, in which MIA lambs showed a tendency towards a higher body weight mass compared to controls. In the literature, the effect of LPS on the body weight of the offspring has already been documented. It has been reported that LPS injection during mid-pregnancy in rats can lead to higher body weight mass [216], or even to obesity in the offspring of mice [207]. On the other hand, existing literature indicates that LPS administration during late pregnancy may lead to a decreased body weight of the rats' offspring in the post-weaning period [218]. Thus, the results obtained in the experiments described here are consistent with the data already documented in the literature. What is worth to note, is that the lambs' body weight gain between 20 and 60 days differed significantly between experiment 1 and the two next experiments, with a more consistent increment in experiment 1 with respect to the other two experiments. Since experiments were performed in different breeding seasons it is possible that modest differences in feeding among the different experiments may have occurred. Considering this and the opposite effect of prenatal LPS on lambs' growth rate observed by comparing different breeding seasons, we speculate that MIA and pre- and postnatal diet may interact to influence the lambs body weight outcomes. Examples of diet-MIA interactions are already reported in literature for other species [219–221], thus it is worth to consider testing this hypothesis also in the ovine model.

The effect of prenatal immune challenge on the behavioral outcome of the lambs

Behavioral symptoms are the main diagnostic criteria of neurodevelopmental disorders. Therefore, in our experiments, lambs obtained from MIA or CTR mothers were subjected to a behavioral screening in order to evaluate those behavioral domains which are pathologic in individuals affected by NDDs. The tests aimed at examining the lamb behavioral responses in three different maze-arenas during social isolation followed by a reunion with the dam, which represents the reward. From the simple Isolation Test to the V-detour and T-maze tests, the lamb is being exposed to increasingly complex spatial navigation tasks, which allowed to measure spatial learning abilities, inhibitory control, memory, and perseveration. Established tests may serve not only to evaluate behaviors specific to NDDs but also to examine learning and cognitive skills, thus modeling other psychiatric diseases.

Isolation Test

The first behavioral assay performed when lambs were 20 days old was the Isolation Test. The measured parameters included the number of jumps, the frequency of entries into the proximity zone as well as the time spent in each of the zones. The number of jumps and the frequency of entries into the proximity zone during maternal separation were examined as a measure of separation-induced social stress and social attachment, as reported previously [288]. No differences in the number of jumps between the groups were observed in any of the experiments. On the other hand, the results obtained from experiment 3 indicate that chronic LPS administration may influence the stress responses of the lambs to maternal separation, especially when they are being isolated for the first time. Moreover, in experiments 1 and 3, lambs from the MIA group display significantly more entries into the proximity zone on the 1st trial, compared to controls. This indicates that the mobility of MIA lambs around the proximity zone is influenced by visual contact with the ewe, while in CTR animals this trend was not observed.

All lambs spent less time and entered with less frequency into the proximity zone when visual contact with the dam was impeded (T2), compared to the trials where the ewe was visible (T1). The results indicate that the lamb has a significant preference to seek their mother in the back and central zones when visual contact is blocked.

Overall, our data indicates that LPS challenge during pregnancy doesn't influence the social attachment of the lamb, although when the administration is chronic, it may mildly affect the stress responses of the lambs to social isolation.

Detour test

Results from the V-detour test concerning habit acquisition trials of Day 1 indicate that all lambs learned quickly to solve the maze, and being able to learn inhibiting visual attractiveness to the mother and detour the obstacle going on the opposite side (inhibitory control). When lambs were exposed again to the same assay on day 2, MIA lambs in experiment 1 and experiment 2 tended to spend more time to solve the task in all 3 trials, although the differences were statistically significant only for experiment 1, and the same trend was not observed in experiment 3. These results show that all lambs learned and remembered how to detour the V-shaped obstacle, although the data indicate subtle effects of MIA on the ability to memorize the task and immediately apply the learned behavior after two days, especially when the LPS challenge was applied in the late gestation (experiment 1). In reversal learning trials, CTR lambs displayed significantly lower time to solve the task already on the 2nd trial after blocking the learned navigation path, showing a better improvement than MIA lambs in this phase of the test. This result let us conclude that MIA during pregnancy, regardless of the developmental time point of the endotoxin administration, may influence the cognitive flexibility of the lambs, as well as their resistance to change an acquired habit (in our case, detouring the obstacle from one side respect to the other).

Another parameter examined in the V-detour test was the frequency of entries into the center zone of the V-shaped obstacle, where the lambs return attempting to reach their mother through the semitransparent wire net. Therefore, the frequency of entries into the center zone of the V-detour can be considered as a measure of the inhibitory control of a visual stimulus, considering that the lamb must go in the opposite direction to finally complete the task [222]. In the habit acquisition trials on day 1 of the test, the number of entries into the center zone substantially decreased after the 1st trial, although the differences were not significant. This is consistent with the decrease observed in the time spent solving the task and indicates that all lambs are able to inhibit the impulse to follow the visual stimulus and move back, in order to reach the dam. In habit acquisition trials on day 2, all lambs from experiments 1 and 3 displayed fewer entries into the center of the "V" after the 1st trial, while lambs from

experiment 2 maintain low frequencies of return since the first trial of the second testing day. This indicates that after the first day of the test, the majority of lambs do not return to the center of the obstacle and they solve the task immediately, remembering the habit acquired on the previous day of the test. In the reversal learning trials, the frequency of entries into the center zone of the obstacle is high in the first trial and tends to decrease for all lambs while subsequent trials are performed. However, the differences between trials are significant only for CTR animals, and MIA lambs tend to return to the center of the "V" at higher rates during subsequent reversal trials. MIA lambs display the worst performance, both in terms of time spent to completely detour the obstacle and of perseverative attempts to reach the dam toward a straight path, manifested by returning to the center of the "V" (induced by the visual stimulus) rather than detouring it.

In reversal learning trials, the frequency of returns into the closed side (previously "chosen" side) is a measure of perseveration, which is often observed in individuals with ASD and is thought to be a measure of cognitive inflexibility [223]. Under our experimental paradigm, all lambs initially return to the closed gate following the path learned previously and keep returning to the same location at high rates, despite this behavior not being useful anymore to reunite with the dam. In further reversal learning trials, lambs displayed decreased frequencies of returns into the closed side compared to the 1st reversal learning trial, indicating that to obtain the reward, the lamb can switch toward a left-right spatial learning route and stop to repeat behavior which is not advantageous anymore. Moreover, in experiment 2, CTR lambs show a decrease in the number of entries into the closed side already in the 2nd trial, while this improvement was significant only in the third trial for MIA lambs. This shows that CTR lambs were able to change the learned habit faster than MIA lambs and indicates that MIA may affect this specific behavioral domain.

Intriguingly, in experiment 2, three male lambs from the MIA group did never solve the reversal learning task of the V-detour test indicating a lack of cognitive flexibility in these individuals and their resistance to change the habit. Moreover, it may suggest that the effects of MIA on the behavior of the offspring might be sex biased. However, the analyses performed with linear regression didn't confirm any influence of the sex of the animal on any behavioral outcome of the lamb observed in the V-detour test.

The results obtained from linear regression analyses indicate that CTR lambs display significant learning improvement in the habit acquisition phases of the test. Moreover, CTR lambs need significantly less time to complete the task on T6. These results indicate that MIA lambs display lower learning and memory skills compared to the CTR group. The analyses suggest that the effect of MIA on offsprings' cognitive flexibility, measured as the lamb's ability to change an acquired habit in the V-detour test, was strongly correlated with maternal inflammatory response to LPS, measured as the levels of inflammatory cytokines. This indicates that MIA lambs may be predisposed to develop perseverative behaviors, which is one of the symptoms characteristics for NDDs.

One of the symptoms observed in human NDDs, especially in ASD patients, is a decreased cognitive flexibility in reversal learning behavioral tasks [85,224,225]. Difficulties in reversal learning following maternal immune activation (MIA), have previously been observed in rodents [153,225–228]. Results obtained from reversal learning indicate that MIA during pregnancy affects the cognitive flexibility of the lambs, making them more resistant to changing the acquired habit. The linear regression analyses revealed also that MIA lambs display higher levels of perseveration in reversal learning. Moreover, the data concerning habit acquisition indicates that LPS administration during late pregnancy may influence the memory of the lambs, making them less efficient in memorizing the learned task. Behavioral evaluation of the lambs revealed that the V-detour test is a robust test to examine learning and memory, as well as cognitive flexibility and resistance to change the habit in the lambs. The behavioral changes observed in the V-detour test reflect some of the symptoms of human neurodevelopmental disorders, confirming the face validity of the presented model in the domains of cognitive functions, learning problems, and repetitive behaviors.

T-maze test

The third behavioral test performed on the lambs was the T-maze test. In this test, the inhibitory control of the lamb was tested in relation to acquired habit in the absence of the visual stimulus, although the lamb may follow the auditory cues (bleating of the mother). During habit acquisition trials on the first day of the T-maze test, MIA lambs from experiment 1, spent significantly more time to complete the trials in the 2nd and 3rd trials, compared to CTR. During the habit acquisition trials on day 2, all lambs solved the trials within less than 20 s. The obtained results indicate that lamb

memorizes the path leading to the mother, and solves the task immediately, without making any mistakes. During reversal learning trials, lambs tend to spend more time solving the first trial after changing the side where the mother is placed. This indicates that changing the acquired habit and finding a new path to reach the mother, despite the availability of auditory cues, is challenging for the lambs and demands more searching attempts. In experiment 3, MIA lambs spent significantly less time solving the task on the 3rd and 4th trial of the reversal learning, compared to the 1st reversal trial, which is not observed in CTR animals. This may indicate that MIA lambs show better improvement in reversal learning following the vocal cue, compared to controls.

The analyses of the frequency of entries into the wrong proximity zone (located on the opposite side respect to the dam) show that on the 1st reversal trial lambs tend to return more times to the wrong proximity zone than in further reversal trials. CTR lambs from experiment 2 display significantly fewer entries into the proximity zone in the 3rd and 4th trials of reversal learning compared to the 1st trial. The same trend was observed in the MIA lambs tested in experiment 3.

Overall, these results indicate that MIA lambs from experiment 3 display significant improvement in completing the trials, as well as the frequency of entries into the proximity zone opposite to the one adjacent to the mother, showing faster learning and lower lever of repetitive behaviors than CTR animals. Nevertheless, the T-maze test appeared to not be as efficient a tool as the V-detour test in evaluating repetitive behaviors and cognitive flexibility of the lambs.

The relationship between the maternal molecular immune response to LPS and the behavioral outcome of the lamb in the V-detour test

To our knowledge, few studies have focused up to date on evaluating the link between the intensity of the maternal immune response and the severity of the behavioral symptoms evoked by MIA in the offspring. Rodent studies showed that increased level of maternal TNF-alpha due to the prenatal immune challenge is associated with weight loss in pregnant females, which is an indication of sickness following MIA [158]. Moreover, the offspring of dams that experienced weight loss after MIA, displayed worse behavioral outcomes compared to the offspring of the dams that gained weight, suggesting a link between the changes in the TNF-alpha levels due to maternal immune challenge and the brain functioning and/or metabolism of the offspring. Another study showed no relationship between the post-treatment levels of

maternal systemic IL-6 and TNF-alpha levels and the behavior displayed by adult female offspring [203]. Moreover, it has been shown that maternal administration of exogenous IL-6 during pregnancy leads to deficits in pre-pulse inhibition (the inhibition of a startle response when the startling stimulus is immediately preceded by a smaller, non-startling stimulus of the same modality and is commonly observed in schizophrenia and autism) [139].

To evaluate the relationship between the changes in the maternal cytokine levels following MIA and the behavioral outcome of the lambs in the V-detour test, the linear regression models were used to analyze datasets obtained from experiment 3. Obtained results show a positive association between maternal IL-6 levels and the lamb improvement rates between the 1st (T4) and the 2nd (T5) trials of habit acquisition on day two. This indicates that specific maternal immune response is connected to changes in the cognitive function of the lambs influencing their memory and learning, but not other behavioral domains. In addition, the results show that the rise of maternal IL-6 after the 1st injection is associated with the frequency of entries into the center of the "V" during the 1st trial of habit acquisition, while lambs are subjected to the test for the first time. This indicates that maternal immune response in terms of IL-6 levels may influence the future behavior of the lamb under stressful conditions.

The rise of TNF-alpha after the 2nd injection has been linked with a higher improvement rate between the first two trials of reversal learning – T7 and T8 and to the higher frequency of entries into the center of the "V" on T8. Obtained results indicate the implication of changes in maternal TNF-alpha with the cognitive flexibility of the lambs and their ability to solve the reversal learning task.

Overall, those results show intriguing connections between the specific maternal immune response to MIA during pregnancy and the behavioral outcome of the lambs. IL-6 and TNF-alpha levels affect different behavioral domains of the lambs. Learning and memory are linked with the levels of IL-6, while the levels of TNF-alpha shows the relation to the cognitive plasticity of the lambs.

Response of the lambs subjected to MIA during prenatal life to isolation-induced stress

Sensitivity to stress and the consequent physiological responses are important factors that may influence behavioral outcomes in different situations. Thus, to evaluate the possible effects of MIA on stress response and exclude their influence on

the other behavioral outcomes, the physiological responses of the lambs to isolation-induced stress were analyzed. Lambs were first socially isolated when aged 20 days in the Isolation Test. The prenatal LPS treatment didn't affect the number of jumps, although the higher frequency of entries into the proximity zone adjacent to the ewe was observed in MIA lambs, compared to CTR in experiment 3, indicating that only chronic immune challenge during mid-gestation may mildly affect the response of the lambs to isolation-induced stress. The time spent in the proximity zone was not influenced by the treatment, showing that prenatal immune challenge does not influence offspring social attachment to the dam. Lambs were later exposed to isolation just before weaning, in this occasion we measured cortisol levels as a biomarker of stress, which is known to rise in response to physical or emotional challenges [25]. No significant changes were found between MIA and CTR lambs, indicating that the LPS challenge during pregnancy doesn't influence the neuroendocrine response to the stress of the lambs. MIA has been previously reported to influence the neuroendocrine response of animals to stress[229], although it has never been examined on sheep. On the other hand, we observed a rise in the temperature in MIA lambs after isolation, which was not observed in controls. Hyperthermia due to stress-inducing situations has been already reported in adult sheep[230]. Nevertheless, big differences among breeds have been observed in the stress responses of ruminants[231]. Thus, the lack of stress-induced hyperthermia in CTR lambs may indicate that the świniarka breed is less reactive in terms of cortisol production towards potentially fear-eliciting events.

