A SINGLE PRENATAL EXPOSURE TO POLY I:C INCREASES SUSCEPTIBILITY TO EPILEPSY IN THE ADULT OFFSPRING

Bio/14

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Anno accademico 2015-2016
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ABSTRACT

In the last years, evidence accumulated showing a direct connection between brain inflammation and neurodevelopmental disorders such as autism and schizophrenia. Epilepsy and seizures episodes, in particular, are associated to enhanced brain inflammation, while the activation of the immune response consequent to infections strongly increases the risk of seizures.

By using the Poly I:C (polyinosinic-polycytidylic acid) mouse model of maternal immune activation (MIA), we demonstrated that a single administration at gestational day 9 (GD9) is able to affect the glutamate-GABA equilibrium in the offspring through a long-lasting deregulation of the chloride transporter KCC2 at cortical level, resulting in an alteration of the hyperpolarizing action of GABA, which endures at mature stages, as highlighted by the increased seizure susceptibility. Furthermore, mice injected with Poly I:C during adult life show no differences in susceptibility to kainate-induced seizures respect to control mice, thus providing the evidence that the increased susceptibility to seizures following prenatal Poly I:C exposure is the consequence of a neurodevelopmental process. We also provide the proof-of-concept that KCC2 expression abnormality and its deleterious physiological consequences can be prevented by dietary maternal supplementation with MgSO₄, already known to reduce inflammation at the maternal-fetal interface.

Notably, the increased binding of the two master regulators of neuronal genes expression, REST and MeCP2, on KCC2 promoter, suggests a possible epigenetic mechanism involved in the regulation of KCC2 expression following inflammation in the mother. Thus, maternal immune activation, through pro-inflammatory cytokines, may lead to epigenetic modifications responsible for KCC2 dysregulation and the consequent pathological outcomes, as suggested also by in vitro experiments.
Negli ultimi anni, numerose evidenze hanno dimostrato come esista un collegamento diretto tra infiammazione nel cervello e disturbi del neurosviluppo, tra cui autismo e schizofrenia. Epilessia e convulsioni, in particolare, sono associati a un’aumentata infiammazione a livello cerebrale, mentre l’attivazione della risposta immunitaria in seguito a infezioni aumenta fortemente il rischio di sviluppare episodi convulsivi.

Mediante l’iniezione di acido polinosinico-policitidilico (Poly I:C), un RNA sintetico a doppio filamento, utilizzato come modello di attivazione immunitaria materna nel topo, siamo stati in grado di dimostrare come una singola somministrazione al nono giorno di gestazione sia in grado di alterare l’equilibrio tra glutammato e GABA nella prole attraverso la deregolazione del trasportatore ionico KCC2, coinvolto nel mantenimento dell’omeostasi dello ione cloruro a livello neuronale, con la conseguente alterazione dell’azione iperpolarizzante del GABA, che perdura nelle fasi mature, come evidenziato dalla maggiore susceptibilità alle convulsioni indotte da acido kainico osservata nella prole adulta. Inoltre, i topi iniettati con la stessa dose di Poly I:C in età adulta non mostrano un’aumentata susceptibility alle crisi epilettiche indotte dall’iniezione di acido kainico rispetto ai topi di controllo, fornendo così la prova che l’aumento della susceptibility alle crisi epilettiche in seguito alla somministrazione prenatale di Poly I:C è la conseguenza di alterazioni a livello del neurosviluppo. Inoltre, siamo stati in grado di dimostrare che le anomalie riscontrate nell’espressione di KCC2 e le sue deleterie conseguenze a livello fisiologico possono essere prevenute grazie all’integrazione, nella dieta materna, di MgSO₄, già noto in letteratura per la sua capacità di ridurre l’infiammazione all’interfaccia materno-fetale.

Inoltre, l’aumento del legame di REST e MeCP2, due fondamentali regolatori dell’espressione di geni neuronal, al promotore del gene codificante per KCC2, suggerisce un possibile meccanismo epigenetico coinvolto nella regolazione dell’espressione di KCC2 nella prole, in seguito a infiammazione prenatale. L’attivazione del sistema immunitario materno, attraverso la produzione di citochine pro-infiammatorie, può quindi portare a modificazioni epigenetiche.
responsabili delle alterazioni dell’espressione di KCC2 e dei conseguenti esiti patologici, come suggerito anche da esperimenti in vitro condotti su colture neuronali primarie ottenute da embrioni sottoposti a Poly I:C prenatale.
Chapter 1

MATERNAL IMMUNE ACTIVATION: IMPLICATIONS AND MODELS

Epidemiological evidence
The developing brain is highly sensitive to several environmental insults, such as maternal stress and nutritional deficiencies, that can induce damaging effects in the future child [1]. Among these insults, infection-induced maternal immune activation (MIA) during pregnancy represent one of the most dangerous. Indeed, MIA can contribute to the onset of causal chains of events potentially leading to several pathophysiological changes in the fetal environment that can contribute to postnatal brain dysfunctions at different ages in the progeny (Figure 1).

Figure 1. Causal chain of events following MIA in humans. MIA can lead to a variety of neuronal dysfunctions and behavioural phenotypes observable in the juvenile, adult or aged progeny. Modified from Knuesel et al. 2014.

Several epidemiological studies, that all together examined from 86000 to 124000 children, confirmed that infections occurring during pregnancy, caused by different pathogenic agents, are associated with an increased incidence of psychiatric and neurological disorders in the offspring. These include neurodevelopmental
disorders such as schizophrenia, autism and epilepsy, as well as neurodegenerative pathologies like Alzheimer and Parkinson’s diseases. Thus, inflammatory processes in the mother can strongly impact on neuronal circuits development in the fetus and the nature, the intensity, the timing and the modality of the exposure to the inflammatory stimulus, together with genetic predispositions, can define the cluster of symptoms and consequently the phenotype of the neurological disorder that the future child can face.

Regarding schizophrenia, that together with autism seems to be particularly sensitive to early inflammatory insults to the central nervous system (CNS), strong epidemiological associations have been found between various infectious agents, including influenza virus, and the number of schizophrenia cases in the progeny. In a paper from Brow et al. published in 2004 [2], it is shown that the risk of developing schizophrenia in the progeny is increased 7-fold following influenza virus exposure during early phases of pregnancy. Increased levels of influenza antibodies and pro-inflammatory cytokines, such as IL6, TNF, CXCL8 [3] as well as C-reactive protein [4] in the mothers serum were strongly associated with the increased risk for schizophrenia in the children. Thanks to these kind of retrospective studies it has been possible to estimate that 14-21% of whole cases of schizophrenia could be avoidable if we could prevent infections in the mothers during pregnancy [5].

Influenza virus is not the only pathogenic agent that shows strong correlation with this pathology; Toxoplasma gondii [6], herpes simplex virus type 2 [7] as well as urinary tract infection and other types of infections have been found to have a strong epidemiological association with schizophrenia when contracted during pregnancy. As for schizophrenia, epidemiological studies support the association between Autistic Spectrum Disorders (ASD) and different pathogenic agents [8, 9]. Birth cohort studies have associated ASD in children with inflammatory markers found in utero [10] and/or maternal hospitalization for infections, as well as with the presence, in the maternal blood, of antibodies against fetal brain antigens [11-13]. Moreover, the CHARGE trial, a big case-control study linked to the University of California–Davis Center for Children’s Environmental Health laboratories, found that women who contracted influenza during pregnancy are more prone to have children with ASD [14]. Moreover, the “winter baby” phenomenon, reported by the California Department of Developmental Services, describes a higher risk for autism
spectrum disorders in babies conceived during the cold winter months [15], possibly due to higher infection rates in the mothers during this period.

**Animal models of MIA**

Epidemiology alone is not able to identify causal links between maternal immune activation and the risk of neurodevelopmental and neurological disorders in the offspring. On the other hand, clinical research is limited in the possibility to identify the molecular pathways involved because of the ethical and technical impossibility to perform invasive experimentation on humans. Furthermore, there is not a successful and valid way to identify at-risk pregnancies in order to investigate specific aspects of infections during pregnancy. Often at-risk pregnancies are symptomless and in any case most of times they lead to healthy children, in which symptoms of CNS disorders appears only after some years, usually exacerbated by “second hits” [16, 17]. Thus, preclinical research based on animal models results essential to overcome these limiting problems and it is the best reason why neurological disorders epidemiology needs neurobiology, as discussed particularly for schizophrenia in a paper from McGrath and Richard in 2009 [18]. Experimental research in animals represents a unique opportunity to identify causal downstream cellular and molecular mechanisms involved, possible diagnostic tools and pharmacological therapies. Indeed, a causal relationship between maternal immune activation and autism/schizophrenia-related behavioural alterations has been clearly demonstrated by using rodent models of MIA and, more recently, non-human primate models.

Generally, these models consist in the administration of specific infectious and inflammatory agents in pregnant animals at specific gestational stages. Fatemi et al. [19] were the first to develop an experimental model of exposure to human influenza virus in mice, consisting in a single intranasal administration of the virus in pregnant dams at gestational day 9. The offspring was then evaluated for behavioural and brain long-term effects respect to control mice. Subsequently, this model was also used to study the impact of prenatal timing by administering the virus at different gestational days [19]. Thanks to this pioneer mouse model, scientists could identify several neuropathological sings in the offspring brains postnatally and interestingly some of these signs were found to be depending upon
timing of prenatal immune activation. These pathological signs include: deficient corticogenesis and brain atrophy, impaired development of the corpus callosum, reduced hippocampal size and decreased expression of GABAergic markers such as Reelin [20, 21], as well as alterations in serotonin production [22]. Exposure of the mother to the human influenza virus also leads to behavioural and cognitive abnormalities in the adult offspring, some of which, like for example reduced sensorimotor gating, social behaviour and exploratory behaviour, are highly relevant for schizophrenia and related psychotic illnesses [23]. This translational research was then extended to non-human primates, in particular to rhesus monkeys, where prenatal corticogenesis is more advanced as compared with mice. Therefore, primate models helped to confirm the relevance of findings obtained in rodents and taken together all the information collected can be taken as experimental evidence supporting causal effects of prenatal infection with influenza virus in long lasting brain defects.

More recently, new animal models of prenatal immune activation have been developed in which the use of living viral or bacterial pathogens have been replaced by immune-activating compounds [23]. These compounds are able to induce a cytokine-associated immune response and were initially used to study the possible key role of cytokines in mediating the link between maternal immune activation and abnormal brain development in the offspring [24, 25]. These “simplified” models, which include also the administration of individual cytokines, enable a more direct study and comprehension of the molecular mechanisms underlying long-lasting modifications in the offspring brains after MIA. They offer the possibility to monitor the response to the compounds by appropriate dose-response and time-dependent studies, thanks to their time-limited effect, usually 24-48 hours from the administration [26, 27]. Moreover, it is important to consider that these compounds, like LPS and Poly I:C, two of the most used, are commercially available and they can be easily handled without particular biosafety precautions. In general, each MIA animal model should possess three important characteristics: face validity – they have to show pathological signs similar to the ones that are found in humans, construct validity – they should have an etiology similar to the human pathology, predictive validity – they should respond to treatments that are already successful in humans [28].
The Poly I:C model of MIA

One of the most commonly used approaches nowadays is represented by the maternal administration of Poly I:C (polyinosinic:polycytidylic acid), a synthetic analogue of double strand RNA that mimics the response to viral infections. During viral infections, double strand RNA is generated as a replication intermediate for single strand RNA. Through the binding to the specific toll-like receptor 3 (TLR3), a member of the super-family of pathogen recognition receptors, Poly I:C leads to the expression of several genes and proteins linked to the innate immune response; different pro-inflammatory molecules including cytokines (IL6 and IL1beta), TNF-alfa as well as type I interferons are induced by Poly I:C administration [27, 29] (Figure 2).

Figure 2. The Poly I:C MIA model in rodents. Responses in the maternal compartment include systemic immune activation characterized by increased levels of proinflammatory cytokines. Consequently, the integrity of the placental barrier could be compromised, allowing entrance of maternally derived cytokines into the fetal circulation and inducing inflammatory responses in the developing fetus, including the brain. This leads to structural and developmental dysfunctions associated with various neuropsychiatric diseases. Modified from Reisinger et al. 2015.
Therefore, Poly I:C efficiently mimics the acute phase response to viral infections, leading to significant inflammatory processes in the mother and consequently in the fetus, when administered systemically [30]. Poly I:C-induced MIA leads to numerous structural, neurochemical but also behavioural and cognitive alterations in adult offspring overlapping with well-known pathophysiological features of psychotic disorders. An interesting aspect to be taken into account is that especially behavioural, cognitive and pharmacological features appear in the offspring only in late adolescence/early adulthood [31, 32], consistent with the post-pubertal onset of psychotic behaviour in schizophrenic patients [33]. Another important aspect of Poly I:C prenatal immune activation is that the severity of the phenotype, in terms of long-term brain and behavioural changes, is strongly dependent upon the intensity of the cytokines-associate immune response [25, 34]. Intense prenatal stimuli are associated with more severe brain abnormalities in the offspring [35] and there are suggestions indicating that a threshold of immune activation is required to induce long-term brain and behavioural changes in the offspring. It is possible that low doses of prenatal Poly I:C may not induce a sufficient maternal cytokine response able to drive the behavioural features in the offspring. This may explain why not all humans exposed to a prenatal infection develop schizophrenia in adulthood [36, 37]. Thus, it could be reasonable to think of Poly I:C and other compounds used in MIA animal models as models of particular neurobehavioral phenotypes, rather than models for specific mental illness [38]. Furthermore, just as Tan et al. wrote in the incipit of their paper in 2008 “genes do not encode for psychopathology” [39] and harmful environmental stimuli (such as prenatal infection) alone do not lead to mental illness. Rather, they can be seen as events which are associated with an increased frequency of alterations in brain morphology and function, underlying particular behavioural manifestations, which are generally associated with particular neuropsychiatric disorders.

**MIA as a disease primer for neurological disorders**

It is well established that in humans most of maternal infections alone are not able to induce neuropsychiatric disorders in the offspring. It is reasonable to consider these pathologies as “multiple hits pathologies”, in which a multiple exposure to more than one risk factor may be necessary for the disease onset [40].
Converging recent findings suggest that MIA can act as a “neurodevelopmental disease primer” making an individual more susceptible to the effect of genetic mutations or environmental stimuli in driving disease-related symptoms later in life [41]. Consistent with this hypothesis, epidemiological data indicate that the incidence of both schizophrenia and ASD is increased in families with auto-immune disorders [42] and the effect of maternal infections is greater in families with a history of schizophrenia [43]. Moreover, low doses of Poly I:C in animal models synergize with mutations in schizophrenia/autism-linked genes including DISC1 (Disrupted in schizophrenia 1), NRG1 (neuregulin1), NR4A2 (Nurr1) and TSC2 (Tuberin), determining a greater effect compared to the inflammatory stimulus alone [17, 44]. Studies of interaction between MIA and environmental risk factors suggest that even subclinical maternal infections can render the offspring more susceptible to second hits. For example, peri-pubertal stress can have synergetic effects with subclinical MIA in a wide range of schizophrenia/autism-related phenotypes [44] and also cannabis exposure during adolescence can exacerbate subclinical effects of MIA in the offspring [45]. Although the molecular basis of MIA are still unclarified, specific epigenetic alterations in loci related to neuropsychiatric disorders (such as DISC1), consequent to prenatal inflammation, may represent a molecular signature of its priming effect [46, 47].

