國立臺灣大學醫學院腦與心智科學研究所

碩士論文

Graduate Institute of Brain and Mind Sciences

College of Medicine

National Taiwan University

Master's Thesis

以思覺失調症小鼠為模式探討聚胞苷酸誘導母體免疫激活和絲氨酸消旋酶缺損間可能的交互作用

Investigation of possible epistatic interactions between poly(I:C)-induced maternal immune activation and serine racemase mutations in schizophrenia

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中華民國 112 年 12 月

December 2023

doi:10.6342/NTU202304492

國立臺灣大學碩士學位論文
口試委員會審定書
MASTER'S THESIS ACCEPTANCE CERTIFICATE
NATIONAL TAIWAN UNIVERSITY

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本論文係 陳圓圓君（學號: r09454012）在國立臺灣大學醫學院腦與心智科學研究所完成之碩士學位論文，於民國 112 年 10 月 25 日承下列考試委員審查通過及口試及格，特此證明。

The undersigned, appointed by the Graduate Institute of Brain and mind sciences on 25th Oct., 2023 have examined a Master's thesis entitled above presented by YUAN-YUAN CHEN (r09454012) candidate and hereby certify that it is worthy of acceptance.

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doi:10.6342/NTU202304492

誌謝

在神經科學領域的探問終於走到一個里程碑了，一開始對於心理疾病機轉神秘的未竟之地感到好奇以及希冀著能對世界上為心理疾病所苦的人們有所貢獻的我勇敢地開啟了這段旅程，沒想到最後受到最多幫助也成長最多的竟是我自己。首先非常感謝指導教授賴文崧老師一路上的提攜指教，謝謝老師給予機會讓研究經驗缺乏的我在這邊開啟一片新天地實現我想做的主題，老師跨領域、多方嘗試的精神也大大地拓展我的視野。感謝黃憲松老師、李立仁老師以及詹銘煥老師擔任我的口委，給予論文方向諸多建議。感謝實驗室所有的學長姊、學弟妹陪伴我度過碩士生涯；特別感謝家源學長、達中學長、如淳學姊、祖儀學姊、弼翔學長，提供我許多研究上的建議和生活上的鼓勵；感謝羿婷、秉翰、忻晴合作無間的扛起實驗室大小事也是我在研究所的溫暖依靠；感謝新血霈翎、浩賢、陳蓉、語喬的加入分擔不少實驗室的辛勞，有你們在好歡樂~感謝在成功大學時我的啟蒙老師陳德祐，以及德祐實驗室台北分部的昀恩、苡蓁、存凱、以欣帶給我許多溫暖。感謝讀書會的成員祥昀、聖傑、嘉容、孮瑋、昀晏，能夠見證彼此的成長至今真是難能的緣分。謝謝我的高中同學們特別是浱展、浩瑋讓我有個自在的出口。謝謝我的好山友太陽、郁茹、育豪、洸含、阿強陪伴我到每個世外桃源讓我在研究之餘能夠有所喘息。衷心的感謝我的父母親全然地支持我，讓我能夠在研究所無後顧之憂並時常體貼我的生活起居。還要謝謝自己，沒有半途而廢完成了學術界的闖蕩，也感念生命中每個貴人和一切相知相遇。最後要感謝所有為了研究默默犧牲奉獻的小鼠，謝謝你們用生命教會我許多課題，你們是真正的英雄。