Overall, the obtained results indicate that lambs subjected to LPS challenge during pregnancy may display higher temperature changes in response to psychological stress, although this is not accompanied by a similar rise in cortisol levels. Thus, basing on the obtained results, we may conclude that lambs subjected to LPS challenge during pregnancy have slightly stronger responses to psychological stress than controls on the physiological level, but not on the hormonal one. However, further analyses will be needed to better clarify this aspect.

Automated video-tracking analyses

To analyze animal movements, localization, and behaviors in the different arenas, the automated video-tracking software EthoVision® XT 11.5 (Noldus Information Technology, Netherlands) was used. To our knowledge, this software has never been

used to analyse the sheep behavior, thus the work performed in this study contributes to establishing a valid protocol for video-tracking of lambs using automated analyses.

The automated video-tracking software is based on the color contrast between the moving animal (in our case: the lamb) and the background (the floor). The EthoVision® software was designed for video-tracking of laboratory animals. Thus, EthoVision® protocols and adjustments have been established here for the first time as a tracking method for large animals. Obtaining an efficient protocol was challenging, although after spotting some limitations and applying specific arrangements, the performed studies led to establishing a valid experimental set-up that works optimally with the EthoVision® software.

One of the limitations spotted during the first experiment was the light grey color of the floors, which was too similar to the color of the lambs, thus disrupting the contrast between the lamb and the background. To solve this problem, since experiment 2 the floor was painted with dark brown, non-toxic paint, which allowed to increase the contrast between the subject and the floor and improve the automated detection of the lamb. Another limitation which has been improved during the study were the lighting conditions. The initial illumination of the arenas was not bright enough and not well-positioned. Thus, not all areas of the testing arenas were equally illuminated, and, in some places, the software was not able to detect the moving lamb. This required additional manual adjustments, which provided satisfactory results. However, in experiment 2 and 3, the number of lightning points was increased, and their position was adjusted to equally illuminate each area, allowing EthoVision® to work automatically with minimal manual adjustments. The limitations mentioned above were solved by the post-tracking corrections, which consisted of manual adjustments of the position detected by the software to the real position of the lamb, performed by an experienced operator whenever needed.

To improve the tracking in future studies it should be considered to paint the floor with black paint in order to further increase the contrast between the lambs and the floor. It should be also avoided to mark the wool with dark dyes, since it may interfere with video-tracking. Moreover, to obtain the optimal lightning condition, testing arenas should be completely isolated from the outside sources of light (the sun coming through the windows), to eliminate the possible shades that may impair the detection of the lamb in the arena.

Despite many limitations, the utilized method led to reliable results, which indicates that it may be used in future experiments to automatize the analyses of sheep and other large animals' behavior, decreasing the time needed to analyze videos and obtain the result, as well as increasing the reproducibility of obtained data.

Differences in the vulnerability of different time windows of fetal development to MIA challenge during pregnancy

In the present study, MIA was elicited by LPS at different gestational windows. However, experiments were performed in different cohorts and breeding seasons, therefore comparisons among gestational periods should be taken with caution. The behavioral changes observed were consistent with an effect of MIA on the memory of inhibitory control, as well as on cognitive flexibility and perseveration, which depended on the gestational period the LPS has been injected. In particular, the effects on memory were observed only when MIA was elicited at 125 DPC, whereas there were no effects on this parameter when MIA was elicited at 85 DPC. Conversely, an effect of MIA on cognitive flexibility and perseveration was observed only when MIA was elicited at 85 DPC. MIA85 lambs spent more time to achieve reversal learning and displayed a higher degree of perseveration (increased frequency of returns in the center and the closed side of the detour test) when compared to controls, whereas MIA125 lambs did not display such a trend.

These observations suggest that during different developmental windows the fetal lamb may display different vulnerability to prenatal immune challenge. Although this is in line with other animal studies, further experiments will be necessary for the proposed ovine model, to systematically investigate the offspring vulnerability to MIA at different gestational time points within a single experiment on the same cohort of ewes.

Summary

The results obtained from this study demonstrate that an LPS immune challenge administered during gestation elicits significant physiological changes in pregnant ewes, which can be assimilable to maternal immune activation-MIA. MIA significantly affect the immune response of pregnant ewes on the molecular level. From both examined cytokines, the most sensitive to LPS administration appeared to

be the IL-6 plasma level, thus making it an optimal marker to measure inflammatory responses in ewes.

The behavioral screening revealed that exposure to MIA during pregnancy affects specific behavioral domains in lambs, which correspond to behavioral domains widely affected in NDDs individuals. Those domains include learning, memory and cognitive flexibility scored by the V-detour test, which appeared to be the most sensitive assay to evaluate such behavioral abnormalities, as compared to the T-maze test. The social behaviors of the lambs, measured during the Isolation Test and evaluated by measuring maternal attachment and the stress response to social isolation, were not affected by the LPS administration during pregnancy under our experimental paradigm. Intriguingly, the analyses of the association between the maternal immune response to MIA challenge and behavioral outcome of the lambs revealed that the plasma levels of IL-6 are strongly associated with the severity of behavioral impairments, especially in the domain of learning and memory.

This study allowed to establish a robust methodology to evaluate the cognitive flexibility of the lambs, as well as their learning and memory skills by the V-detour test. Moreover, this study implemented the use of automated video-tracking analyses with EthoVision® XT software for the analyses of lambs' behavior, reported here for the first time. Established protocols and methodology, which were constantly improved through the course of the study may reduce the time and effort required to analyze sheep behaviors, as well as improve the reproducibility of the data in future studies.

To sum up, the reported experiments allowed to build solid foundations for establishing a new, sheep model of neurodevelopmental disorders. Obtained results may help to study the still unknown etiology of neurodevelopmental disorders in the future and allow the development of new therapeutic and preventive strategies based on immunotherapy.

CONCLUSIONS

1. Behavioral tests developed as part of this thesis were effective in scoring subtle behavioral changes induced by low doses of LPS. Thus, the obtained results let us to conclude that this study contributed to establishing a model of behavioral disorders in sheep, which can be utilized for modeling symptoms of human disorders.
2. MIA in pregnant ewes induces only subtle effects on the offspring behavior. Nevertheless, including a bigger cohort could lead to more statistically significant results.
3. LPS administration in pregnant ewes leads to the occurrence of illness-like symptoms regardless of gestational state. LPS challenge during pregnancy arouses the expression of pro-inflammatory cytokines in pregnant ewes, with the most prominent effect on plasma IL-6 levels.
4. The immune challenge during pregnancy affects specific domains of lambs' behavior, which are commonly affected in NDDs individuals: learning, memory, and cognitive flexibility.
5. The V-detour test shows as a robust tool to examine behavioral domains affected by NDDs in lambs and may be useful to model other psychiatric disorders in sheep.
6. The automatic video-tracking software EthoVision® XT can be successfully implemented in the study of sheep behaviors, as well as other animal models to decrease the time required for the analyses and improve the reproducibility of the obtained data.
2. Performed experiments allowed to lay the foundations for establishing a new animal model of neurodevelopmental disorders, which may have higher translational validity than mice and rises fewer ethical concerns than non-human primates.

BIBLIOGRAPHY

1. Stoner, R., Chow, M.L., Boyle, M.P., Sunkin, S.M., Mouton, P.R., Roy, S., Wynshaw-Boris, A., Colamarino, S.A., Lein, E.S., and Courchesne, E. (2014). Patches of Disorganization in the Neocortex of Children with Autism. New England Journal of Medicine *370*, 1209–1219. 10.1056/NEJMoa1307491.

2. American Psychiatric Association and American Psychiatric Association eds. (2013). Diagnostic and statistical manual of mental disorders: DSM-5 5th ed. (American Psychiatric Association).

3. Whitelaw, C.B.A., Sheets, T.P., Lillico, S.G., and Telugu, B.P. (2016). Engineering large animal models of human disease: Engineering large animal models of human disease. J. Pathol. *238*, 247–256. 10.1002/path.4648.

4. Shihab, N., Bowen, J., and Volk, H.A. (2011). Behavioral changes in dogs associated with the development of idiopathic epilepsy. Epilepsy & Behavior *21*, 160–167. 10.1016/j.yebeh.2011.03.018.

5. Opdam, H.I., Federico, P., Jackson, G.D., Buchanan, J., Abbott, D.F., Fabinyi, G.C.A., Syngeniotis, A., Vosmansky, M., Archer, J.S., Wellard, R.M., et al. (2002). A Sheep Model for the Study of Focal Epilepsy with Concurrent Intracranial EEG and Functional MRI. Epilepsia *43*, 779–787. 10.1046/j.1528-1157.2002.04202.x.

6. Chang, H.T., Rumbeiha, W.K., Patterson, J.S., Puschner, B., and Knight, A.P. (2012). Toxic Equine Parkinsonism: An Immunohistochemical Study of 10 Horses With Nigropallidal Encephalomalacia. Vet Pathol *49*, 398–402. 10.1177/0300985811406885.

7. Lee, S.-H., Jahng, G.-H., Choe, I.-H., Choi, C.-B., Kim, D.-H., and Kim, H.-Y. (2013). Neural Pathway Interference by Retained Acupuncture: A Functional MRI Study of a Dog Model of Parkinson's Disease. CNS Neurosci Ther *19*, 585–595. 10.1111/cns.12108.

8. Lange, M., Williams, W., Bone, H.-G., Van Aken, H., Br??king, K., Morelli, A., Hucklenbruch, C., Daudel, F., Ertmer, C., Stubbe, H., et al. (2007). CONTINUOUSLY INFUSED GLIPIZIDE REVERSES THE HYPERDYNAMIC CIRCULATION IN OVINE ENDOTOXEMIA: Shock *27*, 701–706. 10.1097/01.shk.0000246902.58068.73.

9. Ertmer, C., Bone, H.-G., Morelli, A., Van Aken, H., Erren, M., Lange, M., Traber, D.L., and Westphal, M. (2007). METHYLPREDNISOLONE REVERSES VASOPRESSIN HYPORESPONSIVENESS IN OVINE ENDOTOXEMIA: Shock *27*, 281–288. 10.1097/01.shk.0000235140.97903.90.

10. Barry, J.S., and Anthony, R.V. (2008). The pregnant sheep as a model for human pregnancy. Theriogenology *69*, 55–67. 10.1016/j.theriogenology.2007.09.021.

11. Gluckman, P.D., and Parsons, Y. (1983). Stereotaxic method and atlas for the ovine fetal forebrain. J. Dev. Physiol. *5*, 101–128.

12. Cooley, R.K., and Vanderwolf, C.H. (2004). The sheep brain: a basic guide (A.J. Kirby Co.).

13. Nowak, R., Keller, M., and Lévy, F. (2011). Mother-Young Relationships in Sheep: A Model for a Multidisciplinary Approach of the Study of Attachment in Mammals: mother-young attachment in sheep. Journal of Neuroendocrinology *23*, 1042–1053. 10.1111/j.1365-2826.2011.02205.x.

14. Larsson, K. (1994). The psychobiology of parenting in mammals. Scand J Psychol *35*, 97–143. 10.1111/j.1467-9450.1994.tb00938.x.

15. Lim, M.M., and Young, L.J. (2006). Neuropeptidergic regulation of affiliative behavior and social bonding in animals. Hormones and Behavior *50*, 506–517. 10.1016/j.yhbeh.2006.06.028.

16. Hansen, R.L., Rogers, S.J., and American Psychiatric Publishing eds. (2013). Autism and other neurodevelopmental disorders 1st ed. (American Psychiatric Pub).

17. Thapar, A., Cooper, M., and Rutter, M. (2017). Neurodevelopmental disorders. The Lancet Psychiatry *4*, 339–346. 10.1016/S2215-0366(16)30376-5.

18. Ding, B. (2015). Gene expression in maturing neurons: regulatory mechanisms and related neurodevelopmental disorders. Sheng Li Xue Bao *67*, 113–133.

19. Frederick, A.L., and Stanwood, G.D. (2009). Drugs, biogenic amine targets and the developing brain. Dev. Neurosci. *31*, 7–22. 10.1159/000207490.

20. Hu, W.F., Chahrour, M.H., and Walsh, C.A. (2014). The diverse genetic landscape of neurodevelopmental disorders. Annu Rev Genomics Hum Genet *15*, 195–213. 10.1146/annurev-genom-090413-025600.

21. Rice, D., and Barone, S. (2000). Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. Environ. Health Perspect. *108 Suppl 3*, 511–533. 10.1289/ehp.00108s3511.

22. Bergner, C.L., Smolinsky, A.N., Hart, P.C., Dufour, B.D., Egan, R.J., Laporte, J.L., and Kalueff, A.V. (2010). Mouse models for studying depression-like states and antidepressant drugs. Methods Mol. Biol. *602*, 267–282. 10.1007/978-1-60761-058-8_16.

23. Buckley, P.F., Miller, B.J., Lehrer, D.S., and Castle, D.J. (2009). Psychiatric comorbidities and schizophrenia. Schizophr Bull *35*, 383–402. 10.1093/schbul/sbn135.

24. Krishnan, K.R.R. (2005). Psychiatric and medical comorbidities of bipolar disorder. Psychosom Med *67*, 1–8. 10.1097/01.psy.0000151489.36347.18.

25. Leitner, Y. (2014). The Co-Occurrence of Autism and Attention Deficit Hyperactivity Disorder in Children â€“ What Do We Know? Front. Hum. Neurosci. *8*. 10.3389/fnhum.2014.00268.

26. Homberg, J.R., Kyzar, E.J., Stewart, A.M., Nguyen, M., Poudel, M.K., Echevarria, D.J., Collier, A.D., Gaikwad, S., Klimenko, V.M., Norton, W., et al. (2016). Improving treatment of neurodevelopmental disorders: recommendations based on preclinical studies. Expert Opin Drug Discov *11*, 11–25. 10.1517/17460441.2016.1115834.

27. Ratajczak, H.V. (2011). Theoretical aspects of autism: biomarkers—a review. Journal of Immunotoxicology *8*, 80–94. 10.3109/1547691X.2010.538749.

28. Christensen, D.L., Baio, J., Braun, K.V.N., Bilder, D., Charles, J., Constantino, J.N., Daniels, J., Durkin, M.S., Fitzgerald, R.T., Kurzius-Spencer, M., et al. (2016). Prevalence and Characteristics of Autism Spectrum Disorder Among Children Aged 8 Years — Autism and Developmental Disabilities Monitoring Network, 11 Sites, United States, 2012. MMWR Surveill. Summ. *65*, 1–23. 10.15585/mmwr.ss6503a1.

29. Idring, S., Rai, D., Dal, H., Dalman, C., Sturm, H., Zander, E., Lee, B.K., Serlachius, E., and Magnusson, C. (2012). Autism Spectrum Disorders in the Stockholm Youth Cohort: Design, Prevalence and Validity. PLoS ONE *7*, e41280. 10.1371/journal.pone.0041280.