All considered, two are the possible scenarios by which MIA can work: early-life immune challenge may act as disease primer in offspring with genetic predisposition by exacerbating brain and behavioural abnormalities [48]; secondly MIA may render the offspring more susceptible to the pathological effects of a second postnatal hit [49].

**Timing of prenatal immune activation**

Pregnancy in humans represents a paradox for the maternal immune system which has to protect the mother from infections but at the same time can potentially attack the antigenically foreign fetus [50]. Thus, the maternal immune system exhibits considerable fluctuations as pregnancy proceeds and the changes in cytokine production following an inflammatory stimulus, may therefore differentially impact the developing fetus. This make reasonable to think that depending on the gestational period the developing nervous system of the fetus may not be uniformly
vulnerable to maternal infections. Indeed, several epidemiological studies identified the time-window that goes from the end of the first trimester to the beginning of the second one critical to viral exposure, that could possibly lead to the development of neuropsychiatric disorders in the post-pubertal period [51]. For example, a study of registers, “the Danish medical birth register”, highlighted that maternal infections contracted within the first trimester and resulting in the hospitalization of the pregnant woman, correspond to a 3-fold increase in the incidence of ASD in the progeny [52].

Moreover, considering the highly complex and precisely orchestrated processes involved in fetal neurodevelopment, starting with proliferation and migration of cells, followed by synaptogenesis, myelination but also synapses remodelling through pruning, formation of neural circuits and so on, inflammation in the mother can affect several important aspects of brain development depending upon time of maternal immune activation (Figure 3).

![Figure 3. Steps of human brain development from gestation to adult life. Modified from Knuesel et al. 2014.](image)

Thus, MIA animal models represent a very suitable tool to investigate the impact of infection exposure at different gestational stages on fetal neurodevelopment. This can be easily achieved by exposing pregnant animals to the infectious stimulus at
different times during pregnancy. Because the effects of compounds such as Poly I:C are time-limited (24-48 h depending on the dosage), it results quite easy to investigate the impact of MIA on a specific period of fetal development. Indeed, the gestational stage at which MIA is induced is crucial for the pathological outcome in the offspring; since each gestational period correspond to specific processes involved in the maturation of the CNS. Some anomalous response to classic conditioning protocols such as prepulse inhibition and latent inhibition are associated with schizophrenia and occur usually after the exposure to Poly I:C at gestational day 9, that correspond to the first trimester in women. Instead, defects in cognitive flexibility are more common in subjects exposed to MIA later, during the third trimester of pregnancy [30, 53].

**Acute and long-term effects of prenatal immune activation on fetal brain**

**Cytokine expression in the fetal brain**

It seems no longer a matter of debate that MIA is able to trigger unequivocal inflammatory responses in the mother that could influence fetal CNS development.

However, which component of the acute inflammatory response might be required as mediator of MIA remains an open question. Is an alteration in a specific maternal cytokine, or other inflammatory mediators, fever, etc. necessary to produce effects on the fetal brain? It has been demonstrated that the administration of Poly I:C or LPS to pregnant rodents can clearly increase maternal levels of serum cytokines, including IL-1β, IL-6 and TNFα, and produce fever [30, 54-56]. Moreover, not only the production of pro-inflammatory compounds in the maternal compartment but also changes in cytokines and other mediators at the maternal-fetal interface (the placenta) could be possible mediators MIA-induced changes in fetal brain development and functioning later in life. Inflammation-induced alterations of the placenta may have detrimental consequences on fetal brain development, because increased levels of inflammatory cytokines, such as IL-6 and TNFα, in the placenta, have been implicated in placental dysfunction and trophoblast apoptosis [57-59]. Of course, investigating acute neurochemical changes in the fetal brain results very important in understanding mechanisms mediating long-term changes in the offspring CNS following MIA. One of the most studied parameter in fetuses is
cytokine expression in the fetal brain. Indeed, altered mRNA levels for IL-6, IL-1β and TNFα, the main cytokines involved in the maternal immune response following Poly I:C or LPS administration, were found in fetal brains few hours after MIA challenge [30, 60]. Interestingly, IL-6, which is increased in maternal serum, amniotic fluid, placental tissue and fetal brain, can induced MIA by itself, leading to the same behavioural abnormalities observed upon viral infections, Poly I:C or LPS [61]. Moreover, the combined administration of IL-6 neutralizing antibody and Poly I:C in pregnant dams, prevents behavioural abnormalities and normalizes brain gene expression variations observed upon MIA challenge. Interestingly, IL-6 KO mice challenged with MIA do not display behavioural changes typically observed in MIA offspring [61]. Although lots of studies on MIA animal models provide evidence that MIA could trigger changes in cytokine profile in the fetus, more research needs to be done to explain how these changes are linked to MIA neuropathologies and how cytokines might affect fetal brain development. The ultimate challenge it would be to identify specific immune signatures related to specific disease-related neurodevelopmental and behavioural alterations.

**Synaptic development and neurotransmission**

Cytokines are known to regulate the expression of other immune molecules in the brain, including MHC class 1 molecules [40]. In the immune system MHCI is regulated by cytokines at the very early steps of the immune response while in healthy brains MHCI negatively regulates synapse formation and synaptic plasticity during their initial establishment in neurons [62, 63]. Indeed, alterations in synapse formation and synaptic pruning are associated with a range of neurodevelopmental diseases and are increasingly thought to be involved in the etiology of schizophrenia and ASD [64, 65]. MIA is able to determine a dramatic change in MHCI levels in neurons in the new-born offspring [66], and this seems to be involved in the MIA-induced defective ability of newly born neurons to form synapses and to create the correct connectivity. In fact, neurons from rats exposed to MIA present increased levels of MHCI accompanied by a reduced number of synapses and an impaired neural connectivity [67]. Since MHCI is able to mediate responses to infection in neurons and simultaneously to regulate the activity-dependent plasticity and the circuit formation, these findings support the idea that deregulated MHCI proteins
may mediate synaptic dysfunctions and cognitive impairments, although studies on this topic have to be strongly improved.

**Transcriptional and epigenetic modification following MIA**

Fatemi et al., in 2005, were the first to describe transcriptional alterations in neonatal brains upon viral infection in a mouse model of MIA. These alterations primarily affect genes involved in basic cell functions. In particular, they showed the upregulation of 21 genes and downregulation of 18 genes in the affected neonatal brain, including cytosolic chaperone system, HSC70, bicaudal-D, aquaporin 4, carbonic anhydrase 3, glycine receptor, norepinephrine transporter and myelin basic protein. For the first time they demonstrated that prenatal human influenza infection at GD9 leads to alterations in a subset of genes in the brain of exposed offspring, potentially leading to permanent changes in brain structure and functions [68]. A great history of studies has focused on early life programming as a source of adult mental illnesses; older theories have been increasingly replaced by new ones considering complex interactions between genes and environment. Thanks to these studies, it has recently become clear that early life experiences, like infections, maternal care, nutrition and stress, are able to program the brain in order to confer vulnerability or resilience and this process seems to involve epigenetic mechanisms, including DNA methylation, histone modifications and the actions of small non-coding RNAs, regulating the expression of individual genes or large gene clusters [69]. Several lines of evidence indicate that the timing of the exposure to an environmental risk factor may determine whether the event has limited and specific epigenetic effects or whether it affects the epigenome in a more extended way, as well as how long the effect persists [52]. As epigenetic processes are an integral part of the neurodevelopmental maturation, insults that are experienced during key periods roughly corresponding to early or late gestation, could be involved in reprogramming the epigenome more extensively also through generations, even if incorporated into the germ cells [69] (Figure 4).
Figure 4. Modes of maternal and paternal transgenerational epigenetic transmission. In germline, epigenetic inheritance, an environmental effect occurring during development results in an epigenetic change within the first filial generation (F1) offspring's germ line that is transmitted to F2 offspring, F3 offspring, and so on. Modified from Bale et al. 2015 and Danchin et al 2011

Remarkably, pro-inflammatory cytokines produced by the mother upon MIA may affect fetal somatic cells interacting with the epigenetic machinery. Indeed, the expression of DNA(cytosine5)-methyltransferase 1 is increased by IL-6 which can regulate its promoter [70]. Tang et al., in 2013, detected strong deficits in the expression of genes associated with immune signalling, neuronal development and synaptic transmission in the cortex of juvenile Poly I:C-exposed mice. In particular, they found several genes in the glutamate receptor signalling pathway, including Gria1 and Slc17a7, showing decreased histone acetylation in a promoter-specific way, corresponding to a defective gene expression. In contrast, the expression of these same genes, including also Disc1 and Ntrk3, was increased in the hippocampus of juvenile mice, according with elevated levels of promoter-specific histone acetylation. These results suggested to the authors that early epigenetic changes could contribute to the behavioural abnormalities observed in adult animals, comparable with the symptoms observed in schizophrenia and related disorders [71]. The alteration of neurodevelopmental mechanisms by abnormal expression of these genes may act synergistically with prenatal inflammation to increase the risk of long-lasting neurodevelopmental brain disorders. This scenario would be consistent with the hypothesis that the interaction between environmental and
genetic factors play a fundamental role in the etiology of major neuropsychiatric disorders such as schizophrenia and autism [72, 73].

An important target for epigenetic modifications in models of neurodevelopment is represented by MeCP2 (Methyl CpG-binding protein2) [74]. MeCP2 works as a transcriptional repressor or activator with a very precise timing, by recruiting transcription factor CREB1 (cAMP responsive element-binding protein 1), depending on the methylation status of the DNA [75]. In the brain, it is abundant in neurons and it’s associated with the maturation of the CNS and in the formation of synaptic contacts [76]. MECP2 has a well-established role in Rett syndrome but has also been implied in neurodevelopmental disorders such as autism and schizophrenia [77]. Aberrant promoter methylation of MECP2 itself is thought to contribute to defective biological processes and potentially to the etiology of neurodevelopmental disorders [78]. In a paper from Basil and colleagues published in 2014, it is reported an association between MIA, induced by Poly I:C injection at GD9, and MeCP2 promoter hypomethylation [74]. Since Poly I:C is known to determine an increase in IL6 and MeCP2 in turn may suppress IL6 expression [79], they speculate that hypomethylation of MeCP2 promoter, that leads to higher levels of MeCP2, may be involved in silencing genes that are activated by the immune response. As previously described, changes in MeCP2 could influence the expression of other genes involved in neurodevelopment, already reported to be altered in the MIA models; for example, BDNF (brain-derived neurotrophic factor) [80], reelin and glutamate decarboxylase 67 [81], CREB1 [82] and histone deacetylases and DNA (cytosine-5)-methyltransferase 1 [83].

**Behavioural changes in the MIA offspring**

The most frequently altered behavioural feature in the offspring after prenatal immune activation is the prepulse inhibition (PPI); a neurological phenomenon in which a weak pre-stimulus (prepulse) is able to inhibit the reaction of the subject to a second strongest, sudden and startling stimulus (pulse), administered soon after the prepulse stimulus. The stimuli usually used are acoustic, but also tactile or light stimuli can be used. The startle reflex (startle response) is the response to a sudden and relatively intense stimulus, which is manifested by a contraction of skeletal and facial muscles, usually classified as a defensive reaction. The prepulse inhibition
paradigm consists in the normal suppression of the startle reflex occurring when an intense stimulus (pulse), able to evoke the startle reflex itself, is preceded by a weaker sensory event, the prepulse. The degree by which the prepulse stimulus inhibits the motor response to the pulse stimulus, constitutes one of the simplest measures of the sensorimotor inhibition, or "sensorimotor gating".

To date, the PPI constitutes the most powerful tool for studying not only the modulation of reflexes, but also the sensorimotor gating deficits associated with psychiatric disorders, such as Tourette's syndrome and schizophrenia. One of the major advantages of using this method is represented by the fact that homologous behavioural phenomena can be studied in different species. In humans, the startle reflex is measured with an electromyographic examination of orbicularis oculi muscles (eyeblink), while in rodents a stabilometric force platform is used to measure the stress induced by the startle stimulus.

Deficits in the PPI were found in animal models on MIA following the prenatal administration of either Poly I: C and LPS [84, 85]. Several working groups have pointed out defects in PPI after administration of Poly I: C in early-mid gestation (GD9-GD12.5) [51]. For example, Meyer and colleagues identified defects in the PPI after administration of Poly I: C at GD9 but not at GD17 [86], supporting the hypothesis that the central period of pregnancy in mice represent a critical window of sensitivity to Poly I: C stimuli. Moreover, latent inhibition, exploration in the open filed, spatial working memory evaluated in the Morris water maze and social interactions were found altered in Poly I:C prenatally treated mice (GD12.5/GD17) [30, 61, 86]. Defects in the PPI, latent inhibition and spatial learning in the Morris water maze have also been observed in mice following the administration of influenza virus and IL-6 [26, 61, 87].

In addition, altered spatial learning was observed following LPS injection at GD19, evaluated through Morris water maze test, while no spatial learning impairment were reported in mice exposed to LPS at GD17 [88]. As for defects in memory, Golan and colleagues demonstrated that mice born after prenatal injection of LPS at GD17 prefer the new object respect to the known object in the novel object recognition test (NORT) compared to the control mice [89]. The NORT is used to evaluate cognition, particularly the recognition memory, in rodent models of CNS disorders. This test is based on the physiological and spontaneous tendency of rodents to
spend more time exploring new objects compared to familiar objects. The choice to spend more time exploring a new object reflects the capability of the animal to use learning and recognition memory skills. Offspring born after prenatal treatment with LPS at GD9 show a greater interest in the familiar objects respect to the new one [90], in contrast to what was observed following injection at GD17 [89]. These differences suggest that mice born after LPS injection in the mother do not have deficits in discriminating between familiar objects or new but instead show different preferences according to the period in which the LPS administration takes place.
Chapter 2

THE ROLE OF INFLAMMATION IN EPILEPSY

Medical classification and symptoms of epilepsy
The term “epilepsy” includes a set of heterogeneous neurological disorders characterized by recurrent epileptic seizures. These seizures are caused by an abnormal synchronous firing of groups of neurons (hyper-synchronization), from which derives a periodic neuronal hyper-excitability. Seizures represent the most frequent neurological event in childhood and are characterized by sudden and involuntary alterations in the brain functions, secondary to abnormal neuronal discharges.
As mentioned above, there are many types of epilepsy and equally varied are the ways in which the disease is manifested. An epileptic patient is a person who has experienced at least two unprovoked seizures at a distance of at least 24 hours; since seizures can occur also because of fever, infections of the nervous system, acute trauma or upon fluctuations of electrolytes levels in the blood.

Etiology
Worldwide, about 1% of people is affected by epilepsy, and nearly 4% will experience epilepsy at some point during their lifetime. 75% of people with epilepsy are affected within the first 20 years of life and the incidence in children is around 50 new cases every 100000 children, with an increasing rate during the first year of life. Epilepsy seems to affect equally both sexes and persons belonging to different ethnic groups, with a homogeneous geographical distribution.
Although the majority of patients can benefit from effective pharmacological or surgical treatments, approximately 30% of patients do not respond to the canonical therapies [91]. To date, over 50% of epilepsy cases are considered idiopathic, or primary, without a clear etiology. In the remaining cases, called secondary syndromes or symptomatic, the disease may be related to genetic factors,
neurodevelopmental defects, infections, trauma, stroke or brain tumours. These events can lead to the pathology with latency periods up to 10 years [92]. Based on EEG recordings, epileptic seizures can be classified as partial or generalized [93]. Partial seizures, also called focal seizures, originate from a specific brain area and we can distinguish three different types:

- **Simple**, characterized by a minimum spread of the discharge, so that consciousness and awareness are maintained (10%);
- **Complex**, starting with a localized attack, in which, however, the discharge is more widespread (generally bilateral); almost every time they involve the limbic system and are characterized by loss of consciousness (35%);
- **Secondarily generalized**, partial seizures immediately preceding a tonic-clonic generalized seizure.