doi:10.6342/NTU202304492

摘要

思覺失調症為一種嚴重的心理疾患，且影響全球約 1%的人口，但其致病機轉至今仍尚未被透徹瞭解。過往研究已指出有多種因素可能共同導致思覺失調症，包括遺傳因子和環境因子等等。而思覺失調症的雙重打擊假說提出，遺傳缺損結合早期發展中的環境刺激，最終可能導致思覺失調症發病。在這個前提之下，我們的研究欲以小鼠為模式探討思覺失調症其中之一候選基因—絲氨酸消旋酶（SR）缺損以及產前聚肌胞苷酸（poly(I:C)）所導致免疫活化引起的胎兒感染之間可能存在的交互作用。我們的研究共包括了三個實驗，實驗組為 SR 雜合子孕鼠在懷孕的第 17.5 天注射一劑聚肌胞苷酸（5mg/kg）之子代，對照組則是予以生理食鹽水。在實驗 1 中我們發現不論基因型在聚肌胞苷酸注射 6 小時過後，小鼠胚胎全腦中的三種促炎性細胞因子（IL-6，IL-1β 和 TNF-α）之蛋白質水平皆有升高。在實驗 2 中，則是以胚胎時期接受過聚肌胞苷酸或生理食鹽水介入的 SR 新生小鼠（實驗 2.1）和成鼠（實驗 2.2）執行一系列的行為測試。在新生期所進行的一組行為測驗包括翻身反射 （P3-7），地性反射（P3-7），抓握反射（P5-7）和開闊場測試（P26）。產前接觸聚肌胞苷酸導致其神經運動功能的發展延遲，特別是在 SR 同合子新生小鼠。在成年期，相同的小鼠群體被測試以評估其負性和認知功能。我們在成鼠（P80 及以上）進行了另一組行為測驗，包括開闊場測試、自發交替探索實驗、新物件辨識測驗、注意力測試、社交測試和痕跡恐懼制約測

doi:10.6342/NTU202304492

試。而聚肌胞苷酸影響了特定基因型的 SR 小鼠，明顯導致其運動活動、長期記憶和情境恐懼記憶的缺失。在實驗 3 中，我們測試另一個群體的新生期小鼠，以了解一種新型 D-氨基酸氧化酶抑制劑（RS-D7）可能的拯救效果。而我們的結果發現，不論基因型，RS-D7 有可能改善由聚肌胞苷酸引起的發展延遲。本研究試圖釐清思覺失調症病程中遺傳因素和環境因素複雜的交互作用，提供了有關產前免疫活化和 SR 基因突變在其中扮演腳色的重要發現。而造成這些行為差異背後的神經機轉仍需更進一步實驗研究生化層次的腦部變化才能更加了解。

關鍵字：思覺失調症、絲氨酸消旋酶、聚肌胞苷酸、產前免疫活化、小鼠模式、雙重打擊假說

doi:10.6342/NTU202304492

Abstract

Schizophrenia is a serious mental disorder that is affecting about 1% of the global population, but its pathophysiology is unknown. Previous studies have identified multiple factors, including genetic and environmental, that make contribution to the pathology of schizophrenia. The two-hit hypothesis of schizophrenia posits that schizophrenic symptoms are caused by a genetic susceptibility as well as early developmental injury. In this context, our study investigates potential epistatic interactions between mutations in the serine racemase (SR) gene, a candidate gene for schizophrenia, and prenatal polyinosinic acid-polycytidylic acid (poly(I:C))-induced immune activation, i.e., gestational infections during early development, using mice as a model. SR heterozygous pregnant dams received either a single injection of poly(I:C) (5 mg/kg) or a vehicle on gestation day 17.5, and three experiments were included. In Exp.1, our results revealed that protein levels of three pro-inflammatory cytokines (IL-6, IL-1β, and TNF-α) were elevated in fetal brains 6 hours after poly(I:C) injection, regardless of genotypes. In Exp.2, a battery of behavioral tests was conducted on poly(I:C)- or saline-treated SR offspring in neonates (Exp.2.1) and adulthood (Exp.2.2). During the neonatal period, a set of behavioral tests, including righting reflex (P3-7), geotaxis reflex (P3-7), grasping reflex (P5-7), and open field task (P26), was conducted.