30. Benjak, T., and Vuletic, G. (2013). Prevalence of Pervasive Developmental Disorders – Croatia in Comparison with Other Countries of the World. In Recent Advances in Autism Spectrum Disorders - Volume I, M. Fitzgerald, ed. (InTech). 10.5772/51637.

31. Skonieczna-Żydecka, K., Gorzkowska, I., Pierzak-Sominka, J., and Adler, G. (2017). The Prevalence of Autism Spectrum Disorders in West Pomeranian and Pomeranian Regions of Poland. J Appl Res Intellect Disabil *30*, 283–289. 10.1111/jar.12238.

32. Salari, N., Rasoulpoor, S., Rasoulpoor, S., Shohaimi, S., Jafarpour, S., Abdoli, N., Khaledi-Paveh, B., and Mohammadi, M. (2022). The global prevalence of autism spectrum disorder: a comprehensive systematic review and meta-analysis. Ital J Pediatr *48*, 112. 10.1186/s13052-022-01310-w.

33. Al-Mamri, W., Idris, A.B., Dakak, S., Al-Shekaili, M., Al-Harthi, Z., Alnaamani, A.M., Alhinai, F.I., Jalees, S., Al Hatmi, M., El-Naggari, M.A., et al. (2019). Revisiting the Prevalence of Autism Spectrum Disorder among Omani Children: A multicentre study. Sultan Qaboos Univ Med J *19*, e305–e309. 10.18295/squmj.2019.19.04.005.

34. May, T., Brignell, A., and Williams, K. (2020). Autism Spectrum Disorder Prevalence in Children Aged 12-13 Years From the Longitudinal Study of Australian Children. Autism Res *13*, 821–827. 10.1002/aur.2286.

35. Lundstrom, S., Reichenberg, A., Anckarsater, H., Lichtenstein, P., and Gillberg, C. (2015). Autism phenotype versus registered diagnosis in Swedish children:

prevalence trends over 10 years in general population samples. BMJ *350*, h1961–h1961. 10.1136/bmj.h1961.

36. Persico, A.M., and Bourgeron, T. (2006). Searching for ways out of the autism maze: genetic, epigenetic and environmental clues. Trends in Neurosciences *29*, 349–358. 10.1016/j.tins.2006.05.010.

37. Sandin, S., Lichtenstein, P., Kuja-Halkola, R., Larsson, H., Hultman, C.M., and Reichenberg, A. (2014). The familial risk of autism. JAMA *311*, 1770–1777. 10.1001/jama.2014.4144.

38. Geschwind, D.H., and State, M.W. (2015). Gene hunting in autism spectrum disorder: on the path to precision medicine. The Lancet Neurology *14*, 1109–1120. 10.1016/S1474-4422(15)00044-7.

39. Modabbernia, A., Velthorst, E., and Reichenberg, A. (2017). Environmental risk factors for autism: an evidence-based review of systematic reviews and meta-analyses. Molecular Autism *8*, 13. 10.1186/s13229-017-0121-4.

40. Kim, Y.S., and Leventhal, B.L. (2015). Genetic Epidemiology and Insights into Interactive Genetic and Environmental Effects in Autism Spectrum Disorders. Biological Psychiatry *77*, 66–74. 10.1016/j.biopsych.2014.11.001.

41. Jiang, H., Xu, L., Shao, L., Xia, R., Yu, Z., Ling, Z., Yang, F., Deng, M., and Ruan, B. (2016). Maternal infection during pregnancy and risk of autism spectrum disorders: A systematic review and meta-analysis. Brain, Behavior, and Immunity *58*, 165–172. 10.1016/j.bbi.2016.06.005.

42. Wu, S., Wu, F., Ding, Y., Hou, J., Bi, J., and Zhang, Z. (2017). Advanced parental age and autism risk in children: a systematic review and meta-analysis. Acta Psychiatr Scand *135*, 29–41. 10.1111/acps.12666.

43. Gardener, H., Spiegelman, D., and Buka, S.L. (2009). Prenatal risk factors for autism: comprehensive meta-analysis. Br J Psychiatry *195*, 7–14. 10.1192/bjp.bp.108.051672.

44. Rossignol, D.A., Genuis, S.J., and Frye, R.E. (2014). Environmental toxicants and autism spectrum disorders: a systematic review. Transl Psychiatry *4*, e360–e360. 10.1038/tp.2014.4.

45. Gardener, H., Spiegelman, D., and Buka, S.L. (2011). Perinatal and Neonatal Risk Factors for Autism: A Comprehensive Meta-analysis. PEDIATRICS *128*, 344–355. 10.1542/peds.2010-1036.

46. Dykens, E.M. (2015). Family adjustment and interventions in neurodevelopmental disorders. Curr Opin Psychiatry *28*, 121–126. 10.1097/YCO.0000000000000129.

47. Viding, E., and Blakemore, S.-J. (2007). Endophenotype Approach to Developmental Psychopathology: Implications for Autism Research. Behav Genet *37*, 51–60. 10.1007/s10519-006-9105-4.

48. Willner, P. (1984). The validity of animal models of depression. Psychopharmacology (Berl.) *83*, 1–16. 10.1007/bf00427414.

49. Crawley, J.N. (2004). Designing mouse behavioral tasks relevant to autistic-like behaviors. Ment Retard Dev Disabil Res Rev *10*, 248–258. 10.1002/mrdd.20039.

50. Parikshak, N.N., Luo, R., Zhang, A., Won, H., Lowe, J.K., Chandran, V., Horvath, S., and Geschwind, D.H. (2013). Integrative Functional Genomic Analyses Implicate Specific Molecular Pathways and Circuits in Autism. Cell *155*, 1008–1021. 10.1016/j.cell.2013.10.031.

51. Coe, B.P., Witherspoon, K., Rosenfeld, J.A., van Bon, B.W.M., Vulto-van Silfhout, A.T., Bosco, P., Friend, K.L., Baker, C., Buono, S., Vissers, L.E.L.M., et al. (2014). Refining analyses of copy number variation identifies specific genes associated with developmental delay. Nat Genet *46*, 1063–1071. 10.1038/ng.3092.

52. Pinto, D., Delaby, E., Merico, D., Barbosa, M., Merikangas, A., Klei, L., Thiruvahindrapuram, B., Xu, X., Ziman, R., Wang, Z., et al. (2014). Convergence of Genes and Cellular Pathways Dysregulated in Autism Spectrum Disorders. The American Journal of Human Genetics *94*, 677–694. 10.1016/j.ajhg.2014.03.018.

53. Abrahams, B.S., and Geschwind, D.H. (2008). Advances in autism genetics: on the threshold of a new neurobiology. Nat Rev Genet *9*, 341–355. 10.1038/nrg2346.

54. Spooren, W., Lindemann, L., Ghosh, A., and Santarelli, L. (2012). Synapse dysfunction in autism: a molecular medicine approach to drug discovery in neurodevelopmental disorders. Trends in Pharmacological Sciences *33*, 669–684. 10.1016/j.tips.2012.09.004.

55. Silverman, J.L., and Crawley, J.N. (2014). The promising trajectory of autism therapeutics discovery. Drug Discovery Today *19*, 838–844. 10.1016/j.drudis.2013.12.007.

56. Wöhr, M. (2014). Ultrasonic vocalizations in Shank mouse models for autism spectrum disorders: Detailed spectrographic analyses and developmental profiles. Neuroscience & Biobehavioral Reviews *43*, 199–212. 10.1016/j.neubiorev.2014.03.021.

57. Roullet, F.I., Lai, J.K.Y., and Foster, J.A. (2013). In utero exposure to valproic acid and autism — A current review of clinical and animal studies. Neurotoxicology and Teratology *36*, 47–56. 10.1016/j.ntt.2013.01.004.

58. Engineer, C.T., Centanni, T.M., Im, K.W., Borland, M.S., Moreno, N.A., Carraway, R.S., Wilson, L.G., and Kilgard, M.P. (2014). Degraded auditory processing in a rat model of autism limits the speech representation in non-primary auditory cortex: Speech Processing in a Rat Autism Model. Devel Neurobio *74*, 972–986. 10.1002/dneu.22175.

59. Hamilton, S.M., Green, J.R., Veeraragavan, S., Yuva, L., McCoy, A., Wu, Y., Warren, J., Little, L., Ji, D., Cui, X., et al. (2014). Fmr1 and Nlgn3 knockout rats: Novel tools for investigating autism spectrum disorders. Behavioral Neuroscience *128*, 103–109. 10.1037/a0035988.

60. Modi, M.E., and Young, L.J. (2012). The oxytocin system in drug discovery for autism: animal models and novel therapeutic strategies. Horm Behav *61*, 340–350. 10.1016/j.yhbeh.2011.12.010.

61. Bauman, M.D., Iosif, A.-M., Smith, S.E.P., Bregere, C., Amaral, D.G., and Patterson, P.H. (2014). Activation of the Maternal Immune System During Pregnancy Alters Behavioral Development of Rhesus Monkey Offspring. Biological Psychiatry *75*, 332–341. 10.1016/j.biopsych.2013.06.025.

62. Machado, C.J., Whitaker, A.M., Smith, S.E.P., Patterson, P.H., and Bauman, M.D. (2015). Maternal Immune Activation in Nonhuman Primates Alters Social Attention in Juvenile Offspring. Biological Psychiatry *77*, 823–832. 10.1016/j.biopsych.2014.07.035.

63. Brodkin, E. (2007). BALB/c mice: Low sociability and other phenotypes that may be relevant to autism. Behavioural Brain Research *176*, 53–65. 10.1016/j.bbr.2006.06.025.

64. Moy, S.S., Nadler, J.J., Young, N.B., Perez, A., Holloway, L.P., Barbaro, R.P., Barbaro, J.R., Wilson, L.M., Threadgill, D.W., Lauder, J.M., et al. (2007). Mouse behavioral tasks relevant to autism: phenotypes of 10 inbred strains. Behav. Brain Res. *176*, 4–20. 10.1016/j.bbr.2006.07.030.

65. Moy, S.S., Nadler, J.J., Young, N.B., Nonneman, R.J., Segall, S.K., Andrade, G.M., Crawley, J.N., and Magnuson, T.R. (2008). Social approach and repetitive behavior in eleven inbred mouse strains. Behavioural Brain Research *191*, 118–129. 10.1016/j.bbr.2008.03.015.

66. McFarlane, H.G., Kusek, G.K., Yang, M., Phoenix, J.L., Bolivar, V.J., and Crawley, J.N. (2008). Autism-like behavioral phenotypes in BTBR T+tf/J mice. Genes, Brain and Behavior *7*, 152–163. 10.1111/j.1601-183X.2007.00330.x.

67. Bolivar, V.J., Walters, S.R., and Phoenix, J.L. (2007). Assessing autism-like behavior in mice: variations in social interactions among inbred strains. Behav. Brain Res. *176*, 21–26. 10.1016/j.bbr.2006.09.007.

68. Silverman, J.L., Pride, M.C., Hayes, J.E., Puhger, K.R., Butler-Struben, H.M., Baker, S., and Crawley, J.N. (2015). GABAB Receptor Agonist R-Baclofen Reverses Social Deficits and Reduces Repetitive Behavior in Two Mouse Models of Autism. Neuropsychopharmacol *40*, 2228–2239. 10.1038/npp.2015.66.

69. Scattoni, M.L., Ricceri, L., and Crawley, J.N. (2011). Unusual repertoire of vocalizations in adult BTBR T+tf/J mice during three types of social encounters. Genes Brain Behav. *10*, 44–56. 10.1111/j.1601-183X.2010.00623.x.

70. Scattoni, M.L., Gandhy, S.U., Ricceri, L., and Crawley, J.N. (2008). Unusual Repertoire of Vocalizations in the BTBR T+tf/J Mouse Model of Autism. PLoS ONE *3*, e3067. 10.1371/journal.pone.0003067.

71. Silverman, J.L., Yang, M., Lord, C., and Crawley, J.N. (2010). Behavioural phenotyping assays for mouse models of autism. Nat. Rev. Neurosci. *11*, 490–502. 10.1038/nrn2851.

72. Terranova, M.L., and Laviola, G. (2005). Scoring of Social Interactions and Play in Mice During Adolescence. Current Protocols in Toxicology *26*. 10.1002/0471140856.tx1310s26.

73. Kaidanovich-Beilin, O., Lipina, T., Vukobradovic, I., Roder, J., and Woodgett, J.R. (2011). Assessment of Social Interaction Behaviors. JoVE, 2473. 10.3791/2473.

74. Yang, M., Silverman, J.L., and Crawley, J.N. (2011). Automated Three-Chambered Social Approach Task for Mice. Current Protocols in Neuroscience *56*. 10.1002/0471142301.ns0826s56.

75. Galef, B.G. (2002). Social Learning of Food Preferences in Rodents: Rapid Appetitive Learning. Current Protocols in Neuroscience *21*. 10.1002/0471142301.ns0805ds21.

76. Lijam, N., Paylor, R., McDonald, M.P., Crawley, J.N., Deng, C.-X., Herrup, K., Stevens, K.E., Maccaferri, G., McBain, C.J., Sussman, D.J., et al. (1997). Social Interaction and Sensorimotor Gating Abnormalities in Mice Lacking Dvl1. Cell *90*, 895–905. 10.1016/S0092-8674(00)80354-2.

77. Rapin, I., and Dunn, M. (2003). Update on the language disorders of individuals on the autistic spectrum. Brain and Development *25*, 166–172. 10.1016/S0387-7604(02)00191-2.

78. Chabout, J., Serreau, P., Ey, E., Bellier, L., Aubin, T., Bourgeron, T., and Granon, S. (2012). Adult Male Mice Emit Context-Specific Ultrasonic Vocalizations That Are Modulated by Prior Isolation or Group Rearing Environment. PLoS ONE *7*, e29401. 10.1371/journal.pone.0029401.

79. Ehret, G. (2005). Infant Rodent Ultrasounds ? A Gate to the Understanding of Sound Communication. Behav Genet *35*, 19–29. 10.1007/s10519-004-0853-8.

80. Wang, H., Liang, S., Burgdorf, J., Wess, J., and Yeomans, J. (2008). Ultrasonic Vocalizations Induced by Sex and Amphetamine in M2, M4, M5 Muscarinic and D2 Dopamine Receptor Knockout Mice. PLoS ONE *3*, e1893. 10.1371/journal.pone.0001893.