On the other hand, generalized seizures usually involve both cerebral hemispheres, and are divided into:

- **Tonic-clonic seizures**, or *grand mal* (30%), which are the most dramatic ones characterized by hypertonia of all body ends followed, after 15-30 seconds, by tremor, which represents the interruption of the tone and finally by relaxation;
- **Absences**, characterized by a sudden attack that stops just as suddenly (from 4 to 20 seconds), in which there is an alteration of consciousness. They are also called "Petit mal" because they represent one of the most typical forms of child and juvenile epilepsy [94], involving learning deficits. The distinctive electroencephalographic signs of typical absences are represented by complex spike-wave discharges (SWDs) at 3-4 Hz: regular and symmetrical generalized discharges with normal background rhythm, occurring in the thalamocortical circuits [95].
- **Myoclonic seizures** (4%), characterized by myoclonic jerks, usually caused by sudden involuntary contractions of a muscle or a group of muscles, loss of muscle tone and fall;
- **Atonic seizures**, due to loss of postural tone; if standing the patient suddenly falls to the ground, if sitting the torso and the head can fall forward.
Contribution of inflammation to epilepsy

The CNS is considered a privileged system from the point of view of immunity. In fact, the presence of the blood brain barrier (BBB) protects the brain from inflammatory insults. However, it is well established that immunological and inflammatory reactions can occur in the CNS despite the presence of the BBB, because they can occur also intrinsically, constituting a direct part of the innate immunity. Inflammation in the brain is a condition characterized by the presence of a series of molecules, cytokines – soluble polypeptides acting as inflammatory mediators - and other inflammatory molecules, which are almost undetectable under physiological conditions [96]. Classically, these molecules are produced by cells of the immune system; however, it is known that the brain parenchymal cells, astrocytes, microglia and neurons as well as the choroid plexus and the BBB cells can produce these inflammatory mediators. Experimental evidence indicates a dichotomous role of immune responses in the CNS; these reactions can be both protective as well as directly or indirectly involved in neuronal dysfunctions [97]. Molecules produced both by the innate and by adaptive immune systems are induced in a great number of different neurological disorders, including Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, multiple sclerosis and amyotrophic lateral sclerosis (ALS). In Alzheimer’s disease, a robust inflammatory response of the brain is associated with the deposit of extracellular β-amyloid protein, involved in neurodegeneration. However, a beneficial effect of the inflammatory response in the pathology is highlighted by recent evidence that the activation of microglial cells and the production of transforming growth factor β1 (TGF-β1), can participate in the elimination of deposits of β-amyloid protein, thereby preventing their harmful effects [98]. In addition, peripheral antibodies against specific neuronal targets can be produced in rare inflammatory neurological disease including stiff-man syndrome, Rasmussen’s encephalitis (RE) and paraneoplastic neurological diseases, causing neuronal dysfunctions and cell death by targeting specific antigens [99]. Moreover, inflammatory response play an important role also in acute CNS injury, like stroke and cerebral ischemia, brain trauma as well as in epilepsy.
**Effects of pro-inflammatory cytokines on seizures**

Increasing evidence implicates proinflammatory cytokines as neurochemical mechanisms underlying epilepsy [100]. An inflammatory response in the CNS can trigger seizures and epilepsy, while pro-inflammatory cytokines such as IL-1, TNFα and IL-6 are increased by seizure activity determining a vicious cycle between brain inflammation and epilepsy. Cytokines can directly affect neuronal excitability acting on ionic currents and indirectly by acting on gene expression in glial cells and neurons. Exogenous administration of IL-1β previous to chemically induced seizures by intracerebral application of kainic acid or bicuculline methiodide is able to enhance and prolong seizures in rat models [101], whereas the specific antagonist IL-1Ra, injected intracerebrally, is able to reduce seizure susceptibility [102]. These data are strengthened by the evidence that inhibiting the production of IL-1β by using selective blockers or gene knockout for caspase-1, the enzyme involved in the production of the active form of IL-1β, significantly reduces seizures [103]. For what concerns another important pro-inflammatory cytokine, IL-6, it has been demonstrated that IL-6 levels are increased after seizures, although IL-6 KO mice are more susceptible to seizures than WT mice [104], suggesting a protective role for this cytokine. However, the use of transgenic mice overexpressing IL-6 in astrocytes, indicate an increased sensitivity to seizures induced by glutamatergic agonists [105]. Moreover, also cytokines produced after febrile events have been closely linked to epilepsy, in particular to childhood epilepsies. Several pro-inflammatory cytokines, including IL-1β, act as pyrogens when centrally or systemically administered. Intracerebroventricular injection of IL-1β is able to reduce the threshold for seizures in 14 days old mice subjected to hyperthermia and in 14 days old rats exposed to LPS-induced fever [106, 107]. Moreover, IL-1β receptor-deficient mice or IL-1Ra-injected rats resulted resistant to this type of seizure induction. All together these data suggest that IL-1β signalling displays pro-convulsive properties, although anticonvulsive effects this cytokine have been demonstrated in few works [108, 109].

Also systemic infections seem to play an important role in seizure susceptibility. For example, infections caused by the Enterobacter *Shigella dysenteriae* in mice significantly increases seizure susceptibility upon Pentylenetetrazole administration and IL-1β and TNFα seem to play an important role in CNS
sensitization to infection-induced seizures, because systemic pre-administration of antibodies against these two cytokines in the infected animals is able to prevent the increase in seizure susceptibility [110, 111].

**MIA and epilepsy**

As mentioned above for schizophrenia and autism spectrum disorders, also in epilepsy the prevalence of the pathology in subjects exposed to inflammatory stimuli during the mother's gestational period is significantly increased; considering also the 20-46% of children with autism spectrum syndromes which display sporadic epileptic episodes in adult life [112]. Moreover, the prevalence of epilepsy is significantly increased also in the so called “winter babies” [113]. Furthermore, a recent population-based cohort study conducted by Sun and colleagues and based on data from the Danish National Birth Cohort (1996-2002), demonstrated an association between maternal infection, accompanied by febrile episodes during pregnancy and childhood epilepsy [114]. This and other epidemiological studies showed that maternal cystitis, vaginal yeast infections, coughs and other disorders accompanied by maternal fever are associated with an increased risk for epilepsy in the offspring. A similar epidemiological study, carried out by Nørgaard and colleagues in 2012, on children born between 1998 and 2008 in northern Denmark, showed a 40% increase in the risk of developing epilepsy after prenatal maternal infections [115]. In the work of Nørgaard it was not found any association between the disease and treatment with antifungal, while Sun and colleagues demonstrated an association between vaginal yeast infections and an increased risk of developing epilepsy in the offspring [114]. Children born after prenatal exposure to more than two episodes of fever maternal, with urinary symptoms or with temperatures of 39.0 °C or more, showed increased risk of developing epilepsy, suggesting that the causes of the fever, rather than the high temperature, to have a role in the onset of pathology [116]. Recently, pre-clinical evidence demonstrated that MIA, through Poly I:C immune activation, is able to increase seizure susceptibility [117] and hippocampal excitability [118].
Chapter 3

THE EXCITATORY/INHIBITORY BALANCE: IMPLICATIONS FOR NEUROPSYCHIATRIC DISORDERS

In the context of neurophysiology, the development of correct brain functions depends upon the maintenance of several balances including cell growth/cell proliferation, oxidant/anti-oxidant equilibrium, neurotransmitter release, pro- and anti-inflammatory molecules. Among these balances another important one is represented by excitation and inhibition balance (E/I balance), that refers to the relative contributions of excitatory and inhibitory synaptic inputs responsible for correct transmission, circuit formation, cortical layer organization and neuronal plasticity [119-121]. Deregulation of the E/I balance has been associated with a variety of human neurodevelopmental disorders characterized by cognitive and social deficits, including autism and schizophrenia [122, 123].

In the cerebral cortex, neuronal circuits are constituted by two main classes of neurons: excitatory neurons, mainly using glutamate as neurotransmitter, and inhibitory interneurons, that represent about 20-30% of all cortical neurons, using γ-aminobutyric acid (GABA) as neurotransmitter [124]. Cortical GABAergic interneurons have been shown to play a vital role in controlling the function of cortical networks, in modulating neuronal excitability and integration, in the generation of temporal synchrony and thus oscillation among glutamatergic circuits [125-127]. GABAergic interneurons are also crucial in the regulation of all of the key steps during neurodevelopmental in the cortex, form neuronal proliferation, migration and differentiation to experience-dependent organization and plasticity of local cortical circuits [128, 129]. Thus, not surprisingly, aberrant development of GABAergic transmission has been related to many neurodevelopmental disorders including schizophrenia, autism, Rett syndrome, Down syndrome as well as developmental epilepsy [124, 130, 131].

The formation of the GABAergic network can be divided into distinct developmental steps (Figure 5): generation of specific GABAergic subtypes, migration in the
appropriate brain region, cell axon growth towards specific post-synaptic targets and finally the use-dependent adjustment of GABAergic synapse number and strength.

Figure 5. Time course of GABAergic circuit development. The development of GABAergic circuits is a long process starting during mid-gestation and completed only by the end of adolescence. In the immature brain, GABA-releasing synapses are formed before glutamatergic contacts in a wide range of species and structures and therefore represent the first form of communication between neurons. Early on, GABA action is excitatory and becomes later inhibitory because of the delayed expression of a chloride transporter, leading to a negative shift in the reversal potential for Cl.

Moreover, a striking characteristic of GABAergic interneurons is the exuberance of their innervation field. In rodents and in primates, this rich pattern of innervation is not completed before late adolescence. This time window, during which experience can sculpt GABAergic network architecture, is fundamental. Indeed, sensory deprivation during this critical post-natal period deeply retards GABAergic synapses maturation in visual and somatosensory cortex [132, 133]. The first molecule involved in this process is Brain-derived neurotrophic factor (BDNF) that promotes the formation of GABAergic synapses in hippocampal and cortical cell cultures [134]. More recently, it has been demonstrated that GABA signaling itself can promote GABAergic maturation and innervation in the adolescent cortex [135]. Therefore, dysregulations in one of the crucial stages of GABAergic network development and maturation could lead to an aberrant function of cortical circuits resulting in the manifestation of a pathological phenotype.
**Cation-chloride cotransporters involvement in E/I balance**

Another fundamental process involved in the correct developing of GABAergic circuit and in the maintenance of a proper E/I balance is represented by the “GABA switch”; a process in which GABAergic signalling shifts from depolarization to hyperpolarization through development.

Electrical activity in neurons needs a functional and synergic coupling between ion channels and ion transporters. It has become increasingly evident that one family of ion transporters particularly, the cation-chloride cotransporters (CCCs), play a fundamental role in shaping the GABAergic signalling and neuronal connectivity [136]. Although GABA is known to be the main inhibitory neurotransmitter in the adult brain, during embryonic neurodevelopment it can depolarize neuronal cell membranes depending on the Cl⁻ gradient across them. Indeed, in brain structures such as the hippocampus, the neocortex and the hypothalamus, the inhibitory properties of GABA only emerge after birth [137].

Two members of the SLC12 family, a solute carrier family of electroneutral CCCs, NKCC1 (Na⁺-K⁺-2Cl⁻ cotransporter 1) and KCC2 (K⁺-Cl⁻ cotransporter 2), are known to have cell-autonomous functions in the CNS; they play an important role in setting the reversal potential and the driving force of the anion currents mediated by GABAₐRs, as well as glycine receptors [138] and non-ligand-gated Cl⁻ channels [139, 140]. During the GABA switch the lowering of the resting intracellular chloride concentration is mediated by a progressive downregulation of NKCC1 (chloride importer) and upregulation of KCC2 (chloride extruder) (Figure 6) [141-143].
In immature neurons NKCC1 expression is high while KCC2 is low, resulting in high [Cl\(^-\)]. In the normal mature nervous system, the situation is reversed; expression of NKCC1 is low and that of KCC2 is high, resulting in low [Cl\(^-\)]. This shift has been observed in a large number of different animal species and brain regions, suggesting that it has been conserved throughout evolution [144]. Interestingly, this developmental decrease in intracellular [Cl\(^-\)] does not occur in other cellular types, stressing the point that immature neurons, where intracellular [Cl\(^-\)] is higher, are not an exception as usually thought by neurobiologists, but rather are mature neurons to be an exception respect to other cell types. Chloride is used by cells to maintain basic cellular parameters such as cell volume and intracellular pH and there is a high metabolic cost associated with the maintenance of low intracellular chloride concentrations and generation of hyperpolarizing currents. Thus, the frequently reported downregulation in KCC2 expression, upon neuronal trauma may be a consequence of an adaptive mechanism put in place by neurons to survive by reducing energetic costs [145, 146].

**Figure 6.** (A) Schematic diagram of the alterations in [Cl\(^-\)] in immature and mature neurons. In immature neurons NKCC1 expression is high while KCC2 is low, resulting in high [Cl\(^-\)]. In the normal mature nervous system, the situation is reversed; expression of NKCC1 is low and that of KCC2 is high, resulting in low [Cl\(^-\)]. (B) Line plots showing the average exon array signal intensity of cation-chloride cotransporter (CCC) transcripts in the human neocortex from the early fetal period to late adulthood. Modified from Ben-Ari 2015 and Kaila et al. 2014.
because of its crucial role in maintaining the balance between excitation and inhibition (E/I). With the identification of the molecular mechanisms of synaptic transmission, it has become even clearer that defects in almost every step of synaptic transmission can lead to the onset of seizures. Particularly interesting is the role of ion transporters which, as mentioned above, affect the reversal potential of GABA$_A$-mediated currents ($E_{GABA}$), in what is called “ionic plasticity”. GABAergic signalling has the unique property of “ionic plasticity”, which is based on short-term and long-term changes in the Cl$^-$ and HCO$_3^-$ ion concentrations in the postsynaptic neurons. While short-term ionic plasticity is caused by activity-dependent, channel-mediated anion shifts, long-term ionic plasticity depends on changes in the expression and kinetic of molecules involved in anion homeostasis [147]. Accumulating evidence suggests that this high plastic ion regulation in neurons, contributes to the multiple roles ascribed to GABAergic signalling during epileptogenesis and epilepsy [146].

It has been demonstrated that mice, in which the gene encoding for KCC2 is disrupted, show frequent generalized seizures and die shortly after birth. The most affected regions are the hippocampus, temporal and entorhinal cortices. Moreover, adult heterozygous animals, which are vital, show increased susceptibility to epileptic seizure, thus indicating that KCC2 plays an important role in controlling CNS excitability during both postnatal development and adult life [148]. In addition, progressive downregulation of KCC2 after pilocarpine-induced status epilepticus decreased the inhibition efficacy and abolished the capacity of the dentate gyrus to work as a hippocampal barrier against seizure activity arising in the entorhinal cortex. Indeed, in normal adult dentate granule cells in rats, spiking of these cells is strongly suppressed by KCC2-dependent GABAergic signalling. [149].