doi:10.6342/NTU202304492

Prenatal poly(I:C) exposure led to developmental delays in neuromotor functions, particularly in SR HOM mice during the neonatal period. In adulthood, the same cohort of mice was tested to assess their negative and cognitive functions. Another set of behavioral tests was carried out in adult mice (P80 upwards), including the open field test, spontaneous alternation test, novel object recognition test, object-based attention test, 3-chamber social test, and trace fear conditioning test. In adulthood, poly(I:C) challenges notably affect locomotor activity, long-term memory, and contextual fear memory in adult SR mice of specific genotypes, respectively. A separate cohort of poly(I:C)- or saline-treated SR neonates was evaluated to understand the potential rescue effect of a novel D-amino acid oxidase inhibitor (RS-D7) in Exp. 3. Our findings indicated that RS-D7 had the potential to ameliorate developmental delays induced by poly(I:C) exposure, irrespective of genotypes. This study shed light on the intricate interplay of genetic and environmental factors in schizophrenia, offering valuable insights into the role of prenatal immune challenges and SR mutations. To further examine neural mechanisms underlying behavioral alterations, brain alterations at the biochemical level deserved further study.

Keywords: schizophrenia, serine racemase, poly(I:C), maternal immune activation, mouse model, two-hit hypothesis

doi:10.6342/NTU202304492

Contents

doi:10.6342/NTU202304492

doi:10.6342/NTU202304492

doi:10.6342/NTU202304492

doi:10.6342/NTU202304492

List of Tables and Figures

doi:10.6342/NTU202304492

doi:10.6342/NTU202304492

doi:10.6342/NTU202304492

Chapter 1. Introduction

1.1 An overview of schizophrenia

Schizophrenia, a debilitating mental disorder, is estimated to have a lifetime prevalence of approximately 0.7%. The onset of symptoms tends to peak during early adulthood (Saha et al., 2005). It is a complex mental disorder affecting cognition, behavior and perception. Further, schizophrenia imposes a significant burden on society from health and economic perspectives (Chaiyakunapruk et al., 2016). Over the course of decades, numerous researchers have dedicated their efforts to studying the intricate and heterogeneous nature of schizophrenia (Elert, 2014; McCutcheon et al., 2020). However, despite these endeavors, the mechanisms underlying etiology and pathology of schizophrenia remains largely elusive.

1.1.1 Diverse symptomatology in schizophrenia

Schizophrenia represents a diverse group of illnesses that display a broad spectrum of symptoms and characteristics. Positive symptoms, negative symptoms, and deficits in cognitive domain are the three main categories of schizophrenia symptoms (Andreasen, 1995). Among these, the positive symptoms refer to an excess or

doi:10.6342/NTU202304492

deformation of normal functions including delusions, disorganized thought and hallucinations. The negative symptoms indicate a loss or reduction in typical functions involving anhedonia, a decrease in motivation, diminished emotional expression, and reduced social ability. The cognitive impairments, which occur much earlier than an onset of schizophrenia, are considered a core component of schizophrenia (Wilk et al., 2005). It reflects disturbances in cognitive processing involving attention, memory and executive functions, and has an enormous impact on the overall functioning and quality of life of individuals with the disorder.

In the present clinical context, positive symptoms are the primary focus of available antipsychotic medicines. However, the management of negative symptoms and cognitive impairments, which greatly affect patient's daily lives and social interactions, remains limited (Carbon & Correll, 2014). Moreover, many patients experience symptoms that remain partially controlled despite treatment. On the basis of these unmet medical needs, the current study was organized to focus on the negative symptoms and cognitive impairments of schizophrenia.

1.1.2 Etiology of schizophrenia

As a neurodevelopmental disorder, pathology of schizophrenia is affected by a

doi:10.6342/NTU202304492

complex interplay of genetic, environmental, and interactive factors. Previous studies indicated that no single gene or a single factor can lead to the onset of schizophrenia (Faludi et al., 2011; Lewis & Levitt, 2002). A combination of multiple elements disturbs the normal development of the central nervous system, resulting in neurochemical imbalances and brain abnormalities that may play a part in the emergence of schizophrenia. Neurochemical imbalances in schizophrenia, including the dysregulation of multiple neurotransmitter systems like dopamine and glutamate, represent two of the most well-established hypotheses in the pathogenesis of the disorder, respectively (Howes et al., 2015). Originally, both hypotheses relied primarily on indirect observations derived from pharmacological investigations, and subsequently supported by evidence from post-mortem analysis, brain imaging, and genetic studies (Howes et al., 2015).