81. Sugimoto, H., Okabe, S., Kato, M., Koshida, N., Shiroishi, T., Mogi, K., Kikusui, T., and Koide, T. (2011). A Role for Strain Differences in Waveforms of Ultrasonic Vocalizations during Male–Female Interaction. PLoS ONE *6*, e22093. 10.1371/journal.pone.0022093.

82. Roullet, F.I., Wöhr, M., and Crawley, J.N. (2011). Female urine-induced male mice ultrasonic vocalizations, but not scent-marking, is modulated by social experience. Behavioural Brain Research *216*, 19–28. 10.1016/j.bbr.2010.06.004.

83. Holy, T.E., and Guo, Z. (2005). Ultrasonic Songs of Male Mice. PLoS Biol *3*, e386. 10.1371/journal.pbio.0030386.

84. Crawley, J.N. (2012). Translational animal models of autism and neurodevelopmental disorders. Dialogues Clin Neurosci *14*, 293–305.

85. D'Cruz, A.-M., Ragozzino, M.E., Mosconi, M.W., Shrestha, S., Cook, E.H., and Sweeney, J.A. (2013). Reduced behavioral flexibility in autism spectrum disorders. Neuropsychology *27*, 152–160. 10.1037/a0031721.

86. Kalueff, A.V., Stewart, A.M., Song, C., Berridge, K.C., Graybiel, A.M., and Fentress, J.C. (2016). Neurobiology of rodent self-grooming and its value for translational neuroscience. Nat Rev Neurosci *17*, 45–59. 10.1038/nrn.2015.8.

87. Thomas, A., Burant, A., Bui, N., Graham, D., Yuva-Paylor, L.A., and Paylor, R. (2009). Marble burying reflects a repetitive and perseverative behavior more than novelty-induced anxiety. Psychopharmacology *204*, 361–373. 10.1007/s00213-009-1466-y.

88. Kazdoba, T.M., Leach, P.T., Yang, M., Silverman, J.L., Solomon, M., and Crawley, J.N. (2016). Translational Mouse Models of Autism: Advancing Toward Pharmacological Therapeutics. Curr Top Behav Neurosci *28*, 1–52. 10.1007/7854_2015_5003.

89. PuÅ›cian, A., Å• Ä™ski, S., GÃ³rkiewicz, T., Meyza, K., Lipp, H.-P., and Knapska, E. (2014). A novel automated behavioral test battery assessing cognitive rigidity in two genetic mouse models of autism. Front. Behav. Neurosci. *8*. 10.3389/fnbeh.2014.00140.

90. Sagvolden, T. (2000). Behavioral validation of the spontaneously hypertensive rat (SHR) as an animal model of attention-deficit/hyperactivity disorder (AD/HD). Neuroscience & Biobehavioral Reviews *24*, 31–39. 10.1016/S0149-7634(99)00058-5.

91. Sagvolden, T., Russell, V.A., Aase, H., Johansen, E.B., and Farshbaf, M. (2005). Rodent Models of Attention-Deficit/Hyperactivity Disorder. Biological Psychiatry *57*, 1239–1247. 10.1016/j.biopsych.2005.02.002.

92. Sagvolden, T., Hendley, E.D., and Knardahl, S. (1992). Behavior of hypertensive and hyperactive rat strains: Hyperactivity is not unitarily determined. Physiology & Behavior *52*, 49–57. 10.1016/0031-9384(92)90432-2.

93. Carter, A.M. (2007). Animal models of human placentation--a review. Placenta *28 Suppl A*, S41-47. 10.1016/j.placenta.2006.11.002.

94. Hickey, W.F. (1999). Leukocyte traffic in the central nervous system: the participants and their roles. Seminars in Immunology *11*, 125–137. 10.1006/smim.1999.0168.

95. Pachter, J.S., de Vries, H.E., and Fabry, Z. (2003). The Blood-Brain Barrier and Its Role in Immune Privilege in the Central Nervous System. J Neuropathol Exp Neurol *62*, 593–604. 10.1093/jnen/62.6.593.

96. Ousman, S.S., and Kubes, P. (2012). Immune surveillance in the central nervous system. Nat Neurosci *15*, 1096–1101. 10.1038/nn.3161.

97. Louveau, A., Smirnov, I., Keyes, T.J., Eccles, J.D., Rouhani, S.J., Peske, J.D., Derecki, N.C., Castle, D., Mandell, J.W., Lee, K.S., et al. (2015). Structural and functional features of central nervous system lymphatic vessels. Nature *523*, 337–341. 10.1038/nature14432.

98. Aspelund, A., Antila, S., Proulx, S.T., Karlsen, T.V., Karaman, S., Detmar, M., Wiig, H., and Alitalo, K. (2015). A dural lymphatic vascular system that drains brain interstitial fluid and macromolecules. The Journal of Experimental Medicine *212*, 991–999. 10.1084/jem.20142290.

99. Glynn, M.W., Elmer, B.M., Garay, P.A., Liu, X.-B., Needleman, L.A., El-Sabeawy, F., and McAllister, A.K. (2011). MHCI negatively regulates synapse density during the establishment of cortical connections. Nat Neurosci *14*, 442–451. 10.1038/nn.2764.

100. Stüve, O., Youssef, S., Slavin, A.J., King, C.L., Patarroyo, J.C., Hirschberg, D.L., Brickey, W.J., Soos, J.M., Piskurich, J.F., Chapman, H.A., et al. (2002). The Role of the MHC Class II Transactivator in Class II Expression and Antigen Presentation by Astrocytes and in Susceptibility to Central Nervous System Autoimmune Disease. J Immunol *169*, 6720–6732. 10.4049/jimmunol.169.12.6720.

101. Schwartz, M., Kipnis, J., Rivest, S., and Prat, A. (2013). How Do Immune Cells Support and Shape the Brain in Health, Disease, and Aging? Journal of Neuroscience *33*, 17587–17596. 10.1523/JNEUROSCI.3241-13.2013.

102. Korin, B., Ben-Shaanan, T.L., Schiller, M., Dubovik, T., Azulay-Debby, H., Boshnak, N.T., Koren, T., and Rolls, A. (2017). High-dimensional, single-cell characterization of the brain's immune compartment. Nat Neurosci *20*, 1300–1309. 10.1038/nn.4610.

103. Salter, M.W., and Stevens, B. (2017). Microglia emerge as central players in brain disease. Nat Med *23*, 1018–1027. 10.1038/nm.4397.

104. Cowan, M., and Petri, W.A. (2018). Microglia: Immune Regulators of Neurodevelopment. Front. Immunol. *9*, 2576. 10.3389/fimmu.2018.02576.

105. Howren, M.B., Lamkin, D.M., and Suls, J. (2009). Associations of Depression With C-Reactive Protein, IL-1, and IL-6: A Meta-Analysis: Psychosomatic Medicine *71*, 171–186. 10.1097/PSY.0b013e3181907c1b.

106. Miller, B.J., Buckley, P., Seabolt, W., Mellor, A., and Kirkpatrick, B. (2011). Meta-Analysis of Cytokine Alterations in Schizophrenia: Clinical Status and Antipsychotic Effects. Biological Psychiatry *70*, 663–671. 10.1016/j.biopsych.2011.04.013.

107. Modabbernia, A., Taslimi, S., Brietzke, E., and Ashrafi, M. (2013). Cytokine Alterations in Bipolar Disorder: A Meta-Analysis of 30 Studies. Biological Psychiatry *74*, 15–25. 10.1016/j.biopsych.2013.01.007.

108. Passos, I.C., Vasconcelos-Moreno, M.P., Costa, L.G., Kunz, M., Brietzke, E., Quevedo, J., Salum, G., Magalhães, P.V., Kapczinski, F., and Kauer-Sant'Anna, M. (2015). Inflammatory markers in post-traumatic stress disorder: a systematic review, meta-analysis, and meta-regression. The Lancet Psychiatry *2*, 1002–1012. 10.1016/S2215-0366(15)00309-0.

109. Réus, G.Z., Fries, G.R., Stertz, L., Badawy, M., Passos, I.C., Barichello, T., Kapczinski, F., and Quevedo, J. (2015). The role of inflammation and microglial activation in the pathophysiology of psychiatric disorders. Neuroscience *300*, 141–154. 10.1016/j.neuroscience.2015.05.018.

110. Allan, S.M., and Rothwell, N.J. (2001). Cytokines and acute neurodegeneration. Nat Rev Neurosci *2*, 734–744. 10.1038/35094583.

111. Hassan, W., Noreen, H., Castro-Gomes, V., Mohammadzai, I., Batista Teixeira da Rocha, J., and Landeira-Fernandez, J. (2016). Association of Oxidative Stress with Psychiatric Disorders. CPD *22*, 2960–2974. 10.2174/1381612822666160307145931.

112. Kronfol, Z. (2000). Cytokines and the Brain: Implications for Clinical Psychiatry. American Journal of Psychiatry *157*, 683–694. 10.1176/appi.ajp.157.5.683.

113. Pollak, T.A., Drndarski, S., Stone, J.M., David, A.S., McGuire, P., and Abbott, N.J. (2018). The blood–brain barrier in psychosis. The Lancet Psychiatry *5*, 79–92. 10.1016/S2215-0366(17)30293-6.

114. Schizophrenia Working Group of the Psychiatric Genomics Consortium (2014). Biological insights from 108 schizophrenia-associated genetic loci. Nature *511*, 421–427. 10.1038/nature13595.

115. Lintas, C., Sacco, R., and Persico, A.M. (2012). Genome-wide expression studies in Autism spectrum disorder, Rett syndrome, and Down syndrome. Neurobiology of Disease *45*, 57–68. 10.1016/j.nbd.2010.11.010.

116. Kohane, I.S., McMurry, A., Weber, G., MacFadden, D., Rappaport, L., Kunkel, L., Bickel, J., Wattanasin, N., Spence, S., Murphy, S., et al. (2012). The Co-Morbidity Burden of Children and Young Adults with Autism Spectrum Disorders. PLoS ONE *7*, e33224. 10.1371/journal.pone.0033224.

117. McDougle, C.J., Landino, S.M., Vahabzadeh, A., O'Rourke, J., Zurcher, N.R., Finger, B.C., Palumbo, M.L., Helt, J., Mullett, J.E., Hooker, J.M., et al. (2015).

Toward an immune-mediated subtype of autism spectrum disorder. Brain Research *1617*, 72–92. 10.1016/j.brainres.2014.09.048.

118. Pardo, C.A., Vargas, D.L., and Zimmerman, A.W. (2005). Immunity, neuroglia and neuroinflammation in autism. International Review of Psychiatry *17*, 485–495. 10.1080/02646830500381930.

119. Keil, A., Daniels, J.L., Forssen, U., Hultman, C., Cnattingius, S., Söderberg, K.C., Feychting, M., and Sparen, P. (2010). Parental Autoimmune Diseases Associated With Autism Spectrum Disorders in Offspring: Epidemiology *21*, 805–808. 10.1097/EDE.0b013e3181f26e3f.

120. Xu, G., Jing, J., Bowers, K., Liu, B., and Bao, W. (2014). Maternal Diabetes and the Risk of Autism Spectrum Disorders in the Offspring: A Systematic Review and Meta-Analysis. J Autism Dev Disord *44*, 766–775. 10.1007/s10803-013-1928-2.

121. Schans, J. van der, Çiçek, R., de Vries, T.W., Hak, E., and Hoekstra, P.J. (2017). Association of atopic diseases and attention-deficit/hyperactivity disorder: A systematic review and meta-analyses. Neuroscience & Biobehavioral Reviews *74*, 139–148. 10.1016/j.neubiorev.2017.01.011.

122. Nielsen, P.R., Benros, M.E., and Dalsgaard, S. (2017). Associations Between Autoimmune Diseases and Attention-Deficit/Hyperactivity Disorder: A Nationwide Study. Journal of the American Academy of Child & Adolescent Psychiatry *56*, 234-240.e1. 10.1016/j.jaac.2016.12.010.

123. Boulanger-Bertolus, J., Pancaro, C., and Mashour, G.A. (2018). Increasing Role of Maternal Immune Activation in Neurodevelopmental Disorders. Front. Behav. Neurosci. *12*, 230. 10.3389/fnbeh.2018.00230.

124. Knuesel, I., Chicha, L., Britschgi, M., Schobel, S.A., Bodmer, M., Hellings, J.A., Toovey, S., and Prinssen, E.P. (2014). Maternal immune activation and abnormal brain development across CNS disorders. Nat Rev Neurol *10*, 643–660. 10.1038/nrneurol.2014.187.

125. Brown, A.S., and Derkits, E.J. (2010). Prenatal Infection and Schizophrenia: A Review of Epidemiologic and Translational Studies. AJP *167*, 261–280. 10.1176/appi.ajp.2009.09030361.

126. Patterson, P.H. (2012). Maternal infection and autism. Brain, Behavior, and Immunity *26*, 393. 10.1016/j.bbi.2011.09.008.

127. Atladóttir, H.Ó., Thorsen, P., Østergaard, L., Schendel, D.E., Lemcke, S., Abdallah, M., and Parner, E.T. (2010). Maternal Infection Requiring Hospitalization During Pregnancy and Autism Spectrum Disorders. J Autism Dev Disord *40*, 1423–1430. 10.1007/s10803-010-1006-y.

128. Goines, P.E., Croen, L.A., Braunschweig, D., Yoshida, C.K., Grether, J., Hansen, R., Kharrazi, M., Ashwood, P., and Van de Water, J. (2011). Increased midgestational IFN-γ, IL-4 and IL-5 in women bearing a child with autism: A case-control study. Mol Autism *2*, 13. 10.1186/2040-2392-2-13.

129. Fatemi, S.H., Emamian, E.S., Sidwell, R.W., Kist, D.A., Stary, J.M., Earle, J.A., and Thuras, P. (2002). Human influenza viral infection in utero alters glial fibrillary acidic protein immunoreactivity in the developing brains of neonatal mice. Mol Psychiatry *7*, 633–640. 10.1038/sj.mp.4001046.

130. Fatemi, S.H., Cuadra, A.E., El-Fakahany, E.E., Sidwell, R.W., and Thuras, P. (2000). Prenatal viral infection causes alterations in nNOS expression in developing mouse brains. Neuroreport *11*, 1493–1496.

131. Fatemi, S.H., Emamian, E.S., Kist, D., Sidwell, R.W., Nakajima, K., Akhter, P., Shier, A., Sheikh, S., and Bailey, K. (1999). Defective corticogenesis and reduction in Reelin immunoreactivity in cortex and hippocampus of prenatally infected neonatal mice. Mol Psychiatry *4*, 145–154. 10.1038/sj.mp.4000520.