Temporal lobe epilepsy (TLE) is the most common type of refractory epilepsy in humans. The primary cause leading to TLE is typically a brain insult, such as traumatic brain injury, inflammation or status epilepticus, but in patients the nature of the initial insult remains often unknown because of the long delays between the insult and the appearance of recurrent seizures [150]. It has been observed that in hippocampal brain slices from adult patients with TLE, downregulation of KCC2 and upregulation of NKCC1 leads to depolarizing GABA$_A$R responses in a subpopulation of subicular principal neurons. This has been implicated in the generation of
spontaneous interictal-like (but not ictal-like) activity [151-153]. What was interestingly found is that by inhibiting NKCC1, using diuretic bumetanide, that at low concentration almost specifically inhibits NKCC1 without significantly affect KCC2, it is possible to block interictal activity in vitro [152]. Moreover, patients with TLE showing depolarizing GABAergic neurons in the subicular pyramidal cells as well as increased NKCC1 mRNA levels in the hippocampus [154], show a decreased depolarizing activity of GABA upon treatment with bumetanide [152].

Recently, a rare point mutation (KCC2-R952H) in the SLC12A5 gene (the gene encoding for KCC2) was identified in an Australian family with early childhood onset of febrile seizures [155]. This missense mutation led to defects in neuronal Cl- extrusion capacity as well as defects in cortical dendritic spine formation in rodent neurons, suggesting that this variant represent a good candidate for febrile seizures susceptibility [155]. Support for this conclusion was gained from another point variant, the KCC2-R1049H, found in a French-Canadian cohort with idiopathic generalized epilepsy [156].

Thus, GABA_{A}R signalling appears to resume its immature, depolarizing activity at least in some pyramidal neurons during epileptogenesis.
AIM OF THE STUDY
The developing brain is highly sensitive to several environmental insults, such as maternal stress and nutritional deficiencies, that can induce damaging effects in the future child [1]. Among these insults, infection-induced maternal immune activation (MIA) during pregnancy represent one of the most dangerous. In fact, MIA can contribute to the onset of causal chains of events leading to several pathophysiological changes in the fetal environment and contributing to postnatal brain dysfunctions. Several epidemiological studies confirmed that infections occurring during pregnancy, caused by different pathogenic agents, are associated with an increased incidence of neurodevelopmental disorders in the offspring, such as autism and schizophrenia. As for these two neurodevelopmental disorders, the prevalence of epilepsy is significantly increased in the “winter babies” [113]. Furthermore, a recent population-based cohort study demonstrated an association between maternal infections, accompanied by febrile episodes during pregnancy and childhood epilepsy [114]. This and other epidemiological studies showed that maternal cystitis, vaginal yeast infections, coughs and other diseases accompanied by maternal fever are associated with an increased risk for seizures and epilepsy in the offspring.

It has been recently demonstrated in animal models that prolonged prenatal exposure to Poly I:C (polyinosinic:polycytidylic acid), a viral mimic, leads to increased hippocampal excitability, faster progression of kindled seizures and prolonged persistence of the kindling state, along with impaired social interactions, requiring the activation of both IL-6 and IL-1β [117]. However, the mechanisms directly involved in the increased excitability following prenatal inflammation have not been clarified yet.

The aim of my PhD project was therefore to assess the effects of a single immune challenge, at early stages of gestation, on the susceptibility to epilepsy in the offspring, with particular interest to the mechanisms and the pathways involved. To this purpose, we developed a mouse model of MIA, consisting in a single injection of the immunogenic agent Poly I: C at gestational day 9 (GD9), corresponding to the first trimester of pregnancy in humans. Offspring was than subjected to behavioural analysis, while tissues collected at different times of embryonic development and from young and adult animals were used for functional, biochemical and molecular analyses in order to investigate the mechanism underlying the altered susceptibility.
to seizures observed in adult Poly I:C offspring. The data presented in this thesis and
future experiments will contribute to better understand the link between prenatal
inflammation and epilepsy and to elucidate the molecular mechanisms that may
contribute to the development of seizures following maternal immune activation.
RESULTS
CHOICE OF THE MIA MODEL: INTRAPERITONEAL POLY I:C INJECTION AT GESTATIONAL DAY 9

The first part of this study consisted in setting up the optimal experimental conditions and the proper timing to investigate the pathological effects induced by MIA. Given the extensive literature concerning MIA models, in which a wide variety of substances, doses and times are applied, a real established and accepted model of MIA is still lacking. Nevertheless, in order to mimic a mild pathological infection in the mother, in which animals did not manifest any signs of physical discomfort, we decided to induce MIA by intraperitoneal injections of 2 mg/kg Poly I:C: 40% of the amount commonly used in literature [30, 34, 51]. Poly I:C was injected at gestational day 9 (GD9), corresponding to the end of the first trimester of human pregnancy in terms of fetal development and percentage of pregnancy total duration [157]. This temporal window of embryonic development was chosen because it is believed to constitute a critical window of susceptibility to inflammatory events [85, 158]. The analyses were then carried out both during embryonic stages (3-6-24 hours post-injection and E17) as well as during postnatal adult life (P20 and P90). As mentioned above, many neurodevelopmental disorders manifest the first clinical symptoms in post-pubertal age, having a greater impact on adult life [159, 160]. Therefore, these times allowed us to investigate the events in the offspring following the maternal inflammatory insult till the onset of the disease. We decided to use only male offspring because of the absence of the hormonal cycle, which could introduce different variables difficult to manage, in particular for what concerns behavioural tests but also biochemical analyses. Furthermore, it is also known that both at anatomical and functional levels the brain is deeply influenced by gender and by the action of steroid [161]. In addition, data regarding the prevalence of neurological and psychiatric disorders in humans show a different vulnerability of the two sexes as well as a sexual dimorphism [162]. It was therefore necessary for this study to make a priori choice about the sex to be examined, even if it could be very interesting in the future to study females in order to investigate any possible difference in the two sexes upon MIA challenge.
VALIDATION OF THE MODEL

Since it is known that prenatal Poly I:C administration at GD9 is able to increase IL-6 blood levels in the mother 3 hours after the injection [30], we collected blood from 3-6 months old females 3 hour after Poly I:C or vehicle injection, in order to compare our results with the literature, thus validating our model. The proinflammatory cytokine IL-6 is a multifunctional cytokine with a variety of implications in several pathological conditions. Physiologically its level is less than 1 pg/ml in human blood but it’s subjected to a rapid upregulation in pathophysiological conditions [163]. In our experimental model, 2 mg/kg Poly I:C were able to significantly increase IL-6 plasmatic levels in treated pregnant mothers respect to control mice as detected by ELISA kit (Figure 1). This result is in line with those reported in literature [30, 164], thus confirming the validity of the chosen MIA model.

![Figure 1. IL-6 plasma levels in adult females detected by ELISA kit 3 hours after Poly I:C/vehicle ip. injection. Student’s t-test, **p=0.0014. Ctrl n=4; Poly I:C n=5.](image-url)
SUSCEPTIBILITY TO KAINIC ACID-INDUCED SEIZURES IS INCREASED IN POLY I:C PRENATALLY-TREATED OFFSPRING

Recently it has been demonstrated that increased susceptibility to seizures can be ascribed to inflammatory insults during prenatal life. In fact, the daily administration of Poly I:C in pregnant dams from GD12 to GD16, is able to increase seizure susceptibility in the offspring, examined using the rapid hippocampal kindling model [165]. We therefore aimed to investigate whether an earlier and single intrauterine exposure to Poly I:C, performed at the very beginning of the cortical neuronal layering (GD9), was sufficient to increase offspring susceptibility to seizures in adulthood. For this purpose, MIA offspring was examined at 3 months of age (P90) after ip. administration of 35 mg/kg kainic acid (KA), a potent agonist of a subtype of glutamate receptors capable of inducing seizures in a stereotyped way [166]. The mice were then observed and evaluated over a 3-hour period according to the Racine’s scale. Figure 2 shows that prenatally Poly I:C-treated mice are more susceptible to seizures induced by KA compared to control offspring.

**Figure 2.** Evaluation of the behavioural response of control and Poly I:C prenatally-treated mice to 35 mg/kg kainic acid injection (A) Time course of the behavioural response to 35 mg/kg injection of KA of control mice (n=9) vs Poly I:C mice (n=10). Two-way ANOVA followed by Sidak’s multiple comparisons test; *p<0.05, **p<0.01, ***p<0.001. (B) Mean score variation at the end of the experiment (180 minutes). Student’s t-test, ***p=0.0004.
In all mice, KA was able to induce, within the first 10 minutes, immobility and staring, followed by head bobbing and isolated limbic motor seizures (stage 4 of Racine’s scale), characterized by forelimb clonus and rearing. Latency to the first stage 4 between Poly I:C and Ctrl mice was not statistically different in the two experimental groups even if, in general, Poly I:C mice reached stage 4 earlier than control mice. However, while control animals only displayed isolated limbic motor seizures, without reaching higher stages, Poly I:C mice rapidly progressed to stage 5 (status epilepticus) and showed continuous generalized activity lasting for about 80 minutes (Figure 2A). Thus, Poly I:C mice achieved significantly higher behavioural scores respect to control mice, starting from 60 minutes after KA injection and display a higher mortality rate: 28% vs 10%. Figure 2B shows the comparison between the Racine’s scores in the two experimental groups at the end of the experiment (180 minutes). Notably, no differences in the susceptibility to seizures were detected in dams (Figure 3A) and adult males (Figure 3B) treated with Poly I:C or vehicle during adulthood. All together, these data indicate that a single exposure to an infectious agent at GD9, is sufficient to increase susceptibility to seizures in the adult offspring.

**Figure 3.** (A) Time course of the behavioural response to 35 mg/kg kainic acid injection of control and Poly I:C injected dams. Notably, there are no differences in the behavioural response between the 2 experimental groups. Two-way ANOVA test. Ctrl n=6; Poly I:C n=10. (B) Mean score variation at the end of the 180 minutes observation in adult males who received vehicle or Poly I:C ip. in adult life. No differences were detected in the two experimental groups. Student’s t-test, Ctrl n=5; Poly I:C n=5.
POLY I:C INDUCES SHORT-TERM INFLAMMATORY RESPONSES IN THE EMBRYOS

As already discussed previously, the MIA model consists in the activation of the maternal immune system during pregnancy following an inflammatory challenge. In order to verify whether the inflammatory stimulus we choose was able to induce, directly or indirectly, an immune response also in the embryos, we performed quantitative real-time PCR, for the main proinflammatory cytokines involved in MIA, directly in embryos. It is known that Poly I: C triggers immune responses through its binding with the specific Toll Like Receptor TLR3, which leads to the activation of different transcription factors, including CREB (cyclic AMP-responsive element-binding protein), AP1 (activator protein 1), NF-kB (nuclear factor-kB), IRF3 and IRF7 (interferon-regulatory factors), responsible for the production of several proinflammatory cytokines and interferon type 1 (Figure 4) [167, 168].

![Intracellular TLR signalling pathways (Blasius et al. 2010).](image)

**Figure 4.** Intracellular TLR signalling pathways (Blasius et al. 2010).
To address this point, we analysed a panel of proinflammatory cytokines in embryos collected 3, 6 and 24 hours after Poly I:C treatment in the mother. Embryos were then analysed by means of quantitative real-time PCR. As reported in Figure 5, we observed a transient increase in the transcriptional profile of proinflammatory cytokines IL-6 (Figure 5A) and IL-1β (Figure 5B) 6 hours post-injection, while no statistical difference was observed 3 and 24 hours after the injection for IL-6. Interestingly, IL-1β was significantly reduced 24 h after the injection in Poly I:C offspring, possibly due to a compensatory mechanism. Unlike these cytokines, interferon β (IFNβ) showed no statistically significant variations at any of the analysed times (Figure 5C). The increase in IL-1β and IL-6 mRNAs levels indicates these immune molecules as the principal architects of the pathological phenotypes of MIA and suggests that our experimental procedure is able to activate not only the maternal immune system but also the embryonic one as a result of MIA.

**Figure 5.** Cytokines mRNA levels in control and Poly I:C prenatally-treated embryos collected 3 (Ctrl n=6, Poly I:C n=5), 6 (Ctrl n=9, Poly I:C n=10) and 24 hours (Ctrl n=6, Poly I:C n=7) after the injection of Poly I:C/vehicle in the mother. (A) IL-6 mRNA levels are significantly increased 6 h post-injection in Poly I:C embryos. (B) IL-1β mRNA levels are significantly increased 6 h post-injection and significantly reduced 24 h after the injection in Poly I:C embryos. (C) Interferon β mRNA levels are not affected by MIA stimulus at any time after the immune challenge. Student’s t-test, *p<0,05, **p<0,01.
Although we found differences in timing between the inflammatory response observed in the mother (Figure 1) and that in embryos (Figure 5), we speculate that there could be a potential delay in the embryonic immune response due to the passage of the immune-stimulating agent through the placenta before reaching the fetal tissues. Moreover, the lack of increase of IFNβ argues in favour of a secondary activation of the embryonic immune system. Although different authors reported an increase of IL-1β, TNFα and IL-6 mRNA levels in rodent brains subjected to MIA at different developmental stages and upon different stimuli, there is currently no consensus on the extent of these variations [55, 169, 170]. Indeed, studies performed with Poly I:C at GD9, a paradigm similar to the one used in the present work, show conflicting data; Meyer and colleagues reported that this kind of treatment leads to a reduction of IL-1β mRNA levels 3 hours after the injection and an increase in IL-6 and TNFα levels [30]. It is possible that differences in the animal housing, in the mating timing and in the quality and quantity of the immune-stimulating agent used may originate the observed differences.
INCREASED SUSCEPTIBILITY TO SEIZURES IN ADULT OFFSPRING DOES NOT REFLECT CHRONIC INFLAMMATORY PROCESSES IN THE BRAIN

Given the increased seizure susceptibility in P90 animals and since it is well known that seizures and epilepsy may be the consequences of a chronic inflammatory status in the brain [171], we decided to investigated whether the increased susceptibility to seizures occurring in our model could be associated with an enhanced brain inflammation in adult life. Brains from P90 mice were examined by confocal microscopy in order to quantify the morphological appearance of microglial cells and the expression of proinflammatory markers.

Figure 6. (A) Representative immunofluorescence images obtained from P90 cortical slices showing in green the specific microglial marker IBA1 and in red a marker for activated microglia, CD11b. Quantitative analysis of the total number of microglial cells (B) and of activated microglia (C). No significant differences were found between the two experimental groups. Student’s t-test.

Figure 6B shows that the total number of microglial cells, revealed using the specific marker IBA1 is not altered in Poly I:C offspring respect to controls as well as the number of activated microglial cells, analysed by using the specific marker CD11b (Figure 6C). Moreover, GFAP (Glial fibrillary acidic protein), a marker for reactive
astrocytes, did not differ between the two experimental groups, analysed using western blotting technique (Figure 7).

![Figure 7](image)

**Figure 7.** Biochemical analysis of glial fibrillary acidic protein (GFAP) in the homogenates of adult (P90) Poly I:C prenatally-treated and control mice. **(A)** Quantitative analysis of GFAP protein levels showing no differences between the two experimental groups. Student’s t-test, Ctrl n=4, Poly I:C n=5. **(B)** Representative western blot lanes for GFAP and the housekeeping protein calnexin.

A second parameter used to evaluate microglial activation concerns its ramification. Under physiological conditions microglia in the CNS exists in the ramified or what is generally termed ‘resting’ state. As appreciable in Figure 8A as well as in the quantification (Figure 8C), microglia from MIA offspring possess fewer branches respect to controls, while the length of branches does not change between the two experimental groups (Figure 8B).