According to the dopamine hypothesis, schizophrenia is produced by overstimulation of dopamine receptors. The first antipsychotic medicine, chlorpromazine, has shown efficacy in treating positive symptoms of schizophrenia, however its efficacy in treating negative and cognitive symptoms is limited, indicating the limitations of the dopamine hypothesis. Whereas the glutamate hypothesis that focusing on hypoactivity of N-methyl-D-aspartate receptors have shown potential in interpreting these symptoms (Javitt, 1999; Lim et al., 2016).

doi:10.6342/NTU202304492

A comprehensive genome-wide association study (GWAS) uncovered significant associations in genetic loci regarding the etiology and treatment of schizophrenia. These associations encompassed key genes such as DRD2, the principal target of numerous efficacious antipsychotics, as well as several others, including glutamate ionotropic receptor NMDA type subunit 2A (GRIN2A), metabotropic glutamate receptor 3 (GRM3), and serine racemase ("Biological insights from 108 schizophrenia-associated genetic loci," 2014). All of them play pivotal roles in synaptic plasticity and glutamatergic neurotransmission. Human imaging studies and genetic studies also have been found to support the connection between abnormal glutamate transmission and cognitive dysfunction in schizophrenia (Ohi et al., 2018; Stone et al., 2009). The NMDA receptors, in particular, play an important role in learning and memory procedures, non-competitive NMDAR blockade consequences symptoms resemble the full spectrum of schizophrenia (Nakazawa & Sapkota, 2020). Further studies are still needed to reveal the role of NMDAR in the pathophysiology of schizophrenia. To conclude here, despite numerous efforts that have been made to study the etiology of schizophrenia, the underlying mechanisms are still challenging to apprehend and deserve further research.

doi:10.6342/NTU202304492

1.2 Genetic factors in schizophrenia

1.2.1 The implications of NMDAR and D-serine for schizophrenia etiology

Continuing from the preceding section, further research on the role of NMDAR in schizophrenia is needed to better comprehend the mechanisms causing deficits in negative and cognitive related functions in schizophrenic patients. In the mammalian central nervous system, glutamate is the major excitatory neurotransmitter, activating both ionotropic and metabotropic glutamate receptors. Among these receptors, NMDARs are calcium-permeable ionotropic glutamate-gated channels that extensively distributed across the brain, and are essential for neuroplasticity and cognitive function like learning and memory. In the structure, NMDARs are made up of two obligatory GluN1 (NR1) subunits joined together with either two GluN2 (NR2) subunits or combined with GluN2 (NR2) and GluN3 (NR3) subunits to form a heterotetrameric molecule (Balu, 2016). To activate NMDARs requiring not only the binding of glutamate but also a co-agonist, typically glycine or D-serine (D-serine appears to be the dominant endogenous co-agonist for NMDARs) onto the glycine modulatory site (GMS). One of the

doi:10.6342/NTU202304492

distinctive features of NMDARs is that the GMS must be taken up by glycine and/or D-serine for glutamate to open the channel. D-serine is of paramount importance in regulating synaptic plasticity, learning, memory, and various other crucial neurological functions, primarily because of its role in modulating the functions of NMDARs. In the brain, D-serine is synthesized from L-serine via the enzymatic activity of SR, and it is catabolized by D-amino acid oxidase (DAAO), which is also notably present within astrocytes (Wolosker & Radzishevsky, 2013). Furthermore, evidence suggested that dysfunction in GMS modulators has been a reason to NMDAR hypofunction in schizophrenia (Wu et al., 2021). Importantly, reduced D-serine and SR levels have been found in patients diagnosed with schizophrenia (Bendikov et al., 2007). In summary, dysfunctions in these molecules, including NMDARs and the related modulators and enzymes D-serine, SR, and DAO, may contribute to the disorder, offering potential avenues for future research and treatment.