132. Fatemi, S.H., Earle, J., Kanodia, R., Kist, D., Emamian, E.S., Patterson, P.H., Shi, L., and Sidwell, R. (2002). Prenatal Viral Infection Leads to Pyramidal Cell Atrophy and Macrocephaly in Adulthood: Implications for Genesis of Autism and Schizophrenia. Cellular and Molecular Neurobiology *22*, 25–33. 10.1023/A:1015337611258.

133. Urakubo, A., Jarskog, L.F., Lieberman, J.A., and Gilmore, J.H. (2001). Prenatal exposure to maternal infection alters cytokine expression in the placenta, amniotic fluid, and fetal brain. Schizophrenia Research *47*, 27–36. 10.1016/S0920-9964(00)00032-3.

134. Zuckerman, L., Rehavi, M., Nachman, R., and Weiner, I. (2003). Immune Activation During Pregnancy in Rats Leads to a PostPubertal Emergence of Disrupted Latent Inhibition, Dopaminergic Hyperfunction, and Altered Limbic Morphology in the Offspring: A Novel Neurodevelopmental Model of Schizophrenia. Neuropsychopharmacol *28*, 1778–1789. 10.1038/sj.npp.1300248.

135. Shi, L., Fatemi, S.H., Sidwell, R.W., and Patterson, P.H. (2003). Maternal influenza infection causes marked behavioral and pharmacological changes in the offspring. J. Neurosci. *23*, 297–302.

136. Meyer, U., Feldon, J., Schedlowski, M., and Yee, B.K. (2005). Towards an immuno-precipitated neurodevelopmental animal model of schizophrenia. Neuroscience & Biobehavioral Reviews *29*, 913–947. 10.1016/j.neubiorev.2004.10.012.

137. Patterson, P.H. (2009). Immune involvement in schizophrenia and autism: Etiology, pathology and animal models. Behavioural Brain Research *204*, 313–321. 10.1016/j.bbr.2008.12.016.

138. Boksa, P. (2010). Effects of prenatal infection on brain development and behavior: A review of findings from animal models. Brain, Behavior, and Immunity *24*, 881–897. 10.1016/j.bbi.2010.03.005.

139. Smith, S.E.P., Li, J., Garbett, K., Mirnics, K., and Patterson, P.H. (2007). Maternal Immune Activation Alters Fetal Brain Development through

Interleukin-6. Journal of Neuroscience *27*, 10695–10702. 10.1523/JNEUROSCI.2178-07.2007.

140. Choi, G.B., Yim, Y.S., Wong, H., Kim, S., Kim, H., Kim, S.V., Hoeffer, C.A., Littman, D.R., and Huh, J.R. (2016). The maternal interleukin-17a pathway in mice promotes autism-like phenotypes in offspring. Science *351*, 933–939. 10.1126/science.aad0314.

141. Kuchroo, V.K., and Awasthi, A. (2012). Emerging new roles of Th17 cells. Eur. J. Immunol. *42*, 2211–2214. 10.1002/eji.201242872.

142. Patterson, P.H. (2011). Maternal infection and immune involvement in autism. Trends in Molecular Medicine *17*, 389–394. 10.1016/j.molmed.2011.03.001.

143. Ronovsky, M., Berger, S., Molz, B., Berger, A., and D. Pollak, D. (2016). Animal Models of Maternal Immune Activation in Depression Research. CN *14*, 688–704. 10.2174/1570159X14666151215095359.

144. Reisinger, S., Khan, D., Kong, E., Berger, A., Pollak, A., and Pollak, D.D. (2015). The Poly(I:C)-induced maternal immune activation model in preclinical neuropsychiatric drug discovery. Pharmacology & Therapeutics *149*, 213–226. 10.1016/j.pharmthera.2015.01.001.

145. Meyer, U. (2006). The Time of Prenatal Immune Challenge Determines the Specificity of Inflammation-Mediated Brain and Behavioral Pathology. Journal of Neuroscience *26*, 4752–4762. 10.1523/JNEUROSCI.0099-06.2006.

146. Meyer, U., Yee, B.K., and Feldon, J. (2007). The Neurodevelopmental Impact of Prenatal Infections at Different Times of Pregnancy: The Earlier the Worse? Neuroscientist *13*, 241–256. 10.1177/1073858406296401.

147. Meyer, U. (2014). Prenatal Poly(I:C) Exposure and Other Developmental Immune Activation Models in Rodent Systems. Biological Psychiatry *75*, 307–315. 10.1016/j.biopsych.2013.07.011.

148. Bilbo, S.D., and Schwarz, J.M. (2012). The immune system and developmental programming of brain and behavior. Frontiers in Neuroendocrinology *33*, 267–286. 10.1016/j.yfrne.2012.08.006.

149. Robinson, D.P., and Klein, S.L. (2012). Pregnancy and pregnancy-associated hormones alter immune responses and disease pathogenesis. Hormones and Behavior *62*, 263–271. 10.1016/j.yhbeh.2012.02.023.

150. Kneeland, R.E., and Fatemi, S.H. (2013). Viral infection, inflammation and schizophrenia. Progress in Neuro-Psychopharmacology and Biological Psychiatry *42*, 35–48. 10.1016/j.pnpbp.2012.02.001.

151. Malkova, N.V., Yu, C.Z., Hsiao, E.Y., Moore, M.J., and Patterson, P.H. (2012). Maternal immune activation yields offspring displaying mouse versions of the three core symptoms of autism. Brain Behav. Immun. *26*, 607–616. 10.1016/j.bbi.2012.01.011.

152. Depino, A.M. (2015). Early prenatal exposure to LPS results in anxiety- and depression-related behaviors in adulthood. Neuroscience *299*, 56–65. 10.1016/j.neuroscience.2015.04.065.

153. Fisken, R., and Ward, R.D. (2019). Discrimination difficulty, cognitive burden, and reversal impairments in a maternal immune activation model of schizophrenia risk. Behavioural Processes *167*, 103936. 10.1016/j.beproc.2019.103936.

154. Estes, M.L., and McAllister, A.K. (2016). Maternal immune activation: Implications for neuropsychiatric disorders. Science *353*, 772–777. 10.1126/science.aag3194.

155. Tetreault, N.A., Hakeem, A.Y., Jiang, S., Williams, B.A., Allman, E., Wold, B.J., and Allman, J.M. (2012). Microglia in the Cerebral Cortex in Autism. J Autism Dev Disord *42*, 2569–2584. 10.1007/s10803-012-1513-0.

156. Morgan, J.T., Chana, G., Pardo, C.A., Achim, C., Semendeferi, K., Buckwalter, J., Courchesne, E., and Everall, I.P. (2010). Microglial Activation and Increased Microglial Density Observed in the Dorsolateral Prefrontal Cortex in Autism. Biological Psychiatry *68*, 368–376. 10.1016/j.biopsych.2010.05.024.

157. Pineda, E., Shin, D., You, S.J., Auvin, S., Sankar, R., and Mazarati, A. (2013). Maternal immune activation promotes hippocampal kindling epileptogenesis in mice: Pineda et al: MIA and Epilepsy. Ann Neurol. *74*, 11–19. 10.1002/ana.23898.

158. Missault, S., Van den Eynde, K., Vanden Berghe, W., Fransen, E., Weeren, A., Timmermans, J.P., Kumar-Singh, S., and Dedeurwaerdere, S. (2014). The risk for behavioural deficits is determined by the maternal immune response to prenatal immune challenge in a neurodevelopmental model. Brain, Behavior, and Immunity *42*, 138–146. 10.1016/j.bbi.2014.06.013.

159. Giovanoli, S., Notter, T., Richetto, J., Labouesse, M.A., Vuillermot, S., Riva, M.A., and Meyer, U. (2015). Late prenatal immune activation causes hippocampal deficits in the absence of persistent inflammation across aging. J Neuroinflammation *12*, 221. 10.1186/s12974-015-0437-y.

160. Smolders, S., Smolders, S.M.T., Swinnen, N., Gärtner, A., Rigo, J.-M., Legendre, P., and Brône, B. (2015). Maternal immune activation evoked by polyinosinic:polycytidylic acid does not evoke microglial cell activation in the embryo. Front. Cell. Neurosci. *9*. 10.3389/fncel.2015.00301.

161. Manitz, M.P., Plümper, J., Demir, S., Ahrens, M., Eßlinger, M., Wachholz, S., Eisenacher, M., Juckel, G., and Friebe, A. (2016). Flow cytometric characterization of microglia in the offspring of PolyI:C treated mice. Brain Research *1636*, 172–182. 10.1016/j.brainres.2016.02.004.

162. Perlman, R.L. (2016). Mouse Models of Human Disease: An Evolutionary Perspective. EMPH, eow014. 10.1093/emph/eow014.

163. Of men, not mice (2013). Nat Med *19*, 379–379. 10.1038/nm.3163.

164. Mestas, J., and Hughes, C.C.W. (2004). Of Mice and Not Men: Differences between Mouse and Human Immunology. J Immunol *172*, 2731–2738. 10.4049/jimmunol.172.5.2731.

165. Seok, J., Warren, H.S., Cuenca, A.G., Mindrinos, M.N., Baker, H.V., Xu, W., Richards, D.R., McDonald-Smith, G.P., Gao, H., Hennessy, L., et al. (2013). Genomic responses in mouse models poorly mimic human inflammatory diseases. Proc Natl Acad Sci USA *110*, 3507–3512. 10.1073/pnas.1222878110.

166. Preuss, T.M. (1995). Do Rats Have Prefrontal Cortex? The Rose-Woolsey-Akert Program Reconsidered. Journal of Cognitive Neuroscience *7*, 1–24. 10.1162/jocn.1995.7.1.1.

167. Careaga, M., Murai, T., and Bauman, M.D. (2017). Maternal Immune Activation and Autism Spectrum Disorder: From Rodents to Nonhuman and Human Primates. Biological Psychiatry *81*, 391–401. 10.1016/j.biopsych.2016.10.020.

168. VandeBerg, J.L., and Williams-Blangero, S. (1996). Strategies for using nonhuman primates in genetic research on multifactorial diseases. Lab. Anim. Sci. *46*, 146–151.

169. VandeBerg, J.L., and Williams-Blangero, S. (1997). Advantages and limitations of nonhuman primates as animal models in genetic research on complex diseases. Journal of Medical Primatology *26*, 113–119. 10.1111/j.1600-0684.1997.tb00042.x.

170. DeGrazia, D. (2016). Nonhuman Primates, Human Need, and Ethical Constraints. Hastings Center Report *46*, 27–28. 10.1002/hast.601.

171. Müller, E.E., and Rolla, M. (1996). Aspects of the neuroendocrine control of somatotropic function in calorically restricted dogs and patients with eating disorders: studies with cholinergic drugs. Psychiatry Research *62*, 51–63. 10.1016/0165-1781(96)02989-7.

172. Azizeh, B.Y., Head, E., Ibrahim, M.A., Torp, R., Tenner, A.J., Kim, R.C., Lott, I.T., and Cotman, C.W. (2000). Molecular Dating of Senile Plaques in the Brains of Individuals with Down Syndrome and in Aged Dogs. Experimental Neurology *163*, 111–122. 10.1006/exnr.2000.7359.

173. Lit, L., Schweitzer, J.B., Iosif, A.-M., and Oberbauer, A.M. (2010). Owner reports of attention, activity, and impulsivity in dogs: a replication study. Behav Brain Funct *6*, 1. 10.1186/1744-9081-6-1.

174. Lichter-Peled, A., Polani, S., Stanyon, R., Rocchi, M., and Kahila Bar-Gal, G. (2013). Role of KCNQ2 and KCNQ3 genes in juvenile idiopathic epilepsy in Arabian foals. The Veterinary Journal *196*, 57–63. 10.1016/j.tvjl.2012.08.024.

175. Fureix, C., Jego, P., Henry, S., Lansade, L., and Hausberger, M. (2012). Towards an Ethological Animal Model of Depression? A Study on Horses. PLoS ONE *7*, e39280. 10.1371/journal.pone.0039280.

176. Nurnberg, H.G., Keith, S.J., and Matt Paxton, D. (1997). Consideration of the relevance of ethological animal models for human repetitive behavioral spectrum disorders. Biological Psychiatry *41*, 226–229. 10.1016/S0006-3223(96)00100-X.

177. Moon, J.H., Kim, J.H., Im, H.-J., Lee, D.S., Park, E.J., Song, K., Oh, H.J., Hyun, S.B., Kang, S.C., Kim, H., et al. (2014). Proposed Motor Scoring System in a Porcine Model of Parkinson's Disease induced by Chronic Subcutaneous Injection of MPTP. Exp Neurobiol *23*, 258. 10.5607/en.2014.23.3.258.

178. Salak-Johnson, J.L., Anderson, D.L., and McGlone, J.J. (2004). Differential dose effects of central CRF and effects of CRF astressin on pig behavior. Physiology & Behavior *83*, 143–150. 10.1016/j.physbeh.2004.08.005.

179. Quilter, C.R., Blott, S.C., Wilson, A.E., Bagga, M.R., Sargent, C.A., Oliver, G.L., Southwood, O.I., Gilbert, C.L., Mileham, A., and Affara, N.A. (2007). Porcine maternal infanticide as a model for puerperal psychosis. Am. J. Med. Genet. *144B*, 862–868. 10.1002/ajmg.b.30529.

180. Scheerlinck, J.-P.Y., Snibson, K.J., Bowles, V.M., and Sutton, P. (2008). Biomedical applications of sheep models: from asthma to vaccines. Trends in Biotechnology *26*, 259–266. 10.1016/j.tibtech.2008.02.002.

181. Kuypers, E., Ophelders, D., Jellema, R.K., Kunzmann, S., Gavilanes, A.W., and Kramer, B.W. (2012). White matter injury following fetal inflammatory response syndrome induced by chorioamnionitis and fetal sepsis: Lessons from experimental ovine models. Early Human Development *88*, 931–936. 10.1016/j.earlhumdev.2012.09.011.

182. Gayrard, V., Picard-Hagen, N., Viguié, C., Laroute, V., Andréoletti, O., and Toutain, P.-L. (2005). A possible pharmacological explanation for quinacrine failure to treat prion diseases: pharmacokinetic investigations in a ovine model of scrapie. British Journal of Pharmacology *144*, 386–393. 10.1038/sj.bjp.0706072.

183. Huntington's Disease Sheep Collaborative Research Group, Reid, S.J., Patassini, S., Handley, R.R., Rudiger, S.R., McLaughlan, C.J., Osmand, A., Jacobsen, J.C., Morton, A.J., Weiss, A., et al. (2013). Further Molecular Characterisation of the OVT73 Transgenic Sheep Model of Huntington's Disease Identifies Cortical Aggregates. Journal of Huntington's Disease *2*, 279–295. 10.3233/JHD-130067.