Consistently, quantitative real-time PCR performed for the two main cytokines already assessed in the embryonic age, IL-6 and IL-1β, did not reveal detectable mRNA levels both in Poly I:C and control mice brains (data not shown), indicating that the increase in proinflammatory cytokine levels during embryonic development is transient and does not produce any long term inflammatory condition in adulthood.
Neuroinflammation is usually defined as an innate immune response which needs the contribution of glial cells for its propagation. Numerous studies have demonstrated that inflammation is an important factor in determining neurological and psychiatric disorders, such as major depression, and drugs known for their anti-depressant properties exhibit at the same time anti-inflammatory action [172]. In line with the involvement of an inflammatory component, post-mortem studies of brains of autistic patients permitted to reveal the presence of reactive glia from childhood to adulthood. Microglia represents the immune component of the brain and many insults to the nervous system lead to morphological and/or functional changes in these cells (micro-gliosis). If persistently maintained, the activation of microglia, which initially has the function to restore homeostasis in the brain, may be exacerbated and become detrimental [173]. In addition to microglia, also

Figure 8. (A) Representative immunofluorescence images obtained from P90 cortical slices showing on the left the specific microglial marker IBA 1 (in green) and on the right the same image skeletonized for branches analyses. (B) Quantitative analysis of microglia branches length shows no differences between the two experimental groups. (C) Quantitative analysis of the number of microglial branches per cell shows a significant reduction in the number of microglial branches in Poly I:C offspring respect to controls. Student’s t-test, *p=0.0123. Ctrl n=3 animals, Poly I:C n=3 animals.
astrocytes are involved in the inflammatory processes in the brain through the production of cytokines and through the increase in their number and dimension (astro-gliosis).

Although our results clearly show that the inflammatory insult in the mother at GD9 is able to affect the embryos inducing short-term inflammatory responses, the microglia analysis performed on adults does not show any evidence for the presence of sustained inflammatory responses in P90 brains. Thus, the increased susceptibility to seizures observed upon KA injection in adult mice, does not seem to reflect long-term chronic inflammatory processes occurring in animals prenatally exposed to Poly I:C.
EVALUATION OF THE INVOLVEMENT OF E/I BALANCE IN THE SUSCEPTIBILITY TO SEIZURES IN POLY I:C OFFSPRING

Synaptic proteins levels are not affected by prenatal exposure to Poly I:C

Since we did not found significant inflammatory alteration in adult brains and given that the susceptibility to epilepsy may result from a change in the excitatory/inhibitory (E/I) balance in the brain [174-176], we hypothesized that prenatal immune challenge could modify either the number or the function (or both) of synaptic contacts in the developing (E17) as well as in the adult (P90) brain. To address this issue, we started by analysing the protein expression levels of a battery of synaptic markers, including the vesicular glutamate transporter vGlut1, the GABA vesicular transporter vGAT, the two SNARE proteins Snap25 and syntaxin 1A, as well as the post-synaptic protein PSD95, by western blotting. To this aim E17 and P90 cortical homogenates from Poly I:C and control mice were analysed and, interestingly, no significant differences in synaptic protein expression was found between the two experimental groups at both ages (Figure 9A and 9B). Despite our analysis has been limited to a restricted number of proteins, these data indicate that the increased susceptibility to seizures of Poly I:C offspring is unlikely the result of an alteration of synaptic markers protein expression.
**Figure 9.** Biochemical analysis of excitatory and inhibitory pre- and post-synaptic proteins. Quantitative western blot analysis of the synaptic proteins vGlut1, vGAT, Snap25, Syntaxin 1A (Syntx 1A) and PSD95 in E17 cortical homogenates (A) and in P90 homogenates (B). No differences in all the synaptic proteins tested were found at both ages. Student’s t-test. Ctrl n=5, Poly I:C n=6.

**Network excitability is increased in Poly I:C-prenatally exposed mice**

We then ask whether Poly I:C treatment alters synaptic transmission in cortical neurons. Consistent with the lack of differences in the expression of synaptic markers, patch clamp recordings from P15-28 (that from now for convenience we will call P20) acute cortical slices from Poly I:C and control mice did not reveal any difference in either the frequency or the amplitude of miniature excitatory (mEPSCs) or inhibitory (mIPSCs) post-synaptic currents (Figure 10B).
Figure 10. (A) Representative traces of mEPSCs and mIPSCs recorded from P20 cortical slices obtained from Poly I:C prenatally-treated mice and control mice. (B) Electrophysiological analysis of the frequency and the amplitude of excitatory and inhibitory miniature post-synaptic currents. Student’s t-test. mEPSCs Ctrl n=7, Poly I:C n=7; mIPSCs Ctrl n=6, Poly I:C n=6.

Conversely, and in line with the increased susceptibility to seizures, a higher network excitability was detected by cell-attached recordings, with a significantly higher spontaneous activity in Poly I:C relative to control mice (Figure 11B). Altogether these data indicate that Poly I:C treatment increased spontaneous neuronal activity in cortical neurons in the offspring, without apparently altering neither synaptic basal transmission nor the expression of several synaptic proteins.
**GABA signalling is altered in Poly I:C offspring due to higher intracellular chloride concentrations**

In order to get more insights into the molecular mechanisms responsible for higher neuronal activity in Poly I:C offspring, we focus our attention on GABA signalling. It is now well known that a change in the excitatory/inhibitory balance may result not only from an alteration in glutamatergic and GABAergic synaptic inputs balance, but also from the depolarizing/hyperpolarizing action of GABA. Indeed, at early stages of development, in immature neurons, GABA is depolarizing [177] and the transition of GABA signalling from depolarizing to hyperpolarizing, a process called “GABA switch”, starts soon after birth and is completed, in rodents, by the end of the first postnatal week [178]. It is also known that the developmentally regulated GABA switch is altered in several pathophysiological conditions, thus GABA still retain its immature excitatory role also at adult stages leading to an E/I imbalance in adulthood [122, 179].

To assess whether the process of GABA switch was correctly completed in cortical circuits of Poly I:C offspring, we applied 200 μM GABA directly to cortical slices established from P20 control and Poly I:C offspring and we recorded spontaneous activity through cell-attached configuration. Striking, in contrast to control brain

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**Figure 11.** (A) Representative traces of spiking activity recorded in P20 cortical slices from Poly I:C and control mice. (B) Quantitative analysis of the spiking activity showing an increase in the frequency of action-potential discharge in Poly I:C, prenatally-treated mice respect to controls. Mann-Whitney test, *p=0.0108. Ctrl n=7, Poly I:C n=10.
slices in which GABA application slightly reduce spontaneous neuronal activity, in line with the inhibitory role of GABA signalling at this postnatal stage, Poly I:C slices showed an increase in neuronal activity upon GABA application (Figure 12B). Therefore, in the Poly I:C prenatally-exposed mice, GABA signalling still exerts an excitatory action, indicating the occurrence of a delayed GABA switch.

**Figure 12.** (A) Representative traces of spiking activity recorded from P20 cortical slices obtained from Poly I:C and control offspring, before and after GABA application. (B) Quantitative analysis of the spiking activity upon application of 200 μM GABA. In Poly I:C brain slices neuronal firing is increased when GABA is applied directly on slices. Ctrl n=5, Poly I:C n=8

The excitatory action of GABA signalling results from high intracellular chloride concentrations which force chloride to flow outside the cells through GABA receptors thus leading to membrane depolarization [25]. This prompted us to directly evaluate intracellular chloride concentration ([Cl\(_i_\)]) through two-photon imaging by means of the chloride-sensitive dye MQAE, in order to univocally demonstrate that the GABA switch was altered in Poly I:C cortical circuits. The analyses were performed in P20 cortical slices, a time at which GABA signalling is inhibitory in control mice (Figure 12) reflecting a completed GABA switch in such physiological conditions. As expected, the analysis of MQAE-fluorescence clearly indicated that Poly I:C mice exhibited a higher resting [Cl\(_i_\)] in cortical neurons compared to control mice (Figure 13B), indicating a more immature phenotype in Poly I:C offspring.
Figure 13. Quantitative analysis of the intracellular chloride concentration in cortical neurons from P20 brain slices obtained from Poly I:C and control offspring. (A) Representative two-photon images showing $[\text{Cl}^-]$ in Poly I:C and control cortical neurons. (B) The mean intensity of the fluorescent chloride-sensitive dye MQAE is inversely proportional to the intracellular chloride concentration. MQAE mean intensity is significantly decreased in Poly I:C offspring indicating that $[\text{Cl}^-]$ is significantly increased in Poly I:C cortical neurons respect to controls. Student’s t-test, ***p<0,001, n.cells > 40 for each experimental group.
Prenatal Poly I:C triggers changes in KCC2 and NKCC1 expression leading to a delayed GABA switch

The GABA switch results from a developmentally regulated expression of the Na+ -K+ -2Cl− cotransporter 1 (NKCC1, Cl− importer) and the K+ -Cl− cotransporter 2 (KCC2, Cl− exporter). The NKCC1 reduction and the parallel KCC2 increase during neuronal development is associated with the physiological changes in intracellular chloride concentrations (chloride is higher in immature neurons) leading to the hyperpolarizing effects of GABA in mature neurons [177, 178, 180].

For this reason, we decided to investigate the expression of the two cotransporters NKCC1 and KCC2 in the two conditions. Consistently, western blotting analysis of cortical homogenates from P90 Poly I:C cortices displayed a significantly lower KCC2 amount (Figure 14A), whereas on the contrary the expression of the Cl− importer NKCC1 in homogenates from E17 cortices was found to be increased in Poly I:C offspring respect to controls (Figure 14B). Eventually, no difference in KCC2 expression was detected in cortices obtained from animals acutely treated with Poly I:C in adult stage (Figure 14C), indicating that KCC2 downregulation observed in Poly I:C offspring was the result of a long-lasting effect of an early inflammatory state.
Figure 14. Biochemical analyses of ion cotransporters expression in brain at different ages. (A) KCC2 protein levels are significantly reduced in P90 cortical homogenates from Poly I:C prenatally-treated mice. Student’s t-test, *p=0.0466, Ctrl n=10, Poly I:C n=10. (B) NKCC1 protein levels are increased in E17 cortical homogenates from Poly I:C embryos. Student’s t-test, *p=0.0114, Ctrl n=14, Poly I:C n=15. (C) No difference in KCC2 protein levels are detected when Poly I:C is administered during adulthood, in 3 months old mice. Student’s t-test, Ctrl n=4, Poly I:C n=5.

All together these results indicate that a single challenge to the maternal immune system at GD9 is able to delay the reciprocal change in the expression of NKCC1 and KCC2, leading to higher intracellular chloride concentrations resulting in GABA depolarizing activity in P20 cortical neurons.
Our next goal was to investigate whether the altered GABA switch promoted by maternal immune activation was a process requiring the whole brain environment. To investigate this point, we took advantage of primary cultures of cortical neurons, a model in which whole brain integrity is lost allowing a more precise analysis on neuronal cells. Cortical neuronal cultures were established from E18 embryos, 9 days after exposure to either Poly I:C (Poly I:C cultures) or vehicle (control cultures) and then analysed at 14 days of in vitro (DIV) development. In line with the in vivo results, electrophysiological recording of glutamatergic and GABAergic synaptic basal transmission showed no changes in Poly I:C versus control cultures at 14 DIV (Figure 15B). Accordingly, immunofluorescence analysis through confocal microscopy using antibodies against glutamate (vGlut1) or GABA (vGAT) vesicular transporters, to visualize glutamatergic or GABAergic synapse respectively, revealed no differences in the ratio between vGlut1/vGAT positive puncta (Figure 16). Overall, these results indicate that Poly I:C treatment does not affect the density and functionality of glutamatergic and GABAergic synaptic contacts.
Figure 15. (A) Representative traces of mEPSCs and mIPSCs recorded in DIV 14 primary cortical neurons from Poly I:C prenatally treated embryos and controls. (B) Electrophysiological analysis of the frequency and the amplitude of both excitatory and inhibitory miniature post-synaptic currents shows no differences between the two experimental groups. Student’s t-test. mEPSCs Ctrl n=12, Poly I:C=12; mIPSCs Ctrl n=11, Poly I:C n=14.
Figure 16. Immunocytochemical experiments on 14 DIV primary cortical neurons from Poly I:C and control embryos. (A) Representative confocal images showing presynaptic excitatory marker vGlut1 in blue and the presynaptic inhibitory marker vGAT in red. (B and C) Quantitative analysis of the number of positive vGlut1 and vGAT puncta over the total number of vGlut1 and vGAT positive puncta showing no differences between the two experimental groups. Student’s t-test, n=3 coverslips for each experimental group.

However, and in line with our in vivo data, Poly I:C cultures displayed a significantly higher frequency of both long bursts of action potentials, recorded in whole cell configuration, (Figure 17C), and multi-unit (MU) activity, recorded in cell-attached configuration (Figure 17D), thus suggesting again a possible unbalanced excitatory/inhibitory input onto these neurons.
To investigate whether a delay in the GABA switch could be at the origin of this altered electrophysiological behaviour, we firstly evaluated GABA-mediated calcium transients in both neuronal cultures. Neurons were loaded with the calcium sensitive dye Fura-2AM and subsequently exposed to 100 µM GABA. Since it is described that at DIV 6 about 50-60% of neurons still respond to GABA with depolarizing calcium transients [181], we decided to use 6 DIV neurons as optimal time point for these experiments. Consistent with the observed GABA-mediated depolarizing activity in P20 cortical slices, we found that Poly I:C cultures exhibited
a higher number of GABA-responding neurons than control cultures (Figure 18), thus indicating a delayed GABA switch in neurons established from prenatally Poly I:C-treated embryos respect to controls.

![Bar chart showing GABA responding neurons](image)

**Figure 18.** Quantitative analysis of the number of 6 DIV primary cortical neurons responding to GABA application by depolarization. Student’s t-test *p<0.027. Ctrl n\textsubscript{cells}=538, Poly I:C n\textsubscript{cells}=661

In parallel, cultures were loaded with the chloride-sensitive dye MQAE and intracellular chloride concentration was evaluated in the two conditions. Consistent with this evidence, 6 DIV chloride imaging showed a higher [Cl\textsuperscript{-}] in Poly I:C cultures compared to control cultures (Figure 19B), thus further supporting the evidence of an altered GABA switch in primary cultures established from Poly I:C prenatally-exposed embryos. Furthermore, significantly lower levels of KCC2 and higher levels of NKCC1 were detected by western blotting analysis in 6 DIV Poly I:C cultures relative to controls (Figure 19 C and D).

Therefore, considering all these \textit{in vitro} results, we can conclude that the molecular consequences of an early immune challenge occurring in neurons at GD9 in the vital embryo remain sculpted in the developmental program of neurons even when they are isolated from the brain environment and maintained in primary cultures.
Figure 19. Intracellular chloride measurements in 6 DIV primary cortical neurons established from Poly I:C and control embryos. (A) Representative images showing the fluorescence variation of the chloride sensitive dye MQAE n the 2 type of cultures. (B) Quantitative analysis of the MQAE mean intensity. Like in the in vivo experiments a lower MQAE mean intensity in the Poly I:C cultures indicates an increased [Cl]. Student’s t-test, ****p<0,0001. (C and D) KCC2 and NKCC1 protein levels measured by western blotting experiments. Student’s t-test, *p<0,05, Ctrl n=5, Poly I:C n=5 (E) Representative western blot lanes.
PREVENTIVE EFFECTS OF MAGNESIUM SULFATE ADMINISTRATION DURING PREGNANCY

Magnesium is reported to have several effects at early stages of development, including neuroprotective [182, 183] and anti-inflammatory effects [38-40]. A randomized clinical trial by Rouse and colleagues [184] showed a significantly lower rate of cerebral palsy, a group of permanent movement disorders caused by abnormal development or damages in the brain occurring during pregnancy, in preterm born infants who received magnesium sulfate (MgSO₄) before delivery. This protective association was robust and persisted after controlling for multiple confounders. Moreover, the association was biologically plausible because magnesium is known to reduce vascular instability, hypoxic damage and protect against cytokine or excitatory amino acid damage, all threats to the vulnerable preterm brain [185]. A more recent meta-analysis by Doyle and colleagues confirmed the neuroprotective effects of maternal MgSO₄ administration [186].