1.2.2 Role of SR in schizophrenia

SR has a significant role in schizophrenia because it regulates D-serine levels, which impacts NMDAR activity and glutamatergic neurotransmission. SR is found

doi:10.6342/NTU202304492

mostly in astrocytes, a kind of glial cell found in the brain. Through racemization, SR transforms L-serine, an amino acid prevalent in the brain, to D-serine (Wolosker & Radzishevsky, 2013) . Genetic and biochemical evidence suggests a reduction in SR, as well as a decrease in D-serine levels in individuals with schizophrenia (Hashimoto et al., 2005; Morita et al., 2007). The pathophysiology of schizophrenia has been related to dysregulation of SR activity and associated changes in D-serine levels, making it a target of interest in schizophrenia research and prospective therapeutic approaches.

1.2.3 Animal model with NMDAR deficiency: SR mutant mice

A previous study established an animal model with NMDAR deficiency using genetically modified mice incapable of endogenously producing D-serine (Basu et al., 2009). Importantly, these mutant mice have significant changes in glutamatergic neurotransmission as well as relatively mild but noticeable behavioral impairments such as hyperactivity, poor spatial memory, and elevated anxiety levels (Basu et al., 2009). Furthermore, using HPLC to assess D-serine levels in the cortex, researchers discovered that levels in SR HOM mice are around one-tenth of those in WT mice,

doi:10.6342/NTU202304492

and levels in SR HET mice are about seven-tenth of those in WT mice (Basu et al., 2009). These biochemical features replicate those found in patients with schizophrenia (Coyle, 2006).

1.2.4 Indirect GMS modulators: D-amino acid oxidase inhibitor (DAOI), a novel approach to NMDAR modulation in the treatment of schizophrenia

Because of the substantial potential for managing not only positive but also negative symptoms in schizophrenia, several agents that directly or indirectly target GMS have been found (Pei et al., 2021). Unfortunately, prior attempts to directly modulate NMDAR functions using D-serine or glycine have yielded unsatisfactory results, suggesting limited therapeutic potential. These limitations are caused by the need for high dosages, a small therapeutic window, and poor CNS penetration (Pei et al., 2021). Thus, an alternative approach involves indirectly targeting NMDARs' GMS by boosting glycine and D-serine levels. This offers a novel means of modulating NMDAR functions to address the requirements of schizophrenia patients. Among these treatments, one of the indirect ways targeting GMS is D-

doi:10.6342/NTU202304492

amino acid oxidase inhibitors (DAOI). D-amino acid oxidase (DAO) is an enzyme in astrocytes that transforms D-serine to hydroxypyruvate, whereas DAOI impede D-serine metabolism, thereby increasing the synaptic concentration of D-serine to restore the functions of NMDARs. In addition, the expression and activity of DAO are markedly elevated in individuals with schizophrenia (Madeira et al., 2008; Verrall et al., 2007). In rodents, D-serine levels have been found to rise after DAOI treatment, which suggests the therapeutic potential of DAOI (Adage et al., 2008).

1.3 Early-life infections and Inflammations in schizophrenia

1.3.1 Schizophrenia risk factors

Although the etiology of schizophrenia remains elusive, recent research has identified various risk factors. The development of schizophrenia is influenced by numerous risks, encompassing environmental and genetic influences. In the genetic side, the heredity of schizophrenia has been reported to be as high as eighty percent (Owen et al., 2016). Furthermore, multiple potential genes associated to the dopaminergic system, glutamatergic system, and immunological system have been discovered in earlier genome-wide association studies ("Biological insights from

doi:10.6342/NTU202304492

108 schizophrenia-associated genetic loci," 2014).

On the other hand, several environment factors have been identified relevant to pathology of schizophrenia including infectious diseases, substance abuse and psychosocial stresses (Janoutová et al., 2016; Scherr et al., 2012). Environmental insults, particularly during pregnancy, may interfere with the development of the CNS in the child from the perspective of development, and therefore give rise to schizophrenia and other neurodevelopmental diseases.