184. Watari, H., Born, D.E., and Gleason, C.A. (2006). Effects of First Trimester Binge Alcohol Exposure on Developing White Matter in Fetal Sheep. Pediatr Res *59*, 560–564. 10.1203/01.pdr.0000203102.01364.de.

185. Kramer, B.W. (2012). Effects of in utero endotoxemia on the ovine fetal brain A model for schizophrenia. Front Biosci *E4*, 2745–2753. 10.2741/e588.

186. Campbell, K.H.S., McWhir, J., Ritchie, W.A., and Wilmut, I. (1996). Sheep cloned by nuclear transfer from a cultured cell line. Nature *380*, 64–66. 10.1038/380064a0.

187. Partridge, E.A., Davey, M.G., Hornick, M.A., McGovern, P.E., Mejaddam, A.Y., Vrecenak, J.D., Mesas-Burgos, C., Olive, A., Caskey, R.C., Weiland, T.R., et al. (2017). An extra-uterine system to physiologically support the extreme premature lamb. Nat Commun *8*, 15112. 10.1038/ncomms15112.

188. Enkhbaatar, P., Nelson, C., Salsbury, J.R., Carmical, J.R., Torres, K.E.O., Herndon, D., Prough, D.S., Luan, L., and Sherwood, E.R. (2015). Comparison of Gene Expression by Sheep and Human Blood Stimulated with the TLR4 Agonists Lipopolysaccharide and Monophosphoryl Lipid A. PLoS ONE *10*, e0144345. 10.1371/journal.pone.0144345.

189. Bohs, C.T., Fish, J.C., Miller, T.H., and Traber, D.L. (1979). Pulmonary vascular response to endotoxin in normal and lymphocyte depleted sheep. Circ. Shock *6*, 13–21.

190. Godsoe, A., Kimura, R., Herndon, D., Flynn, J.T., Schlag, G., Traber, L., and Traber, D. (1988). Cardiopulmonary changes with intermittent endotoxin administration in sheep. Circ. Shock *25*, 61–74.

191. Noshima, S., Noda, H., Herndon, D.N., Traber, L.D., and Traber, D.L. (1993). Left ventricular performance during continuous endotoxin-induced hyperdynamic endotoxemia in sheep. Journal of Applied Physiology *74*, 1528–1533. 10.1152/jappl.1993.74.4.1528.

192. Traber, D.L., Redl, H., Schlag, G., Herndon, D.N., Kimura, R., Prien, T., and Traber, L.D. (1988). Cardiopulmonary responses to continuous administration of endotoxin. American Journal of Physiology-Heart and Circulatory Physiology *254*, H833–H839. 10.1152/ajpheart.1988.254.5.H833.

193. Morrison, J.L., Berry, M.J., Botting, K.J., Darby, J.R.T., Frasch, M.G., Gatford, K.L., Giussani, D.A., Gray, C.L., Harding, R., Herrera, E.A., et al. (2018). Improving pregnancy outcomes in humans through studies in sheep. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology *315*, R1123–R1153. 10.1152/ajpregu.00391.2017.

194. Guesdon, V., Nowak, R., Meurisse, M., Boivin, X., Cornilleau, F., Chaillou, E., and Lévy, F. (2016). Behavioral evidence of heterospecific bonding between the lamb and the human caregiver and mapping of associated brain network. Psychoneuroendocrinology *71*, 159–169. 10.1016/j.psyneuen.2016.05.020.

195. Hein, W.R., and Griebel, P.J. (2003). A road less travelled: large animal models in immunological research. Nat Rev Immunol *3*, 79–84. 10.1038/nri977.

196. Back, S.A., Riddle, A., Dean, J., and Hohimer, A.R. (2012). The Instrumented Fetal Sheep as a Model of Cerebral White Matter Injury in the Premature Infant. Neurotherapeutics *9*, 359–370. 10.1007/s13311-012-0108-y.

197. Shen, H.H. (2016). News Feature: Better models for brain disease. Proc Natl Acad Sci USA *113*, 5461–5464. 10.1073/pnas.1605358113.

198. Dean, J.M., van de Looij, Y., Sizonenko, S.V., Lodygensky, G.A., Lazeyras, F., Bolouri, H., Kjellmer, I., Huppi, P.S., Hagberg, H., and Mallard, C. (2011).

Delayed cortical impairment following lipopolysaccharide exposure in preterm fetal sheep. Ann Neurol. *70*, 846–856. 10.1002/ana.22480.

199. Svedin, P., Kjellmer, I., Welin, A.-K., Blad, S., and Mallard, C. (2005). Maturational Effects of Lipopolysaccharide on White-Matter Injury in Fetal Sheep. J Child Neurol *20*, 960–964. 10.1177/08830738050200120501.

200. Keogh, M.J., Bennet, L., Drury, P.P., Booth, L.C., Mathai, S., Naylor, A.S., Fraser, M., and Gunn, A.J. (2012). Subclinical exposure to low-dose endotoxin impairs EEG maturation in preterm fetal sheep. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology *303*, R270–R278. 10.1152/ajpregu.00216.2012.

201. Gantert, M. (2010). Increased EEG delta frequency corresponds to chorioamnionitis-related brain injury. Front Biosci *S2*, 432–438. 10.2741/s76.

202. Dean, J.M., Shi, Z., Fleiss, B., Gunn, K.C., Groenendaal, F., van Bel, F., Derrick, M., Juul, S.E., Tan, S., Gressens, P., et al. (2015). A Critical Review of Models of Perinatal Infection. Dev Neurosci *37*, 289–304. 10.1159/000370309.

203. Lins, B.R., Marks, W.N., Zabder, N.K., Greba, Q., and Howland, J.G. (2019). Maternal Immune Activation during Pregnancy Alters the Behavior Profile of Female Offspring of Sprague Dawley Rats. eNeuro *6*, ENEURO.0437-18.2019. 10.1523/ENEURO.0437-18.2019.

204. Blair, J., Adaway, J., Keevil, B., and Ross, R. (2017). Salivary cortisol and cortisone in the clinical setting: Current Opinion in Endocrinology & Diabetes and Obesity *24*, 161–168. 10.1097/MED.0000000000000328.

205. Ralph, C.R., and Tilbrook, A.J. (2016). The hypothalamo-pituitary-adrenal (HPA) axis in sheep is attenuated during lactation in response to psychosocial and predator stress. Domestic Animal Endocrinology *55*, 66–73. 10.1016/j.domaniend.2015.11.003.

206. Long, N.M., Nathanielsz, P.W., and Ford, S.P. (2012). The impact of maternal overnutrition and obesity on hypothalamic-pituitary-adrenal axis response of offspring to stress. Domestic Animal Endocrinology *42*, 195–202. 10.1016/j.domaniend.2011.12.002.

207. Zhang, Z., Ji, M., Liao, Y., Yang, J., and Gao, J. (2018). Endotoxin tolerance induced by lipopolysaccharide preconditioning protects against surgery-induced cognitive impairment in aging mice. Mol Med Report. 10.3892/mmr.2018.8370.

208. Zila, I., Mokra, D., Kopincova, J., Kolomaznik, M., Javorka, M., and Calkovska, A. (2015). Heart rate variability and inflammatory response in rats with lipopolysaccharide-induced endotoxemia. Physiol Res *64 Suppl 5*, S669-676.

209. Kabaroff, L., Boermans, H., and Karrow, N.A. (2006). Changes in ovine maternal temperature, and serum cortisol and interleukin-6 concentrations after

challenge with Escherichia coli lipopolysaccharide during pregnancy and early lactation1. Journal of Animal Science *84*, 2083–2088. 10.2527/jas.2005-625.

210. McClure, L., O'Connor, A.E., Hayward, S., Jenkin, G., Walker, D.W., and Phillips, D.J. (2005). Effects of age and pregnancy on the circulatory activin response of sheep to acute inflammatory challenge by lipopolysaccharide. Journal of Endocrinology *185*, 139–149. 10.1677/joe.1.06051.

211. Tian, C., Cheng, L., and Gu, X. (2017). Cord blood TNF-α and IL-6 levels as diagnostic indicators of brain damage in neonates with non-asphyxia fetal distress. Arch Gynecol Obstet *295*, 337–342. 10.1007/s00404-016-4241-4.

212. Wattegedera, S.R., Corripio-Miyar, Y., Pang, Y., Frew, D., McNeilly, T.N., Palarea-Albaladejo, J., McInnes, C.J., Hope, J.C., Glass, E.J., and Entrican, G. (2017). Enhancing the toolbox to study IL-17A in cattle and sheep. Vet Res *48*, 20. 10.1186/s13567-017-0426-5.

213. Xu, D.-X., Wang, H., Zhao, L., Ning, H., Chen, Y.-H., and Zhang, C. (2007). Effects of low-dose lipopolysaccharide (LPS) pretreatment on LPS-induced intra-uterine fetal death and preterm labor. Toxicology *234*, 167–175. 10.1016/j.tox.2007.02.010.

214. Schlafer, D.H., Yuh, B., Foley, G.L., Elssaser, T.H., Sadowsky, D., and Nathanielsz, P.W. (1994). Effect of Salmonella Endotoxin Administered to the Pregnant Sheep at 133-142 Days Gestation on Fetal Oxygenation, Maternal and Fetal Adrenocorticotropic Hormone andCortisol, and Maternal Plasma Tufnor Necrosis Factor a Concentrations. Biology of reproduction, 1297–1302.

215. Samarasinghe, T.D., Sands, S.A., Skuza, E.M., Joshi, M.S., Nold-Petry, C.A., and Berger, P.J. (2015). The effect of prenatal maternal infection on respiratory function in mouse offspring: evidence for enhanced chemosensitivity. Journal of Applied Physiology *119*, 299–307. 10.1152/japplphysiol.01105.2014.

216. Wei, Y., Li, X., and Zhou, J. (2007). Prenatal exposure to lipopolysaccharide results in increases in blood pressure and body weight in rats. Acta Pharmacologica Sinica *28*, 651–656. 10.1111/j.1745-7254.2007.00593.x.

217. Qin, S., Chen, X., Gao, M., Zhou, J., and Li, X. (2017). Prenatal Exposure to Lipopolysaccharide Induces PTX3 Expression and Results in Obesity in Mouse Offspring. Inflammation *40*, 1847–1861. 10.1007/s10753-017-0626-1.

218. Bakos, J., Duncko, R., Makatsori, A., Pirnik, Z., Kiss, A., and Jezova, D. (2004). Prenatal Immune Challenge Affects Growth, Behavior, and Brain Dopamine in Offspring. Annals of the New York Academy of Sciences *1018*, 281–287. 10.1196/annals.1296.033.

219. Chen, Y., Mou, D., Hu, L., Zhen, J., Che, L., Fang, Z., Xu, S., Lin, Y., Feng, B., Li, J., et al. (2017). Effects of Maternal Low-Energy Diet during Gestation on Intestinal Morphology, Disaccharidase Activity, and Immune Response to Lipopolysaccharide Challenge in Pig Offspring. Nutrients *9*, 1115. 10.3390/nu9101115.

220. Basil, P., Li, Q., McAlonan, G.M., and Sham, P.-C. (2019). Genome-wide DNA methylation data from adult brain following prenatal immune activation and dietary intervention. Data in Brief *26*, 104561. 10.1016/j.dib.2019.104561.

221. Li, Q., Leung, Y.O., Zhou, I., Ho, L.C., Kong, W., Basil, P., Wei, R., Lam, S., Zhang, X., Law, A.C.K., et al. (2015). Dietary supplementation with n-3 fatty acids from weaning limits brain biochemistry and behavioural changes elicited by prenatal exposure to maternal inflammation in the mouse model. Transl Psychiatry *5*, e641–e641. 10.1038/tp.2015.126.

222. Kabadayi, C., Bobrowicz, K., and Osvath, M. (2018). The detour paradigm in animal cognition. Anim Cogn *21*, 21–35. 10.1007/s10071-017-1152-0.

223. Landry, O., and Al-Taie, S. (2016). A Meta-analysis of the Wisconsin Card Sort Task in Autism. J Autism Dev Disord *46*, 1220–1235. 10.1007/s10803-015-2659-3.

224. Coldren, J.T., and Halloran, C. (2003). Spatial Reversal as a Measure of Executive Functioning in Children With Autism. The Journal of Genetic Psychology *164*, 29–41. 10.1080/00221320309597501.

225. Lins, B.R., Hurtubise, J.L., Roebuck, A.J., Marks, W.N., Zabder, N.K., Scott, G.A., Greba, Q., Dawicki, W., Zhang, X., Rudulier, C.D., et al. (2018). Prospective Analysis of the Effects of Maternal Immune Activation on Rat Cytokines during Pregnancy and Behavior of the Male Offspring Relevant to Schizophrenia. eNeuro *5*. 10.1523/ENEURO.0249-18.2018.

226. Kleinmans, M., and Bilkey, D.K. (2018). Reversal learning impairments in the maternal immune activation rat model of schizophrenia. Behav. Neurosci. *132*, 520–525. 10.1037/bne0000275.

227. Han, X., Li, N., Meng, Q., Shao, F., and Wang, W. (2011). Maternal immune activation impairs reversal learning and increases serum tumor necrosis factor-α in offspring. Neuropsychobiology *64*, 9–14. 10.1159/000322455.

228. Wallace, J., Marston, H.M., McQuade, R., and Gartside, S.E. (2014). Evidence that aetiological risk factors for psychiatric disorders cause distinct patterns of cognitive deficits. Eur Neuropsychopharmacol *24*, 879–889. 10.1016/j.euroneuro.2013.12.005.

229. French, S.S., Chester, E.M., and Demas, G.E. (2013). Maternal immune activation affects litter success, size and neuroendocrine responses related to behavior in adult offspring. Physiology & Behavior *119*, 175–184. 10.1016/j.physbeh.2013.06.018.

230. Pedernera-Romano, C., Ruiz de la Torre, J.L., Badiella, L., and Manteca, X. (2010). Effect of perphenazine enanthate on open-field test behaviour and stress-induced hyperthermia in domestic sheep. Pharmacology Biochemistry and Behavior *94*, 329–332. 10.1016/j.pbb.2009.09.013.

231. Boissy, A., Fisher, A.D., Bouix, J., Hinch, G.N., and Le Neindre, P. (2005). Genetics of fear in ruminant livestock. Livestock Production Science *93*, 23–32. 10.1016/j.livprodsci.2004.11.003.

232. Karimi, P., Kamali, E., Mousavi, S., and Karahmadi, M. (2017). Environmental factors influencing the risk of autism. J Res Med Sci *22*, 27. 10.4103/1735-1995.200272.