Besides the clinical evidence of the neuroprotective activity of magnesium, preclinical studies on rodents demonstrated that magnesium sulfate is able to reduce inflammation at the maternal-fetal interface following LPS administration in the mother during pregnancy [187]. Moreover, short- and long-term changes in the inflammatory status in pregnancies complicated by pathologies such as pre-eclampsia are reported [188] and magnesium seems to have beneficial effects by reducing IL-6 [189] and IL-1β levels [190] in the mother circulation as well as in the placental compartment. Not less important is the fact that one of the largest clinical study on eclamptic events prevention during pregnancy, the Magnesium Sulfate for Prevention of Eclampsia or Magpie Trial, demonstrated that treatment with magnesium sulfate is able to lower the number of patients who developed eclampsia by about half, without any serious harmful effects on either the mother or fetus [191]. It seems clear that, despite the exact mechanism through which magnesium exerts its beneficial effects is not yet known, its neuroprotective, anti-inflammatory and vasodilatory properties are supported by several studies.

All these knowledges about magnesium prompt us to investigate whether the effects of prenatal immune activation on the offspring could be prevented by using magnesium sulfate in the mother. First, we investigated whether the treatment with MgSO₄, prior to Poly I:C injection, could prevent the very rapid increase of IL-6 in
the maternal plasma. Surprisingly we found that IL-6 levels in females pre-treated with magnesium were comparable to control levels (Figure 20A).

**Figure 7.** (A) Quantitative analysis of IL-6 plasmatic levels in adult females 3 hours after Poly I:C injection alone or MgSO₄ treatment prior to Poly I:C. MgSO₄ is able to prevent the increase in IL-6 plasmatic levels observed with the Poly I:C alone. Ordinary one-way ANOVA followed by Tukey’s multiple comparisons test, ***p<0.001. (B). Quantitative analysis of IL-6 mRNA levels in whole embryos 6 hours after Poly or MgSO₄+Poly I:C in the mother. MgSO₄ is able to prevent the increase in IL-6 mRNA levels observed in the Poly I:C embryos. Ordinary one-way ANOVA followed by Tukey’s multiple comparisons test, *p<0.05, **p<0.01. Ctrl n=8, Poly I:C n=10, MgSO₄ alone n=5, MgSO₄+Poly I:C n=14.

In addition, we investigated the IL-6 mRNA levels in embryos exposed to MgSO₄ pre-treatment in the mother and, very interestingly, we found that MgSO₄ is able to prevent IL-6 increase in whole embryos 6 hours after Poly I:C injection. Magnesium itself seems to have no effects on IL-6 expression (Figure 20B).

We then asked whether magnesium administration before Poly I:C injection in the mother could somehow prevent the increased susceptibility to seizures observed in the Poly I:C offspring. Strikingly, P90 offspring from Magnesium-Poly I:C treated dams resulted less susceptible to KA induced seizures respect to Poly I:C mice, with a behavioural trend fully comparable with the control offspring (Figure 21).
Figure 8. Time course of the behavioural response to 35 mg/kg kainate injection in control, Poly I:C and MgSO₄+Poly I:C injected offspring. Notably, MgSO₄ is able to prevent increase seizure susceptibility when administered in the mother before Poly I:C.

We then wanted to investigate whether the restoration of a physiological susceptibility of epileptic by MgSO₄ was associated with a normalization of intracellular chloride concentrations in the Poly I:C offspring cortical neurons. We then performed two-photon imaging also in P20 cortical slices from offspring prenatally treated with magnesium prior to Poly I:C. In line with what expected, we found that MgSO₄ treatment could prevent the increased intracellular chloride concentration observed in Poly I:C offspring (Figure 22A), by restoring these levels to values comparable to control mice. Consistently with this data, we found no changes in KCC2 protein levels in P90 Poly I:C offspring pre-treated with MgSO₄ respect to control mice (Figure 22B), further suggesting that magnesium, probably via cytokine-activated pathways or via other pathways that still need to be investigated, is able to prevent the altered intracellular chloride concentrations and KCC2 protein expression observed in Poly I:C offspring.
Figure 9. (A) Quantitative analysis of intracellular [Cl\(^-\)] by two-photon imaging on P20 brain slices. MgSO\(_4\) prior to Poly I:C is able to prevent the increase in [Cl\(^-\)] observe in the offspring prenatally treated with Poly I:C alone. Ordinary one-way ANOVA followed by Tukey’s multiple comparisons test *p<0.05, **p<0.01, ***p<0.001, \(n_{\text{cells}} > 40\) for each experimental group. (B) KCC2 protein levels are reduced by Poly I:C prenatal treatment but not by combined administration of both MgSO\(_4\) and Poly I:C. One-way ANOVA followed by Tukey’s multiple comparisons test, *p<0.05. Ctrl n=10, Poly I:C n=10, MgSO\(_4\)+Poly I:C n=4.

Besides providing the direct demonstration that the increased susceptibility to seizures following prenatal immune activation stems from an altered GABA switch due to aberrant intracellular chloride homeostasis, these data open the possibility of a potential strategy to prevent harmful effects of prenatal immune activation on the fetal brain.
TIME OF PRENATAL IMMUNE CHALLENGE DIFFERENTIALLY AFFECTS INTRACELLULAR CHLORIDE CONCENTRATION

The development of the human CNS begins early during the embryonic period and proceeds through a series of very complex processes long after delivery. Although the neurodevelopmental processes are genetically determined, their complexity implicates the vulnerability of the CNS to several environmental factors [192]. As extensively discussed in the introduction, timing of prenatal immune activation may differentially impact the developing fetus, since the maternal immune system exhibits considerable fluctuations as pregnancy proceeds. The associated changes in cytokines production may therefore critically modulate the specificity of the inflammatory events in the developing fetus, possibly leading to aberrant brain development and related behavioural and cognitive alterations later in life [30]. Therefore, depending on the gestational period the developing nervous system of the fetus may not be uniformly vulnerable to maternal infections.

In order to assess whether GD9 represents a specific window of vulnerability for the onset of the behavioural but also biochemical and functional alterations observed following Poly I:C challenge, we administered the same dosage (2 mg/kg) at later developmental stages during pregnancy. With the same modality GD15 pregnant dams were injected with either Poly I:C or vehicle ip. and intracellular chloride concentrations were examined in cortical neurons from P20 acute brain slices from Poly I:C and control offspring. We interestingly found that unlike GD9 administration, Poly I:C administration at GD15 results in the opposite phenotype. In fact, two-photon microscopy showed a decrease in $[\text{Cl}^-]$, in Poly I:C offspring as compared to control, as highlighted by the corresponding increase in the MQAE mean lifetime (Figure 23).
Figure 23. Quantitative analysis of the intracellular chloride concentration in cortical neurons from P20 brain slices obtained from Poly I: C and control offspring at the two gestational ages, GD9 and GD15. The mean lifetime of the fluorescent chloride-sensitive dye MQAE is inversely proportional to the intracellular chloride concentration. MQAE mean lifetime is significantly decreased in GD9-Poly I: C offspring, indicating that [Cl\(^-\)] is significantly increased in Poly I: C cortical neurons respect to controls. On the other hand, the [Cl\(^-\)] is significantly decreased in offspring from GD15-Poly I: C dams. Ordinary one-way ANOVA followed by Tukey’s multiple comparisons test, \(^{***}p<0.001; n_{\text{cells}} > 40\) for each experimental group.

Although further experiments need to be performed in order to clarify the causes underlying the opposite effect induced by Poly I: C at the two gestational times, it seems clear that two distinct temporal windows corresponding to mid- (GD9) and late- (GD15) gestation, are crucial in determining specific outcomes of prenatal immune activation.
REST AND MeCP2 BINDING TO KCC2 PROMOTER IS INCREASED IN POLY I:C OFFSPRING

Our evidence clearly indicates that a single immune activation at early stages of fetal brain development is sufficient to induce long-lasting modifications in the offspring brain, thus suggesting possible genetic or epigenetic mechanisms underlying. In an attempt to explore the molecular underpinnings of this phenomenon, we pointed our attention on possible molecular players which are known to have a fundamental role in modulating chromatin state during brain development [193]. Indeed, chromatin modifiers, such as DNA methyltransferases, histone methyltransferases and histone acetyltransferases, are recruited to specific genomic loci by DNA binding proteins, altering the access of specific transcription factors to chromatin thus modulating gene expression [194]. A good candidate for orchestrating epigenetic events in the nervous system is the DNA binding protein REST (Repressor Element 1 Silencing Transcription Factor), also known as the Neuron-restrictive Silencer Factor (NRSF). REST was firstly discovered in 1995 as a repressor of neuronal genes containing a 23 bp conserved motif known as RE-1 (repressor element 1 or NRSE) sequences. It plays a pivotal role in maintaining the transcriptional silencing of a large number of neuronal genes in non-neuronal differentiated cells, as well as in un-differentiated neuronal cells during early stages of neurogenesis, by recruiting a co-repressor complex containing CoREST [195] histone deacetylases (HDACs) [196-198] and methyl CpG-binding protein MeCP2 [199]. Downregulation of REST during transition from progenitors to post-mitotic neurons is necessary to allow neuronal gene expression [200], so that neuronal development can occur properly. Because RE-1 sequences was initially found only in neuronal genes, REST was assumed to be a master regulator of neurogenesis [201-203], but subsequently it has been implicated in more complex gene regulation such as the maintenance of embryonic stem cell pluripotency and self-renewal and regulation of mitotic fidelity in non-neural cells [193, 204]. In addition, REST dysfunction has been implicated in several diseases ranging from Down's syndrome to cardiomyopathies and cancer, as well as in epilepsy and ischemia [205-207] emphasizing its importance also as a master regulator of normal gene expression programs [208]. Interestingly, REST repressor complex is known to be involved in the regulation of KCC2 expression in neurons by binding to both RE-1 elements.
located in the KCC2 gene, one in the promoter of the gene, the second one downstream the TSS (transcriptional start site), in the intronic region of the gene [209].

Since we found reduced levels of KCC2 and associated alterations in intracellular chloride concentration following prenatal Poly I:C exposure, both in vitro and in vivo, we investigated the possible involvement of REST in our model. Taking advantage of ChIP (chromatin immunoprecipitation) experiments we investigated the possible interaction between REST and the KCC2 promoter in P20 cortices from Poly I:C and control offspring. Remarkably, we found that prenatal exposure to Poly I:C results in an increased binding of REST to the RE-1 element in the promoter of KCC2 gene (fragment 509), as well as to the RE-1 element located downstream the TSS (fragment 524) (Figure 24B). These results are in line with the downregulation of KCC2 mRNA levels observed by quantitative real-time PCR in the same Poly I:C animals compared to controls (Figure 24C).
Figure 24. (A) Schematic representation of mouse KCC2 promoter with the transcriptional start site (TSS) and the double RE-1 repressor sites (modified from Yeo et al. 2009 [209]). (B) ChIP analysis on P20 cortical tissue from Poly I:C and control offspring. An upward trend was observed in the binding of REST to both the RE-1 sites in the KCC2 gene. Ctrl n=3, Poly I:C n=5. (C) KCC2 mRNA expression levels analysed by quantitative real-time PCR in P20 mice prenatally treated with Poly I:C or vehicle. KCC2 expression is significantly decreased in Poly I:C animals respect to controls. Ctrl n=11, Poly I:C n=13, Student’s t-test *p=0.0401.
As previously discussed, another important target for epigenetic modifications in models of neurodevelopment is represented by MeCP2 [74]. MeCP2 deregulation has a well-established role in Rett’s syndrome but it has also been implicated in other neurodevelopmental disorders such as autism and schizophrenia [77]. Moreover, a first experimental demonstration that inflammation during prenatal life is associated with epigenetic changes, including MeCP2 promoter hypomethylation, has been reported in 2014 by Basil and colleagues [74]. Given the possible implications of MeCP2 in the regulation of neuronal genes in neurodevelopmental pathologies, we decided to investigate MeCP2 binding to KCC2 promoter in the same samples used for REST ChIP analyses. Unexpectedly, we found an increase also in MeCP2 binding in both regions of the gene (509 and 524), containing the two RE-1 sites (Figure 25).

![Figure 25. ChIP analysis of P20 cortical tissue from Poly I:C and control offspring. An upward trend was observed in the binding of MeCP2 to both the RE-1 sites in the KCC2 gene. Ctrl n=3, Poly I:C n=5.](image)

Although further experiments are required for shedding light into such mechanisms, we can speculate that prenatal Poly I:C could induce epigenetic modifications on KCC2 gene, leading to an increased binding of the transcriptional regulators, REST and MeCP2, to the gene. This increased binding could result in the KCC2 downregulation we observed, leading to the pathological deregulation of neuronal
chloride concentrations, thus resulting in increased network excitability and seizure susceptibility.
Animal models based on maternal exposure to immune stimuli allowed to explore the consequences of prenatal immune activation on brain development, thus shedding light on the molecular and cellular mechanisms underlying such phenomenon. The most common immunogens used in pregnant rodents are lipopolysaccharide (LPS), a component of the Gram-negative bacteria cell wall that binds to toll-like receptor (TLR) 4 mimicking bacterial infections, or polyinosinic:polycytidylic acid (Poly I:C), a synthetic double-strand RNA which, as viral nucleic acids, binds TLR3. These immunogens initiates a signalling cascade leading to the production of inflammatory mediators such as cytokines (IL-1β, IL-6, TNFα), chemokines, and complement proteins [210]. Interestingly, the injection of these inflammation-triggering molecules at different timepoints during gestation leads to different neuropathological features [30] and gene expression profiles [21]. Consistently, the time of prenatal immune challenge critically influences the pattern of behavioural abnormalities displayed by the offspring in the adult age [30]. As in human infections, the effects of the immune challenge on fetal brain development are not due to the immunogen itself but rather to the induction of pro-inflammatory cytokines in the maternal circulation or placenta [26, 56], including IL-6 [61]. Notably, existing data indicate that the inflammatory cytokine IL-6 might be a pivotal immunological mediator of the link between maternal immune activation and altered brain development including schizophrenia, autism and epilepsy [211-213]. Indeed, the administration of exogenous IL-6 alone to pregnant animals is sufficient to induce long-lasting structural and functional abnormalities in the adult offspring, some of which are highly comparable to those induced by prenatal exposure to other immune activating agents such as Poly I:C [61, 214].

A recent population-based cohort study demonstrated an association between maternal infections during pregnancy and childhood epilepsy [115]. This is particularly relevant given that epilepsy is highly comorbid with different neurodevelopmental diseases [215]. Consistent with the epidemiological data, a recent study demonstrates that offspring of mice which have been chronically exposed to Poly I:C (GD12-GD16) during pregnancy show increased hippocampal excitability, faster progression of kindled seizures and prolonged persistence of the kindling state, along with impaired social interactions [117, 164].
Our model provides further support to these data adding the key information that even a single intrauterine exposure to 2 mg/kg Poly I:C at GD9 is able to significantly increase offspring susceptibility to seizures induced by kainic acid injection (Figure 2).