1.3.2 Maternal immune activation as a risk factor for schizophrenia pathogenesis

Maternal immune activation (MIA) is one of the environmental risks that may raise the likelihood of neurodevelopmental disorders develop, including schizophrenia (Brown & Derkits, 2010). The associations between MIA and neurodevelopmental disorders is contributed to disturbed physiological mechanisms during development of the fetal brain. Maternal factors which disturbed the physiological processes, especially occurs prenatally, including obesity, psychosocial stress and infection (Han et al., 2021).

doi:10.6342/NTU202304492

Following reports on psychosis symptoms of infants born throughout the devastating 1918 Spanish influenza pandemic (Kępińska et al., 2020), growing numbers of epidemiological investigations have yielded evidence indicating that prenatal infection exposure has made a contribution to the etiology of schizophrenia. Besides the reports of epidemics in populations, further confirmation has been made using bio-markers to characterize birth cohorts and diagnose the offspring systematically (Brown & Derkits, 2010). Based on one of the largest birth cohort, which born from 1959 through 1966, maternal virus infection from early to mid-gestation has been linked to a threefold rise in schizophrenia susceptibility (Brown et al., 2004). As a result, the potential implications of infections in prenatal life to psychotic disorder have received a lot of attention within this neuroimmune paradigm.

1.3.3 Modeling maternal immune activation in animal to uncover the underlying neurodevelopmental mechanisms

So far, human investigations based on the epidemiological data have revealed

doi:10.6342/NTU202304492

a significant connection between MIA and schizophrenia. However, human epidemiological research cannot clearly demonstrate causality for the connection in between let alone to define the downstream mechanisms affecting brain development for ethical and technological reasons.

Animal models, on the other hand, filled this need and provided a mechanism for exploring the precise effects of MIA during development of fetal brain. The prenatal period which the central nervous system still developing, seems to be more sensitive to the environmental insults such as infections. Experimental studies in rodents and monkeys shows strong evidence supports the presence of long-lasting brain alterations in function or structure following prenatal exposure to certain infectious or inflammatory factors (Meyer, 2014). Particularly, these long-lasting brain alterations origins from early neurodevelopmental period are relevant for disorders with developmental etiologies, such as schizophrenia.

1.3.4 Mouse models of maternal immune activation induced by poly(I:C)

In animal, systemically maternal injection of polyriboinosinic-polyribocytidilic

doi:10.6342/NTU202304492

acid [poly(I:C)] is one of the most common and frequently utilized ways presently to induce maternal immune activation. Poly(I:C) is a double-stranded RNA analog that is identified and causes an immunogenic response in mice via the transmembrane protein toll-like receptor 3 (TLR3), which can imitate the acute responses of a viral infection. These responses including releasing of various pro-inflammatory and anti-inflammatory cytokines, such as interleukin-10 (IL-10), interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) (Möller et al., 2015).

Pregnant dams in timed-mating conditions are subjected to exposure to poly(I:C) on a particular gestational day in the mouse prenatal poly(I:C) model. The subsequent evaluation involves comparing the brain or behavioral outcomes of the offspring born to these dams with those born to mothers treated with a control vehicle. Since its initial application in mouse developmental biology, the mouse prenatal poly(I:C) model has played a pivotal role in guiding scientists investigating the neuro-developmental and neuro-immunological underpinnings of complex human disorders, such as schizophrenia (Meyer, 2014). Intriguingly, these behavioral phenotypes seem to only emerges after late adolescence or early adulthood of the offspring and vary dependent on the gestational period (Guma et al., 2021; Meyer, 2014). Numerous studies have been put forward to understand the

doi:10.6342/NTU202304492

mechanisms underlying these long-lasting impacts of maternal immune activation induced by poly(I:C), however, a substantial amount still remains unknown.