233. Karimi, P., Kamali, E., Mousavi, S., and Karahmadi, M. (2017). Environmental factors influencing the risk of autism. J Res Med Sci *22*, 27. 10.4103/1735-1995.200272.

234. Choi, G.B., Yim, Y.S., Wong, H., Kim, S., Kim, H., Kim, S.V., Hoeffer, C.A., Littman, D.R., and Huh, J.R. (2016). The maternal interleukin-17a pathway in mice promotes autism-like phenotypes in offspring. Science *351*, 933–939. 10.1126/science.aad0314.

235. Sandin, S., Lichtenstein, P., Kuja-Halkola, R., Larsson, H., Hultman, C.M., and Reichenberg, A. (2014). The Familial Risk of Autism. JAMA *311*, 1770. 10.1001/jama.2014.4144.

236. Jeste, S.S., and Tuchman, R. (2015). Autism Spectrum Disorder and Epilepsy: Two Sides of the Same Coin? J Child Neurol *30*, 1963–1971. 10.1177/0883073815601501.

237. Perera, F., and Herbstman, J. (2011). Prenatal environmental exposures, epigenetics, and disease. Reproductive Toxicology *31*, 363–373. 10.1016/j.reprotox.2010.12.055.

238. Brown, A.S. (2012). Epidemiologic studies of exposure to prenatal infection and risk of schizophrenia and autism. Devel Neurobio *72*, 1272–1276. 10.1002/dneu.22024.

239. Rose, D.R., Careaga, M., Van de Water, J., McAllister, K., Bauman, M.D., and Ashwood, P. (2017). Long-term altered immune responses following fetal priming in a non-human primate model of maternal immune activation. Brain, Behavior, and Immunity *63*, 60–70. 10.1016/j.bbi.2016.11.020.

240. Gantert, M. (2010). Increased EEG delta frequency corresponds to chorioamnionitis-related brain injury. Front Biosci *S2*, 432–438. 10.2741/s76.

241. Wöhr, M. (2014). Ultrasonic vocalizations in Shank mouse models for autism spectrum disorders: Detailed spectrographic analyses and developmental profiles. Neuroscience & Biobehavioral Reviews *43*, 199–212. 10.1016/j.neubiorev.2014.03.021.

242. Moy, S., Nadler, J., Young, N., Perez, A., Holloway, L., Barbaro, R., Barbaro, J., Wilson, L., Threadgill, D., and Lauder, J. (2007). Mouse behavioral tasks relevant to autism: Phenotypes of 10 inbred strains. Behavioural Brain Research *176*, 4–20. 10.1016/j.bbr.2006.07.030.

243. McFarlane, H.G., Kusek, G.K., Yang, M., Phoenix, J.L., Bolivar, V.J., and Crawley, J.N. (2008). Autism-like behavioral phenotypes in BTBR T+tf/J mice. Genes Brain Behav *7*, 152–163. 10.1111/j.1601-183X.2007.00330.x.

244. Antonson, A.M., Radlowski, E.C., Lawson, M.A., Rytych, J.L., and Johnson, R.W. (2017). Maternal viral infection during pregnancy elicits anti-social behavior in neonatal piglet offspring independent of postnatal microglial cell activation. Brain, Behavior, and Immunity *59*, 300–312. 10.1016/j.bbi.2016.09.019.

245. McClure, L., O'Connor, A.E., Hayward, S., Jenkin, G., Walker, D.W., and Phillips, D.J. (2005). Effects of age and pregnancy on the circulatory activin response of sheep to acute inflammatory challenge by lipopolysaccharide. Journal of Endocrinology *185*, 139–149. 10.1677/joe.1.06051.

246. Kabaroff, L., Boermans, H., and Karrow, N.A. (2006). Changes in ovine maternal temperature, and serum cortisol and interleukin-6 concentrations after challenge with Escherichia coli lipopolysaccharide during pregnancy and early lactation1. Journal of Animal Science *84*, 2083–2088. 10.2527/jas.2005-625.

247. Onore, C., Careaga, M., and Ashwood, P. (2012). The role of immune dysfunction in the pathophysiology of autism. Brain, Behavior, and Immunity *26*, 383–392. 10.1016/j.bbi.2011.08.007.

248. Atladóttir, H.Ó., Thorsen, P., Østergaard, L., Schendel, D.E., Lemcke, S., Abdallah, M., and Parner, E.T. (2010). Maternal Infection Requiring Hospitalization During Pregnancy and Autism Spectrum Disorders. J Autism Dev Disord *40*, 1423–1430. 10.1007/s10803-010-1006-y.

249. Malkova, N.V., Yu, C.Z., Hsiao, E.Y., Moore, M.J., and Patterson, P.H. (2012). Maternal immune activation yields offspring displaying mouse versions of the three core symptoms of autism. Brain, Behavior, and Immunity *26*, 607–616. 10.1016/j.bbi.2012.01.011.

250. Bauman, M.D., Iosif, A.-M., Smith, S.E.P., Bregere, C., Amaral, D.G., and Patterson, P.H. (2014). Activation of the Maternal Immune System During Pregnancy Alters Behavioral Development of Rhesus Monkey Offspring. Biological Psychiatry *75*, 332–341. 10.1016/j.biopsych.2013.06.025.

251. Stoner, R., Chow, M.L., Boyle, M.P., Sunkin, S.M., Mouton, P.R., Roy, S., Wynshaw-Boris, A., Colamarino, S.A., Lein, E.S., and Courchesne, E. (2014). Patches of Disorganization in the Neocortex of Children with Autism. N Engl J Med *370*, 1209–1219. 10.1056/NEJMoa1307491.

252. Mestas, J., and Hughes, C.C.W. (2004). Of Mice and Not Men: Differences between Mouse and Human Immunology. J Immunol *172*, 2731–2738. 10.4049/jimmunol.172.5.2731.

253. Seok, J., Warren, H.S., Cuenca, A.G., Mindrinos, M.N., Baker, H.V., Xu, W., Richards, D.R., McDonald-Smith, G.P., Gao, H., Hennessy, L., et al. (2013). Genomic responses in mouse models poorly mimic human inflammatory diseases. Proc Natl Acad Sci USA *110*, 3507–3512. 10.1073/pnas.1222878110.

254. Hein, W.R., and Griebel, P.J. (2003). A road less travelled: large animal models in immunological research. Nat Rev Immunol *3*, 79–84. 10.1038/nri977.

255. Stryker, J.A., Fisher, R., You, Q., Or-Rashid, M.M., Boermans, H.J., Quinton, M., McBride, B.W., and Karrow, N.A. (2013). Effects of dietary fish meal and soybean meal on the ovine innate and acquired immune response during pregnancy and lactation. Animal *7*, 151–159. 10.1017/S175173111200136X.

256. Fisher, R.E., Or'Rashid, M., Quinton, M., AlZahal, O., Boermans, H.J., McBride, B.W., and Karrow, N.A. (2014). Maternal supplementation with fishmeal protects against late gestation endotoxin-induced fetal programming of the ovine hypothalamic-pituitary-adrenal axis. J Dev Orig Health Dis *5*, 206–213. 10.1017/S2040174414000191.

257. Gantert, M. (2010). Increased EEG delta frequency corresponds to chorioamnionitis-related brain injury. Front Biosci *S2*, 432–438. 10.2741/s76.

258. Crawley, J.N. (2012). Translational animal models of autism and neurodevelopmental disorders. Dialogues Clin Neurosci *14*, 293–305.

259. Gould, T.D., and Gottesman, I.I. (2006). Psychiatric endophenotypes and the development of valid animal models. Genes Brain Behav *5*, 113–119. 10.1111/j.1601-183X.2005.00186.x.

260. Coe, B.P., Witherspoon, K., Rosenfeld, J.A., van Bon, B.W.M., Vulto-van Silfhout, A.T., Bosco, P., Friend, K.L., Baker, C., Buono, S., Vissers, L.E.L.M., et al. (2014). Refining analyses of copy number variation identifies specific genes associated with developmental delay. Nat Genet *46*, 1063–1071. 10.1038/ng.3092.

261. Silverman, J.L., Yang, M., Lord, C., and Crawley, J.N. (2010). Behavioural phenotyping assays for mouse models of autism. Nat Rev Neurosci *11*, 490–502. 10.1038/nrn2851.

262. Arakawa, H., Arakawa, K., Blanchard, D.C., and Blanchard, R.J. (2008). A new test paradigm for social recognition evidenced by urinary scent marking behavior in C57BL/6J mice. Behavioural Brain Research *190*, 97–104. 10.1016/j.bbr.2008.02.009.

263. Roullet, F.I., Wöhr, M., and Crawley, J.N. (2011). Female urine-induced male mice ultrasonic vocalizations, but not scent-marking, is modulated by social experience. Behavioural Brain Research *216*, 19–28. 10.1016/j.bbr.2010.06.004.

264. Kozłowska, A., Wojtacha, P., Równiak, M., Kolenkiewicz, M., and Huang, A.C.W. (2019). ADHD pathogenesis in the immune, endocrine and nervous systems of juvenile and maturating SHR and WKY rats. Psychopharmacology *236*, 2937–2958. 10.1007/s00213-019-5180-0.

265. Lit, L., Schweitzer, J.B., Iosif, A.-M., and Oberbauer, A.M. (2010). Owner reports of attention, activity, and impulsivity in dogs: a replication study. Behav Brain Funct *6*, 1. 10.1186/1744-9081-6-1.

266. Wu, J., Platero Luengo, A., Gil, M., Suzuki, K., Cuello, C., Morales Valencia, M., Parrilla, I., Martinez, C., Nohalez, A., Roca, J., et al. (2016). Generation of human organs in pigs via interspecies blastocyst complementation. Reprod Dom Anim *51*, 18–24. 10.1111/rda.12796.

267. Caine, E., Jagger, B., and Diamond, M. (2018). Animal Models of Zika Virus Infection during Pregnancy. Viruses *10*, 598. 10.3390/v10110598.

268. Missault, S., Van den Eynde, K., Vanden Berghe, W., Fransen, E., Weeren, A., Timmermans, J.P., Kumar-Singh, S., and Dedeurwaerdere, S. (2014). The risk for behavioural deficits is determined by the maternal immune response to prenatal immune challenge in a neurodevelopmental model. Brain, Behavior, and Immunity *42*, 138–146. 10.1016/j.bbi.2014.06.013.

269. Long, N.M., Nathanielsz, P.W., and Ford, S.P. (2012). The impact of maternal overnutrition and obesity on hypothalamic-pituitary-adrenal axis response of offspring to stress. Domestic Animal Endocrinology *42*, 195–202. 10.1016/j.domaniend.2011.12.002.

270. Missault, S., Van den Eynde, K., Vanden Berghe, W., Fransen, E., Weeren, A., Timmermans, J.P., Kumar-Singh, S., and Dedeurwaerdere, S. (2014). The risk for behavioural deficits is determined by the maternal immune response to prenatal immune challenge in a neurodevelopmental model. Brain, Behavior, and Immunity *42*, 138–146. 10.1016/j.bbi.2014.06.013.

271. Lins, B.R., Marks, W.N., Zabder, N.K., Greba, Q., and Howland, J.G. (2019). Maternal Immune Activation during Pregnancy Alters the Behavior Profile of Female Offspring of Sprague Dawley Rats. eNeuro *6*, ENEURO.0437-18.2019. 10.1523/ENEURO.0437-18.2019.

272. Smith, S.E.P., Li, J., Garbett, K., Mirnics, K., and Patterson, P.H. (2007). Maternal Immune Activation Alters Fetal Brain Development through Interleukin-6. Journal of Neuroscience *27*, 10695–10702. 10.1523/JNEUROSCI.2178-07.2007.

273. Fisken, R., and Ward, R.D. (2019). Discrimination difficulty, cognitive burden, and reversal impairments in a maternal immune activation model of schizophrenia risk. Behav. Processes *167*, 103936. 10.1016/j.beproc.2019.103936.

274. Han, X., Li, N., Meng, Q., Shao, F., and Wang, W. (2011). Maternal Immune Activation Impairs Reversal Learning and Increases Serum Tumor Necrosis Factor-α in Offspring. Neuropsychobiology *64*, 9–14. 10.1159/000322455.

275. Epstein, D.H., Preston, K.L., Stewart, J., and Shaham, Y. (2006). Toward a model of drug relapse: an assessment of the validity of the reinstatement procedure. Psychopharmacology *189*, 1–16. 10.1007/s00213-006-0529-6.

276. Sarter, M., and Bruno, J.P. (2002). Animal Models in Biological Psychiatry. In Biological Psychiatry, H. D'Haenen, J. A. den Boer, and P. Willner, eds. (John Wiley & Sons, Ltd), pp. 37–44. 10.1002/0470854871.chiii.

277. Holmes, P.V. (2003). Rodent Models of Depression: Reexamining Validity Without Anthropomorphic Inference. Crit Rev Neurobiol *15*, 143–174. 10.1615/CritRevNeurobiol.v15.i2.30.

278. Silva, F. (1993). Psychometric foundations and behavioral assessment (Sage Publications).

279. Davis, K.L., and American College of Neuropsychopharmacology eds. (2002). Neuropsychopharmacology: the fifth generation of progress: an official publication of the American College of Neuropsychopharmacology (Lippincott Williams & Wilkins).

280. Geyer, M.A., and Moghaddam, B. (2002). Animal models relevant to schizophrenia disorders. In Neuropsychopharmacology: The Fifth Generation of Progress. (American College of Neuropsychopharmacology), pp. 689–701.

281. Wright, C. (2002). Animal models of depression in neuropsychopharmacology qua Feyerabendian philosophy of science. In Advances in psychology research (Nova Science Publishers), pp. 129–148.

282. Kaplan, R.M., and Saccuzzo, D.P. (1997). Psychological testing. Principles, applications, and issues (Pacific Grove: Brooks/Cole Publishing Company).

283. Noldus, L.P.J.J., Spink, A.J., and Tegelenbosch, R.A.J. (2001). EthoVision: A versatile video tracking system for automation of behavioral experiments. Behavior Research Methods, Instruments, & Computers *33*, 398–414. 10.3758/BF03195394.

284. Domadenik, A. (2018). Overview of current mouse models of autism and strategies for their development using CRISPR/Cas9 technology. Acta Agric. Slov. *112*, 19. 10.14720/aas.2018.112.1.3.

285. Zhang, J., Sadowska, G.B., Chen, X., Park, S.Y., Kim, J.-E., Bodge, C.A., Cummings, E., Lim, Y.-P., Makeyev, O., Besio, W.G., et al. (2015). Anti-IL-6 neutralizing antibody modulates blood-brain barrier function in the ovine fetus. FASEB J. *29*, 1739–1753. 10.1096/fj.14-258822.