The evidence collected in this work about the absence of a chronic inflammatory condition in the brain of adult offspring is quite relevant, since different papers reported the occurrence of major brain alterations following maternal immune activation. Indeed, complex and long lasting changes in inflammatory cytokines levels (including IL-6 and IL-1β) have been reported in the neocortex and hippocampus [216, 217] in offspring from mother subjected to MIA, with relevant consequences on brain structure, neurogenesis and microglia activation [218, 219]. Furthermore, it is also known that pro-inflammatory molecules per se may increase epileptic susceptibility. Indeed, high IL-1β levels in the brain act as pro-convulsive in the kainic acid model of temporal lobe epilepsy [101] and increased levels of serum IL-6 occur in epileptic patients, with IL-6 levels directly correlating with seizures frequency [220]. In contrast to a possible inflammation-based hypothesis underlying the enhanced susceptibility to seizures observed in MIA offspring, we failed in detecting any significant variations of either IL-6 or IL-1β mRNA levels in MIA offspring mice. Although the apparent discrepancy between our data and that of other groups might be the result of different dosage and timing of Poly I:C injection, our data clearly indicates that the enhanced susceptibility to seizures does not result from an enduring elevation in cytokine expression.

The results reported in this study, point to alterations in neuronal chloride transporters expression, KCC2 and NKCC1 (Figure 14A), as the main causes of the neuronal network hyperexcitability observed in cortical neurons of Poly I:C offspring (Figure 11). In particular, several works indicate that KCC2 reduction results in an increased neuronal network excitability. Indeed, reduction of surface expression of KCC2 has been described to render neuronal networks hyperexcitable and susceptible to seizures triggered by excitatory stimuli [221], while mutations in the Drosophila melanogaster KCC gene kazachoc (kcc) confer increased seizure susceptibility [222]. Moreover, KCC2 heterozygous mice, expressing ~50% of the
wild-type KCC2 protein, show increased susceptibility to pentylenetetrazole-induced seizures [148] and a KCC2 co-segregating variant (KCC2-R952H), which reduces neuronal Cl\(^{-}\) extrusion, has been described in an Australian family with febrile seizures [155].

Interestingly, the alteration of intracellular chloride concentrations upon Poly I: C administration, which is responsible for the excitatory role of GABA signalling in our model, is tightly dependent on the developmental stages of Poly I: C injection. In fact, when administered at GD9, Poly I: C induces an increase in intracellular chloride concentrations of cortical neurons (Figure 14), while in contrast and unexpectedly, at GD15 the effect is the opposite (Figure 23), probably leading to different outcomes in the offspring. Certainly, GD9 and GD15 represent two very distinct stages of brain development. In mice, GD9 corresponds to a very precocious stage of neurodevelopment, when cortical neurogenesis is about to get started; cortical layer I is the first to be shaped during neurogenesis from E10.5 to E12.5 [223] while layers IV, V and VI are formed during E11.5 - E14. Layers II and III are the last to be formed during corticogenesis around E13.5 to E16 and include pyramidal neurons, stellate neurons and radial glial cells [224] as layer IV, V and VI. On the other hand, at GD15 the cortical layering is almost completed and the mouse blood brain barrier is almost completely functional (E15.5) [225]. When administered at GD9 or at GD15, Poly I: C impact on two very different stages of embryonic development and it would not be so surprising if the maternal response to Poly I: C was different at the two times. In this regard, it has been widely demonstrated that the maternal innate immune system is very dynamic during gestation and that TLRs are differentially expressed in non-pregnant, compared with pregnant tissues [226]. Moreover, TLR3 expression is increased from the first to the third trimesters in humans and Poly I: C is able to reduce its expression during the third trimester with no effects in the first trimester. Thus, it would be very interesting to investigate the pattern of inflammatory cytokines expressed both by the mothers and the embryos when Poly I: C is administered at GD15 rather than at GD9. Moreover, the precise timing of prenatal immune challenge critically determines the specificity of behavioural pathology, a concept which is clearly demonstrated in preclinical models. Poly I: C challenge on GD9 suppresses spatial exploration, whereas the same treatment
conducted on GD17 leads to perseverative behaviour [30]. Hence, since the specificity of the offspring vulnerability may depend on the gestational time windows at which the maternal immune system is challenged, further experiments will help us to clarify the causes underlying the opposite phenotypes observed at the two gestational ages in our model.

Despite our results exclude a contribution of persistent elevated cytokine levels in the brain of adult offspring, we believe that the transient increase in IL-6 and IL-1β occurring in the fetuses (Figure 5), few hours after Poly I:C injection in the mother, is at the root of the developmental trajectory leading to KCC2 dysregulation. Indeed, we observed that maternal dietary supplementation with MgSO\textsubscript{4} was sufficient to prevent fetal cytokines transient increase (Figure 20) as well as the increase in [Cl\textsuperscript{-}] in cortical neurons and KCC2 deregulation (Figure 22), thus also avoiding the increase in seizures susceptibility (Figure 21) observed upon prenatal Poly I:C administration. MgSO\textsubscript{4} is reported to have several effects at early developmental stages, including neuroprotective [182, 183] and anti-inflammatory properties [38-40] and in our model resulted to be effective in preventing the early inflammatory state associated to maternal immune activation.

It is known that KCC2 is the only one of the nine KCC isoforms to be exclusively expressed in central neurons [227] and its lack in non-neuronal cells relies on REST repression through the binding to the RE-1 site located in the KCC2 promoter [228]. In addition, REST repressor complex regulates the expression of KCC2 in neurons by binding to two RE-1 sites in the KCC2 gene [209], allowing its progressive increase during neurodevelopment, necessary for correct brain maturation and functioning. It has been reported that the alteration of KCC2 expression through REST activity can modify the chloride switch in cortical neurons [209]. Accordingly, by ChIP experiments we found an increase in REST binding to KCC2 gene following prenatal Poly I:C administration respect to controls (Figure 24), consistent with a downregulation of KCC2 mRNA expression levels. Moreover, another important master regulator of neuronal genes transcription, MeCP2, was found to be tightly bound to KCC2 RE-1 sites in Poly I:C offspring respect to controls (Figure 25). It is known that MeCP2 binds to the N-terminus domain of REST in the REST complex,
(Figure 26) and it has been demonstrated that both upstream and intronic RE-1 sites are bounded by REST, as well as by the corepressors MeCP2 and CoREST with a relative binding of MeCP2 and CoREST to the intronic RE-1 higher than that measured for the upstream RE-1 by ChIP analyses; suggesting a tighter association of REST and MeCP2 with the intronic RE-1 [209].

Since MeCP2 is part of the REST repressor complex responsible for the regulation of KCC2 expression, it is possible that REST and MeCP2 could cooperate together in the regulation of KCC2 expression rather than compete for the binding to the RE-1 sites as recently suggested in a paper by Tang et al. [229]. Thus, we hypothesize that prenatal Poly I:C could lead to an increase in the repression of KCC2, probably by epigenetic modifications on chromatin in the KCC2 gene thus altering the binding of the REST complex (including MeCP2). Further experiments, like for example analyses of changes in chromatin structure, like histone methylation levels, in the KCC2 gene, must be performed in order to further investigate the mechanism underlying KCC2 deregulation.

Despite several studies on KCC2 expression, no data are reported about a possible role of REST in regulating NKCC1 expression. Since both NKCC1 and KCC2 are involved in chloride homeostasis and in the GABA switch during neurodevelopment, and since in our model we found not only a decrease in KCC2 levels but also an
increase in NKCC1 protein levels in E17 cortices from Poly I:C offspring and in 6 DIV Poly I:C-established cultures, it will be necessary to investigate why and how NKCC1 is dysregulated in our Poly I:C model.

Since at the root of the phenotype observed upon Poly I:C immune challenge there could be epigenetic modifications leading to KCC2 dysregulation responsible for increased network excitability, we can speculate that maternal immune activation could change neuron developmental trajectories through epigenetic processes which are intrinsically “sculpted” in the neuron, independently from the brain environment, as demonstrated by \textit{in vitro} experiments from Poly I:C cultures recapitulating \textit{ex vivo} and \textit{in vivo} observations (Figure 15-19).

A number of studies have examined the possible role of furosemide and bumetanide, two widely-used diuretic drugs, in the treatment of epileptic disorders, since these two drugs can inhibit cation-chloride cotransporters [230]. While these and other observations indicate an anticonvulsant action of these drugs, novel compounds, also including KCC2 activators, are needed for further research on CCCs as pharmacotherapeutic targets. Indeed, recent reports indicate that enhancing KCC2 activity may be the best therapeutic strategy to restore inhibition and normal function in pathological conditions involving impaired Cl\textsuperscript{−} transport [231, 232]. Gagnon and colleagues identified a KCC2-selective analogue (CLP257) able to reduce \([\text{Cl}^-]\), restoring the impaired Cl\textsuperscript{−} transport in neurons with diminished KCC2 activity. The compound rescued KCC2 plasma membrane expression, renormalized stimulus-evoked responses in spinal nociceptive pathways sensitized after nerve injury and alleviated hypersensitivity in a rat model of neuropathic pain [233]. These results validate KCC2 as a novel therapeutic target for CNS diseases, opening the possibility to use this kind of compounds for the treatment of a wide range of neurological and psychiatric disorders, since it is known that KCC2 dysfunction and Cl\textsuperscript{−} homeostasis are central to many CNS disorders including epilepsy, stress, anxiety and schizophrenia. Thus, it could be interesting to evaluate if such compounds could be effective in the treatment of the pathological conditions we observed following prenatal Poly I:C in our model.
In conclusion, using the Poly I:C model of maternal immune activation, we provide here the evidence that a single prenatal stimulus, during a selected time window of pregnancy (GD9), affects the glutamate-GABA equilibrium in the offspring through a long-lasting alteration of the chloride transporter KCC2 expression at cortical level, resulting in an alteration of the hyperpolarizing action of GABA which endures at mature stages, as highlighted by increased seizure susceptibility. We also provide the proof-of-concept that such protein expression abnormality and its deleterious physiological consequences can be prevented by dietary maternal supplementation with MgSO$_4$, already known to reduce inflammation at the maternal-fetal interface. Notably, the increased binding of the two master regulators of neuronal genes expression, REST and MeCP2, on KCC2 promoter, suggests a possible epigenetic mechanism involved in the regulation of KCC2 expression following inflammation in the mother. Thus, maternal immune activation, through pro-inflammatory cytokines, may lead to epigenetic modification responsible for KCC2 dysregulation and the consequent pathological outcomes, as suggested also by in vitro experiments.
EXPERIMENTAL PROCEDURES
ANIMALS

Wild type C57BL/6 mice (Charles River) were used. Animals were housed at constant temperature (22-18°C) and relative humidity (50%) under a regular light-dark schedule (lights on 7 a.m. to 7 p.m.) with food and water ad libitum. All the experimental procedures were carried out following the guidelines established by the Italian Council on Animal Care and were approved by the Italian Government Decree No. 27/2010 and the Italian Legislation (L.D. No 26/2014). All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

MATERNAL IMMUNE ACTIVATION

Three to six months old females were mated for 24 hours with a single male. Starting from gestational day 0 (GD0) females were weighed every 2 days till GD9. At GD9 females who gained at least 1,5 grams, gradually in time, were injected with 2 mg/kg body weight Poly I:C (polyinosinic:polycytidylic acid, Sigma-Aldrich) or vehicle intraperitoneally (ip.). Pregnant females were then used to obtain embryos at different stages of embryonic development or the offspring was allowed to be born and grow till postnatal day 20 (P20) or P90.

ELISA FOR IL6

Three months old females were treated with vehicle or 2 mg/kg Poly I:C ip. Three hours after the injection the animals were sacrificed and the blood was collected and placed in tubes containing 4.1 mM EDTA used as anticoagulant. After centrifugation at 2000 g for 15 minutes the plasma was collected and used to evaluate IL-6 levels using a IL-6 specific ELISA kit (Thermo Fisher Scientific), with 50-2000 pg/ml sensitivity, according to the instructions from the manufacturer. The absorbance was then measured at 450 and 550 nm using Victor² spectrophotometer (Wallac).
BEHAVIOURAL OBSERVATION OF KAINIC ACID-INDUCED SEIZURES

Kainic Acid (KA, Sigma-Aldrich) was dissolved in saline solution and administered ip. 35 mg/kg body weight. P90 control and Poly I:C-offspring mice were used. In all experiments, the experimenter was blind to the treatment of the animals. Seizure severity was determined according to the Racine’s scale [234]: stage 0: normal behaviour; stage 1: immobility; stage 2: forelimb and/or tail extension, rigid posture; stage 3: repetitive movements, head bobbing; stage 4: forelimb clonus with rearing and falling (limbic motor seizure); stage 5: continuous rearing and falling; stage 6: severe whole body convulsions (tonic-clonic seizures); stage 7: death. For each animal, the rating scale value was scored every ten minutes for a maximum of 3 hours after KA administration. The data were used to calculate the time-course of seizure severity for each experimental group.

BRAIN SLICE PREPARATION FOR IMMUNOFLOURESCENCE STAINING

P90 brains from Poly I:C prenatally-treated mice and controls were used. Animals were first euthanized and then perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2 as described by Frassoni et al. [235]. Forebrains were dissected out and coronally cut with a VT1000S vibratome (Leica Microsystems) in serial sections. The following primary antibodies were used: anti-rabbit IBA1 (1:200; Wako), anti-rat CD11b (1:1000, Biolegend). Free-floating sections were preincubated for 45 min in 0.01 M phosphate buffered saline pH 7.4, containing 10% normal goat serum and 0.1% Triton X-100 and then incubated with primary antibodies. Subsequently the sections were incubated with the corresponding secondary fluorescent antibodies conjugated with Alexa-488, Alexa-555 or Alexa-633 fluorophores (Invitrogen). Slices were then put on coverslips by the use of the aqueous mounting medium Fluorsave (Millipore) and maintained at -20°C.
SKELETON ANALYSIS
The skeleton analysis is a quantitative method that enables to analyze morphological features of microglial cells through IF images obtained from brain slices acquired by confocal microscopy. We obtained a projection of the maximum intensity of the IBA1-positive signal to be able to fully visualize all the microglial cell processes. The resulting image was then converted to a binary image and skeletonized before using the plugin from ImageJ software: AnalyzeSkeleton plugin. The data from this analysis were then normalized to the number of cells present in the image as described [236].

BRAIN SLICE PREPARATION FOR ELECTROPHYSIOLOGY
Poly I:C and control offspring, aged 17-28 days were deeply anesthetized with ether and decapitated. Brains were removed and placed in ice-cold solution containing the following (in millimolar): 87 NaCl, 21 NaHCO₃, 1.25 NaH₂PO₄, 7 MgCl₂, 0.5 CaCl₂, 2.5 KCl, 25 D-glucose and 7 sucrose, equilibrated with 95% O₂ and 5% CO₂ (pH 7.4). Coronal slices (300 µm thick) were cut with a VT1000S vibratome (Leica Microsystems) from medial prefrontal cortex. Slices were incubated at room temperature for at least 1 h, in the same solution as above, before being transferred to the recording chamber. During experiments, slices were superfused at 1.0 ml/min with artificial cerebrospinal fluid containing the following (in millimolar): 135 NaCl, 21 NaHCO₃, 0.6 CaCl₂, 3 KCl, 1.25 NaH₂PO₄, 1.8 MgSO₄, and 10 D-glucose, aerated with 95% O₂ and 5% CO₂ (pH 7.4). Cells were examined with a BX51WI upright microscope (Olympus) equipped with a water immersion differential interference contrast (DIC) objective and an infrared (IR) camera (XM10r Olympus).