1.3.5 The timing of poly(I:C)-induced maternal immune activation determines consequent brain and behavioral phenotypes

The prenatal injection of poly(I:C) in mice results in a range of biological alterations that replicate the neurobiology, symptomatology, and epidemiology characteristic of schizophrenia. Furthermore, numerous experiments have suggested that the specific timing of prenatal immune challenge can result in particular difficulties in maturity (Guma et al., 2021). The behavioral outcomes of offspring resulting from maternal immune activation occurring during early, middle, or late gestation days have been an interest in research. For example, previous studies have shown that adult phenotypes in the offspring of poly(I:C)-induced maternal immune activation on gestation day 9 (GD 9) are correlated with the positive symptoms of schizophrenia. Alternatively, exposure on GD 17 results in long-term changes primarily associated with negative and cognitive symptoms of

doi:10.6342/NTU202304492

schizophrenia (da Silveira et al., 2017). In addition to behavioral phenotypes, pathological changes in mouse brains also seem to be influenced by the timing of immune activation during pregnancy. In Meyer's study, the researchers found that poly(I:C)-induced prenatal immune challenge on GD 9 negatively impacted functions in sensorimotor gating and prefrontal dopamine D1 receptors in adulthood, while late gestational immune activation affected working memory, and hippocampal NMDA-receptor subunit 1 expression (Meyer et al., 2008). These findings from behavioral and biochemical perspectives emphasize that prenatal immune challenge at specific times determines subsequent pathological development in adulthood.

1.4 Schizophrenia's two-hit theory

A multifactorial or "two-hit" model of schizophrenia proposes that the illness develops as a result of a combination of genetic susceptibility, early life exposure to environmental stressors or inflammatory processes, and a subsequent distinct developmental insult. This combination may predispose an individual to subsequent events that result in the onset of schizophrenia, showing a broad spectrum of symptoms (Möller et al., 2015). In the framing of the two-hit theory, genetic vulnerability or early-

doi:10.6342/NTU202304492

life environmental insults usually constitute the '1st hit', while factors affected later such as infections, drug abuse, and trauma during development (van Os et al., 2010) may be viewed as the '2nd hit'. Critically, the 'two-hit' model of schizophrenia presumes that the disorder arises from the interplay of genetic and environmental factors, and this combination has been seen in animal models as in human studies (Feigenson et al., 2014).

1.4.1 The genetic and environmental factors interplay in schizophrenia

While schizophrenia is a mental illness with robust genetic underpinnings, over 100 loci have now been associated with predisposition to schizophrenia through the identification of single nucleotide polymorphisms (SNPs) through genome-wide association studies (GWAS). However, each of these SNPs have only limited effects individually and contributed to just a minor percentage of the genetic susceptibility. The remaining part is most likely due to multiple more loci, uncommon variations, and gene-environment interactions (Harrison, 2015). Twin studies for those with schizophrenia have also found that genetic factors account for almost half of their susceptibility to develop schizophrenia. These findings

doi:10.6342/NTU202304492

emphasize the involvement of environmental variables that add on and interact with genetic factors to develop the disorder (Moran et al., 2016).

1.5 Modeling schizophrenia in animals

While the extent to which animal models accurately imitate human psychiatric diseases is debatable, it is widely known that animals cannot fully contain the complexities of human disorders. However, dissecting the symptom characterization into more manageable features or endophenotypes is a useful approach for resolving this difficulty and provide causal links between etiological variables and phenotypic outcomes (Laura & Anthony, 2007).

On the other hand, assessing gene-environment interactions in human and clinical studies is challenging, due to ethical constraints, sample size limits, and the intricate nature of environmental factors. Animal models are invaluable tools for uncovering the complicated role of genes, environmental variables, and their interplay in the etiology of mental illnesses (Kannan et al., 2013). Using animal models enables precise control of environmental conditions and experimental manipulations to address the causal relationships between specific environmental and genetic factors, thus aiding the investigation of gene-environment interactions

doi:10.6342/NTU202304492

(Laura & Anthony, 2007).