286. Guzman, E., and Montoya, M. (2018). Contributions of Farm Animals to Immunology. Front. Vet. Sci. *5*, 307. 10.3389/fvets.2018.00307.

287. Galeana, L., Orihuela, A., Aguirre, V., and Vázquez, R. (2007). Mother-young spatial association and its relation with proximity to a fence separating ewes and lambs during enforced weaning in hair sheep (Ovis aries). Applied Animal Behaviour Science *108*, 81–88. 10.1016/j.applanim.2006.10.016.

288. Barnard, S., Matthews, L.R., Messori, S., Podaliri Vulpiani, M., and Ferri, N. (2015). Behavioural reactivity of ewes and lambs during partial and total social isolation. Applied Animal Behaviour Science *163*, 89–97. 10.1016/j.applanim.2014.11.016.

289. Vandenheede, M., Bouissou, M.F., and Picard, M. (1998). Interpretation of behavioural reactions of sheep towards fear-eliciting situations. Applied Animal Behaviour Science *58*, 293–310. 10.1016/S0168-1591(98)00088-4.

290. Viérin, M., and Bouissou, M.-F. (2003). Responses of weaned lambs to fear-eliciting situations: Origin of individual differences: Fear Reactions in Lambs. Dev. Psychobiol. *42*, 131–147. 10.1002/dev.10090.

291. Barnard, S., Matthews, L.R., Messori, S., Podaliri Vulpiani, M., and Ferri, N. (2015). Behavioural reactivity of ewes and lambs during partial and total social isolation. Applied Animal Behaviour Science *163*, 89–97. 10.1016/j.applanim.2014.11.016.

292. Dodd, C.L., Pitchford, W.S., Hocking Edwards, J.E., and Hazel, S.J. (2012). Measures of behavioural reactivity and their relationships with production traits in sheep: A review. Applied Animal Behaviour Science *140*, 1–15. 10.1016/j.applanim.2012.03.018.

293. Boissy, A., Bouix, J., Orgeur, P., Poindron, P., Bibé, B., and Le Neindre, P. (2005). Genetic analysis of emotional reactivity in sheep: effects of the genotypes of the lambs and of their dams. Genet Sel Evol *37*, 381. 10.1186/1297-9686-37-5-381.

294. Zimmerberg, B., Sukel, H.L., and Stekler, J.D. (1991). Spatial learning of adult rats with fetal alcohol exposure: deficits are sex-dependent. Behavioural Brain Research *42*, 49–56. 10.1016/S0166-4328(05)80039-7.

295. Wan, R.-Q., Pang, K., and Olton, D.S. (1994). Hippocampal and amygdaloid involvement in nonspatial and spatial working memory in rats: Effects of delay and interference. Behavioral Neuroscience *108*, 866–882. 10.1037/0735-7044.108.5.866.

296. Nagahara, A.H., and Handa, R.J. (1997). Fetal alcohol exposure produces delay-dependent memory deficits in juvenile and adult rats. Alcohol. Clin. Exp. Res. *21*, 710–715.

297. Olton, D.S., and Papas, B.C. (1979). Spatial memory and hippocampal function. Neuropsychologia *17*, 669–682. 10.1016/0028-3932(79)90042-3.

298. Riley, E.P., Lochry, E.A., Shapiro, N.R., and Baldwin, J. (1979). Response perseveration in rats exposed to alcohol prenatally. Pharmacology Biochemistry and Behavior *10*, 255–259. 10.1016/0091-3057(79)90097-2.

299. Davis, K.E., Burnett, K., and Gigg, J. (2017). Water and T-maze protocols are equally efficient methods to assess spatial memory in 3xTg Alzheimer's disease mice. Behavioural Brain Research *331*, 54–66. 10.1016/j.bbr.2017.05.005.

300. Deibel, S.H., Lehr, A.B., Maloney, C., Ingram, M.L., Lewis, L.M., Chaulk, A.-M.P., Chaulk, P.D., Skinner, D.M., and Thorpe, C.M. (2017). Rats in a levered T-maze task show evidence of time–place discriminations in two different measures. Learn Behav *45*, 184–190. 10.3758/s13420-016-0254-x.

301. Park, J.-J., and Baum, M.J. (1999). Intracerebroventricular infusion of the galanin antagonist M40 attenuates heterosexual partner preference in ferrets. Behavioral Neuroscience *113*, 391–400. 10.1037/0735-7044.113.2.391.

302. Burgess, J.W., Villablanca, J.R., and Levine, M.S. (1986). Recovery of functions after neonatal or adult hemispherectomy in cats. III. Complex functions: Open field exploration, social interactions, maze and holeboard performances. Behavioural Brain Research *20*, 217–230. 10.1016/0166-4328(86)90005-7.

303. Peretti, P.O., and Baird, M. (1976). Experimental Studies on Food Selective Behavior in Squirrel Monkeys Fed on Riboflavin Deficient Diet. The Journal of General Psychology *94*, 135–144. 10.1080/00221309.1976.9711599.

304. Heird, J.C., Lennon, A.M., and Bell, R.W. (1981). Effects of Early Experience on the Learning Ability of Yearling Horses. Journal of Animal Science *53*, 1204–1209. 10.2527/jas1981.5351204x.

305. Johnson, T.B., Stanton, M.E., Goodlett, C.R., and Cudd, T.A. (2012). T-maze learning in weanling lambs. Dev. Psychobiol. *54*, 785–797. 10.1002/dev.20624.

306. Hosoi, E., Swift, D.M., Rittenhouse, L.R., and Richards, R.W. (1995). Comparative foraging strategies of sheep and goats in a T-maze apparatus. Applied Animal Behaviour Science *44*, 37–45. 10.1016/0168-1591(95)00572-A.

307. Elmore, M.R.P., Dilger, R.N., and Johnson, R.W. (2012). Place and direction learning in a spatial T-maze task by neonatal piglets. Anim Cogn *15*, 667–676. 10.1007/s10071-012-0495-9.

308. Köhler, W. (1925). Köhler W. The mentality of apes. London: Routledge and Kegan Paul; 1925.

309. Köhler, W., and Winter, E. (2019). The mentality of apes.

310. Smith, B.P., and Litchfield, C.A. (2010). How well do dingoes, Canis dingo, perform on the detour task? Animal Behaviour *80*, 155–162. 10.1016/j.anbehav.2010.04.017.

311. Ellen, P., and Thinus-Blanc, C. (1987). Cognitive Processes and Spatial Orientation in Animal and Man Volume I Experimental Animal Psychology and Ethology (Springer Netherlands).

312. Vlamings, P.H.J.M., Hare, B., and Call, J. (2010). Reaching around barriers: the performance of the great apes and 3–5-year-old children. Anim Cogn *13*, 273–285. 10.1007/s10071-009-0265-5.

313. Diamond, A. (1990). Developmental Time Course in Human Infants and Infant Monkeys, and the Neural Bases of, Inhibitory Control in Reaching. Ann NY Acad Sci *608*, 637–676. 10.1111/j.1749-6632.1990.tb48913.x.

314. Wallis, J.D., Dias, R., Robbins, T.W., and Roberts, A.C. (2001). Dissociable contributions of the orbitofrontal and lateral prefrontal cortex of the marmoset to performance on a detour reaching task: Dissociable PFC contributions to detour reaching. European Journal of Neuroscience *13*, 1797–1808. 10.1046/j.0953-816x.2001.01546.x.

315. Uslaner, J.M., Eddins, D., Puri, V., Cannon, C.E., Sutcliffe, J., Chew, C.S., Pearson, M., Vivian, J.A., Chang, R.K., Ray, W.J., et al. (2013). The muscarinic M1 receptor positive allosteric modulator PQCA improves cognitive measures in rat, cynomolgus macaque, and rhesus macaque. Psychopharmacology *225*, 21–30. 10.1007/s00213-012-2788-8.

316. Taylor, J.R., Elsworth, J.D., Roth, R.H., Sladek, J.R., and Redmond, D.E. (1990). COGNITIVE AND MOTOR DEFICITS IN THE ACQUISITION OF AN OBJECT RETRIEVAL/DETOUR TASK IN MPTP-TREATED MONKEYS. Brain *113*, 617–637. 10.1093/brain/113.3.617.

317. Taylor, J.R., Roth, R.H., Sladek, J.R., and Redmond, D.E. (1990). Cognitive and motor deficits in the performance of an object retrieval task with a barrier-detour in monkeys (Cercopithecus aethiops sabaeus) treated with MPTP: Long-term performance and effect of transparency of the barrier. Behavioral Neuroscience *104*, 564–576. 10.1037/0735-7044.104.4.564.

318. Roitberg, B.Z., Emborg, M.E., Sramek, J.G., Palfi, S., and Kordower, J.H. (2002). Behavioral and Morphological Comparison of Two Nonhuman Primate Models of Huntington's Disease. Neurosurgery *50*, 137–146. 10.1097/00006123-200201000-00022.

319. Cannon, C.E., Puri, V., Vivian, J.A., Egbertson, M.S., Eddins, D., and Uslaner, J.M. (2013). The nicotinic α7 receptor agonist GTS-21 improves cognitive performance in ketamine impaired rhesus monkeys. Neuropharmacology *64*, 191–196. 10.1016/j.neuropharm.2012.05.003.

320. Jentsch, J.D., Roth, R.H., and Taylor, J.R. (2000). Object retrieval/detour deficits in monkeys produced by prior subchronic phencyclidine administration: evidence for cognitive impulsivity. Biological Psychiatry *48*, 415–424. 10.1016/S0006-3223(00)00926-4.

321. Jentsch, J.D., Taylor, J.R., Elsworth, J.D., Redmond, D.E., and Roth, R.H. (1999). Altered frontal cortical dopaminergic transmission in monkeys after subchronic phencyclidine exposure: involvement in frontostriatal cognitive deficits. Neuroscience *90*, 823–832. 10.1016/S0306-4522(98)00481-3.

322. Jentsch, J.D., Taylor, J.R., Redmond Jr, D.E., Elsworth, J.D., Youngren, K.D., and Roth, R.H. (1999). Dopamine D 4 receptor antagonist reversal of subchronic phencyclidine-induced object retrieval/detour deficits in monkeys. Psychopharmacology *142*, 78–84. 10.1007/s002130050865.

323. Smith, S.M., Uslaner, J.M., Cox, C.D., Huszar, S.L., Cannon, C.E., Vardigan, J.D., Eddins, D., Toolan, D.M., Kandebo, M., Yao, L., et al. (2013). The novel phosphodiesterase 10A inhibitor THPP-1 has antipsychotic-like effects in rat

and improves cognition in rat and rhesus monkey. Neuropharmacology *64*, 215–223. 10.1016/j.neuropharm.2012.06.013.

324. Jentsch, J.D. (1997). Enduring Cognitive Deficits and Cortical Dopamine Dysfunction in Monkeys After Long-Term Administration of Phencyclidine. Science *277*, 953–955. 10.1126/science.277.5328.953.

325. Edgin, J.O., Inder, T.E., Anderson, P.J., Hood, K.M., Clark, C.A.C., and Woodward, L.J. (2008). Executive functioning in preschool children born very preterm: Relationship with early white matter pathology. J Int Neuropsychol Soc *14*, 90–101. 10.1017/S1355617708080053.

326. Hughes, C., and Russell, J. (1993). Autistic children's difficulty with mental disengagement from an object: Its implications for theories of autism. Developmental Psychology *29*, 498–510. 10.1037/0012-1649.29.3.498.

327. Lockman, J.J. (1984). The development of detour ability during infancy. Child Dev *55*, 482–491.

328. Wynne, C.D.L., and Leguet, B. (2004). Detour behavior in the Quokka (Setonix brachyurus). Behavioural Processes *67*, 281–286. 10.1016/j.beproc.2004.04.007.

329. Frank, H., and Frank, M.G. (1982). Comparison of problem-solving performance in six-week-old wolves and dogs. Animal Behaviour *30*, 95–98. 10.1016/S0003-3472(82)80241-8.

330. Pongrácz, P., Miklósi, Á., Kubinyi, E., Gurobi, K., Topál, J., and Csányi, V. (2001). Social learning in dogs: the effect of a human demonstrator on the performance of dogs in a detour task. Animal Behaviour *62*, 1109–1117. 10.1006/anbe.2001.1866.

331. Lockman, J.J. (1987). Detour ability in infants and toddlers. In Cognitive Processes and Spatial Orientation in Animal and Man (Dordrecht: Martinus Nijhoff Publishers), pp. 250–256.

332. Lacreuse, A., Chang, J., Metevier, C.M., LaClair, M., Meyer, J.S., and Ferris, C.M. (2014). Oestradiol Modulation of Cognition in Adult Female Marmosets (*Callithrix jacchus*). J Neuroendocrinol *26*, 296–309. 10.1111/jne.12147.

333. Pryce, C.R., Dettling, A., Spengler, M., Spaete, C., and Feldon, J. (2004). Evidence for Altered Monoamine Activity and Emotional and Cognitive Disturbance in Marmoset Monkeys Exposed to Early Life Stress. Annals of the New York Academy of Sciences *1032*, 245–249. 10.1196/annals.1314.030.

334. Meyer, U., Feldon, J., Schedlowski, M., and Yee, B.K. (2005). Towards an immuno-precipitated neurodevelopmental animal model of schizophrenia. Neuroscience & Biobehavioral Reviews *29*, 913–947. 10.1016/j.neubiorev.2004.10.012.

335. Bissonette, G.B., and Powell, E.M. (2012). Reversal learning and attentional set-shifting in mice. Neuropharmacology *62*, 1168–1174. 10.1016/j.neuropharm.2011.03.011.

336. Tait, D., Chase, E., and Brown, V. (2014). Attentional Set-Shifting in Rodents: A Review of Behavioural Methods and Pharmacological Results. CPD *20*, 5046–5059. 10.2174/1381612819666131216115802.

337. Barak, S., and Weiner, I. (2011). Putative cognitive enhancers in preclinical models related to schizophrenia: The search for an elusive target. Pharmacology Biochemistry and Behavior *99*, 164–189. 10.1016/j.pbb.2011.03.011.

338. South, M., Newton, T., and Chamberlain, P.D. (2012). Delayed Reversal Learning and Association With Repetitive Behavior in Autism Spectrum Disorders: Reversal learning in autism. Autism Res *5*, 398–406. 10.1002/aur.1255.

339. Reddy, L.F., Waltz, J.A., Green, M.F., Wynn, J.K., and Horan, W.P. (2016). Probabilistic Reversal Learning in Schizophrenia: Stability of Deficits and Potential Causal Mechanisms. SCHBUL *42*, 942–951. 10.1093/schbul/sbv226.