IN VIVO WHOLE-CELL RECORDINGS
Neurons were voltage (or current) clamped with a Multiclamp 700B patch-clamp amplifier (Molecular Devices, Union City, CA) at room temperature. Low-resistance micropipettes (2-3 MΩ) were pulled from borosilicate. The cell capacitance and series resistance were always compensated. Experiments in which series resistance
did not remain below 10 MΩ (typically 5-8 MΩ) were discarded. Input resistance was generally close to 100-200 MΩ. Synaptic currents were low-pass filtered at 2 kHz, sampled at 10 kHz and analysed with pClamp/Digidata 1440A (Molecular Devices). Pipettes contained (in millimolar) 135 K gluconate, 5 KCl, 2 MgCl₂, 10 HEPES, and 2 MgATP (pH 7.2). During experiments, slices were superfused at 1.0 mL/min with artificial cerebrospinal fluid containing the following (in millimolar): 135 NaCl, 21 NaHCO₃, 0.6 CaCl₂, 3 KCl, 1.25 NaH₂PO₄, 1.8 MgSO₄, and 10 D-glucose, aerated with 95% O₂ and 5% CO₂ (pH 7.4). Recordings were made from cortical layer II/III or V pyramidal neurons. For miniature excitatory post-synaptic current (mEPSC) recordings TTX was added to the solution. For cell-attached recordings, glass micropipettes (resistance, 2–5 MΩ) were filled with ACSF, and slices were bath perfused with the same solution. After a giga-ohm seal had been obtained, each cell was recorded under different conditions depending on the experiments for 4 min each: baseline in ACSF, GABA 200 μM. All cell-attached recordings were performed in the voltage-clamp mode, and the recording pipette was kept at 0 mV for all such recordings. Data from the whole 4 min of recording were analysed for quantification of spiking activity.

**TWO-PHOTON CHLORIDE IMAGING**

Imaging of neuronal intracellular Cl⁻ in acute cortical slices was performed using the fluorescent indicator MQAE [N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide]. MQAE dye detects Cl⁻ ions via diffusion-limited collisional quenching, resulting in a concentration-dependent decrease in fluorescence emission after an increase in Cl⁻ concentration. Ex vivo brain slices (prepared as described above for patch-clamp experiments) were incubated at 37 °C in the dark for 30 minutes in oxygenated ACSF containing 5 mM MQAE (Biotium). FLIM z-stacks were acquired with a TrimScope II two-photon upright microscope (LaVision BioTec) and a 20x NA 1.0 objective (XLUMPFLN 20x, Olympus). Samples were excited with Ti:Sa pulsed laser tuned at 740 nm (Chameleon Ultra II, Coherent), and all emission light was collected with a TCSPC detector (FLIM x16, LaVision Biotec). Z-stacks started at least -30 μm from slice top position, in order to avoid any possible artifact due to cell non-
viability, with z-step 3 μm and total z-stack size 80 μm. In a setup phase, standard z-stack time-lapses with the same excitation conditions, collected with a standard PMT and 420/50 filter were recorded over 10-15 minutes in order to check slices viability and to assure that acquisition conditions did not induce any phototoxicity. We then drew a ROI for each cell, and FLIM data for each ROI was fitted to a single-exponential lifetime curve with calculated offset (analysis software ImSpector Pro, LaVision Biotec). Data for each group was then statistically tested for Gaussian distribution and either t-test, parametric or non-parametric ANOVA depending on the datasets (Prism, GraphPad).

**BICINCHONIC ACID (BCA) ASSAY**

Protein content of brain cortices at different ages was assessed by bicinchoninic acid (BCA) assay, using BCA protein assay kit (Thermo Fischer Scientific). In the BCA method, BCA molecules chelate cuprous ion Cu⁺¹, formed from Cu²⁺ reduction by proteins in alkaline environment. This chelation generates a purple-colored reaction product which has a strong absorbance at 562 nm, proportional to protein concentration in the sample. Sample absorbance was read through a spectrophotometer (Victor² - 1420 multilabel counter, Wallac) set to 550 nm.

**WESTERN BLOTTING**

Cortices from E17 and P90 mice brains were homogenized in a glass-teflon potter using a solubilizing mix composed by sodium dodecyl sulphate 1% (SDS), 62.5 mM Tris-HCl at pH 6.8 and 290 mM sucrose. The homogenates were then centrifuged at 1600 g at 4 °C for 15 minutes and the low speed supernatant was stored at -20 °C and subsequently used for SDS PAGE experiments. Cortex homogenates were separated by electrophoresis, blotted, and incubated with primary antibody, followed by HRP-conjugated secondary antibody (Jackson ImmunoResearch) and developed by Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific). The following primary antibodies were used: monoclonal antibodies directed against PSD95 1:10000 (NeuroMab), Syntaxin1A 1:1000 (Sigma-Aldrich), SNAP25 1:1000
(Chemicon) and polyclonal antibodies directed against Calnexin 1:2000 (Sigma-Aldrich), GAPDH 1:1000 (Synaptic Systems) vGLUT1 1:1000 (Synaptic Systems), vGAT 1:1000 (Synaptic Systems), KCC2 1:1000 (Millipore), NKCC1 1:1000 (Millipore), MeCP2 1:1000 (Sigma-Aldrich).

**QUANTITATIVE REAL-TIME PCR**

Whole embryos obtained from GD9 injected dams and brain tissues from E17, P20 and P90 offspring were used for real-time PCR analysis. Samples were homogenized prior to RNA extraction in 500 μl of TRI-reagent (Zymo research). Total RNA was isolated using the Direct-zol RNA MiniPrep isolation kit (Zymo research) according to the manufacturer’s protocol. The RNA was eluted with 25 μL DNase/RNase-free water, quantified using NANOdrop 2000c spectrophotometer (Thermo Fisher Scientific) and optical density 260/280 nm ratios were determined. Reverse transcription was performed using 1 μg RNA with a High Capacity cDNA RT kit (Applied Biosystems). Real-time polymerase chain reaction (qRT-PCR) was performed using RT-PCR Viia7 (Applied Biosystems) in a final volume of 10 μl for SYBR Green technique (SensiFAST SYBR Lo-ROX, Bioline) and 20 μl for TaqMan technique (Gene Expression Master Mix, Life Technologies). Each gene was analysed at least in duplicate and data analysis was performed with the ΔΔCt method. All RNA levels were normalized to GAPDH or Actin where indicated.

**MATERNAL MAGNESIUM SULFATE SUPPLEMENTATION**

GD9 pregnant dams were used. The magnesium sulfate (MgSO₄, Sigma-Aldrich) injection protocol included an initial subcutaneous loading dose of 270 mg/kg followed by maintenance doses of 57 mg/kg every 40 minutes for 4 hours. A second loading dose of 270 mg/kg was given at the end of the injection session. Control mice were injected with saline solution at the same volume and schedule (protocol adapted from Hallak 1999, [237]). Magnesium sulfate supplementation was performed just before Poly I:C/vehicle ip. injection at GD9. Four experimental groups were evaluated to assess the potential preventive effects of magnesium by
acting as immunomodulator and reducing inflammation at the maternal-fetal interface: MgSO₄ (sc)+vehicle (ip); MgSO₄ (sc)+Poly I:C (ip); vehicle (sc)+vehicle (ip); vehicle (sc)+Poly I:C (ip).

PRIMARY CORTICAL CULTURES
Primary neuronal cultures were prepared from E17 mice from Poly I:C or vehicle injected dams. Once obtained, cortices were chemically dissociated by treatment with 0.25% trypsin (Invitrogen) for 15 min at 37°C, followed by mechanical dissociation with a fire-polished Pasteur pipette. Dissociated cells were then plated on poly-L-lysine-treated (1 mg/ml, Sigma-Aldrich) 24 mm glass coverslips in Neurobasal (Invitrogen) containing 2% B27 (Invitrogen), 100U/ml penicillin, 100 μg/ml streptomycin, 200 mM glutamine, 10 nM glutamate, as described in Brewer et al. [238], at densities ranging from 10x10³ to 20x10³ cells/cm². Neuronal cultures were kept at constant temperature (37°C) in the presence of 5% CO₂. At 3 days in vitro (DIV), half of the culture medium was replaced with fresh medium without glutamate to avoid excitotoxicity phenomena. Cells were then used for calcium imaging and chloride imaging at 6 DIV and electrophysiological recordings and immunocytochemical staining at 14 DIV.

CELL CULTURE ELECTROPHYSIOLOGY
Miniature excitatory and inhibitory post-synaptic currents (mEPSCs and mIPSCs) were recorded in the presence of tetrodotoxin (TTX, Tocris); a reversible blocker of sodium channels that avoids the generation of action potentials. In presence of TTX the mPSCs incur spontaneously, induced by the release of a single vesicle. Thus, they correspond to the event generated from a quantum of neurotransmitter that interacts with postsynaptic receptors. Whole-cell patch-clamp recordings were obtained from 13-14 DIV cortical neurons with an Axopatch 200B amplifier and pClamp-10 software (Axon Instruments). Recordings were performed in the voltage-clamp mode. Currents were sampled at 2 kHz and filtered at 2-5 kHz. External solution [Krebs'-Ringer's-HEPES (KRH)] had the following composition (in
mM): 125 NaCl, 5 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 2 CaCl₂, 6 glucose, and 25 HEPES-NaOH, pH 7.4. mEPSCs and mIPSCs were recorded in the presence of 1 μM tetrodotoxin (TTX). Recording pipettes were fabricated from capillary glass (World Precision Instruments) using a two-stage puller (Narishige) and had tip resistances of 3-5M when filled with the intracellular solution of the following composition (in mM): 130 K-gluconate (or Cs-Gluconate for IPSCs & mEPSCs), 10 KCl, 1 EGTA, 10 HEPES, 2 MgCl₂, 4 MgATP and 0.3 Tris-GTP. Neurons were held at -70 or +10 mV to identify, respectively, excitatory or inhibitory miniature events. Recordings were performed at room temperature. Off-line analysis of mEPSCs and mIPSCs have been performed using Clampfit-pClamp-10 software and events had to exceed a threshold of two times the SD of the baseline noise. Spontaneous burst activity was recorded in whole-cell voltage-clamp configuration in the absence of TTX while Multi Units were recorded in cell-attached configuration.

**INTRACELLULAR CALCIUM MEASUREMENTS IN NEURONAL CULTURES**

5-6 DIV cortical cultures were loaded with 5 μM Fura-2 pentacetoxymethylester in KRH for 30 minutes at 37 °C, washed in the same solution and transferred to the recording chamber of an inverted microscope (Axiovert 100, Zeiss, Oberkochen, Germany) equipped with a calcium imaging unit. After a period of baseline acquisition, neurons were stimulated with GABA 100 μM and responses were recorded. Fura-2 fluorescence images were analysed with TILLvision software (TILL Photonics). After excitation at 340 and 380 nm wavelengths, emitted light was acquired at 505 nm at a rate of 1–4 Hz. Temporal Ca²⁺ intensity profiles (expressed as F340/380 fluorescence ratio) were calculated in discrete areas of interest from image sequences. “Ratio changes” indicate the amplitude of the peak of Ca⁺⁺ responses, thus indicating the depolarizing GABA activity.
INTRANECLLULAR CHLORIDE MEASUREMENTS IN NEURONAL CULTURES

6 DIV cortical cultures were loaded with 5 μM MQAE (Biotium) in KRH. MQAE is a 6-methoxyquinolinium derivative and it is used as a fluorescent indicator for intracellular chloride. This dye detects the ion via diffusion-limited collisional quenching. MQAE has greater sensitivity to Cl⁻ and a higher fluorescence quantum yield than SPQ; consequently, it is currently the more widely used of the two indicators. After 1 hour incubation at 37 °C, neurons were washed in KRH and transferred to the recording chamber of an inverted microscope (Olympus) equipped with a CellR imaging station. All excitation and acquisition parameters were kept constant throughout the experiments. Image analysis was performed using Xcellence software (Olympus) by measuring the mean fluorescent intensity of regions of interest.

IMMUNOCYTOCHEMICAL STAINING

Primary cortical cultures were fixed with 4% paraformaldehyde and 4% sucrose in 0.12 M phosphate buffer for 20 min at 37°C and immunofluorescence staining was carried out as previously described [239]. Images were acquired using a Zeiss LSM 510 META confocal microscope with 60X objective. Immunofluorescence staining was carried out using the following antibodies: guinea pig anti-vGlut1 (1:1000), rabbit anti-vGAT (1:1000), both from Synaptic System. Secondary antibodies were conjugated with Alexa-488, Alexa-555 or Alexa-633 fluorophores (Invitrogen). The number of vGAT and vGlut1 positive puncta have been counted after the detection of an appropriate threshold which was set to 2.5-fold the level of background fluorescence referring to diffuse fluorescence within dendritic shafts. Fluorescence images processing and analyses were performed with ImageJ Software (National Institutes of Health).
CHROMATIN IMMUNOPRECIPITATION

Cortices of P20 mice have been dissected and rapidly incubated in 1% formaldehyde for 15 minutes at RT for cross-linking and then transferred in 0.125M glycine for 10 minutes and homogenized in the appropriate lysis buffer (10 mM Tris- HCl pH 8; 1 mM EDTA, 0.5 mM EGTA, 100 mM NaCl, 0.1% Na-deoxy-cholate, 0.5% N-laurylsarcosine) containing protease inhibitors and PMSF 0.2 mM. Lysates were then sonicated to generate fragments with an average length of ~500-200 bp, as determined empirically by agarose gel electrophoresis of the fragmented chromatin sample. The samples were then incubated with protein G Dynabeads (Invitrogen) bound to REST (Millipore) and MeCP2 pAb (Sigma-Aldrich) at 4°C for 6 hours. Immunoprecipitation was then performed overnight with 25 µg of sonicated chromatin in 600 µl of lysis buffer containing Triton 1%, PMSF 0.2 mM and 1.2 µg of anti-MeCP2 pAb bound to. A sample without Igg was included as a control. The beads were then washed sequentially at 4°C (for 7 minutes each) with 800 µl of low salt buffer (0.1% SDS, 2 mM EDTA, 1% Triton, 20 mM Tris-HCl pH 8, 150 mM NaCl), high salt buffer (0.1% SDS, 2 mM EDTA, 1% Triton, 20 mM Tris-HCl pH 8, 500 mM NaCl and TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA pH 8.0), then again with low salt buffer and with high salt buffer. At the end the beads were washed with TE-NaCl buffer (10 mM Tris-HCl, 1 mM EDTA, 50 mM NaCl). Elution was performed in 100µl of fresh elution buffer (1% SDS, 0.1 M NaHCO₃). Cross-linking was reversed overnight at 65°C. After cross-link reversal, 240 µg of Ribonuclase A (Sigma-Aldrich) were added to each sample to completely eliminate the RNA and the samples were incubated for 40 minutes at 37°C. The samples were then digested with 20 µg of proteinase K (Sigma-Aldrich) for 1 hour at 56°C, and DNA was recovered by standard methods in 20 µl of 10 mM Tris-HCl pH 8. KCC2 promoter was then analysed by quantitative real-time PCR.

STATISTICAL ANALYSIS

Pairwise comparisons between treatments were assessed with Student’s t-test. One-way ANOVA with repeated measures or 2-way ANOVA as between subject factor were used. Post hoc analysis was done using Tukey’s Bonferroni’s or Holm Sidak’s
post hoc tests. Unless otherwise stated, average data are expressed as mean ± SEM. Data were expressed as means ± SEM for the number of cells or animals (n). The differences were considered to be significant, if p< 0.05 (indicated by one asterisk), p< 0.01 (double asterisks), p< 0.005 (triple asterisks). All statistical analyses were done with software Prism, version 6 (GraphPad).
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