1.6 Aims of the current study

Schizophrenia is a complex disorder, and our understanding of its underlying pathology remains limited. This is particularly true for the negative and cognitive symptoms, which have posed persistent challenges in meeting the medical needs of individuals with schizophrenia. However, it is important to note that not only one risk factor contributes to the development of schizophrenia, instead the genetic varibles, the environmental insults, and the interactions in between play roles in the pathogenesis of schizophrenia. In this regard, animal models have been considered valuable tools for exploring gene-environment interactions in schizophrenia.

For genetic factors, researchers have identified significant roles played by both glutamatergic genes and immune-related genes ("Biological insights from 108 schizophrenia-associated genetic loci," 2014). Furthermore, drawing from the hypothesis of NMDAR hypofunction, deficiency in SR, a candidate gene for schizophrenia and the enzyme responsible for producing D-serine to modulate NMDAR function, shows a high association with the pathogenesis of schizophrenia (Jacobi et al., 2019). However, a restricted number of researches

doi:10.6342/NTU202304492

have investigated the phenotypes of SR mutant mice, including both heterozygous and homozygous mutants (Basu et al., 2009). Studying the characteristics of mice with mutations in the SR gene, including heterozygous and homozygous mutants, can provide us with a better knowledge of the genetic factors associated with schizophrenia, potentially opening up new avenues for treatment research in this complex disorder.

On an environmental point, since the influenza pandemic in 1900's, for decades, researchers have been studying the contribution of inflammation in the development of schizophrenia. Recently, researchers have discovered a reciprocally affecting relationship between disruptions in glutamate functioning and inflammation, both of which results in impaired glutamate regulation and reduced NMDAR function (de Bartolomeis et al., 2022). Animal models of prenatal immunological activation generated by poly(I:C) injection have further provided a possible explanation for the environmental risk factor in schizophrenia pathogenesis. Moreover, prenatal immunological activation in the late gestational phase has been linked to both negative and cognitive deficits of schizophrenia (Bitanihirwe et al., 2010; Macêdo et al., 2012). Previous studies also identified reduced levels of glutamate and changes in NMDAR subunits in mice exposed to poly(I:C) (Bitanihirwe et al., 2010; Forrest et al., 2012).

doi:10.6342/NTU202304492

However, despite extensive research into the individual roles of SR (a genetic factor) and poly(I:C)-induced maternal immune activation (an environmental factor) in the pathogenesis of schizophrenia, there remains a notable gap in our understanding regarding their potential interactions and their cumulative impact on NMDAR function. Exploring the interplay between these two factors and their collective effects on NMDAR regulation represents a promising avenue for future research in unraveling the complex mechanisms underlying schizophrenia. This study aims to investigate the possible gene-environment interactions between SR mutations and poly(I:C)-induced neurodevelopmental abnormalities in a mouse model of the two-hit hypothesis, shedding light on potential therapeutic approaches for addressing the negative and cognitive symptoms of the disorder.

The following section outlines the 3 specific aims and corresponding experiments in this thesis guiding our investigation into the potential interplay between prenatal immune activation, SR mutations, and neurodevelopmental outcomes. The aims of each experiments were summarized in Table 2.

(1) To assess the acute effects of poly(I:C) injection during late prenatal immune activation on cytokine responses in the offspring (Experiment 1).

(2) To characterize behavioral phenotypes in SR mutant mice with prenatal immune activation (Experiment 2).

doi:10.6342/NTU202304492

(3) To explore the potential therapeutic effect of RS-D7, a novel DAOI, on neuromotor functions in neonatal mice (Experiment 3).

In Experiment 1, we selected a commonly used prenatal challenge paradigm with poly(I:C), which is more likely to be associated with human scenario and to imitate maternal immune activation after virus infections in people (de Bartolomeis et al., 2022; Han et al., 2021; Meyer, 2014). We would like to investigate how poly(I:C) injection via intravenous route (5 mg/kg, *i.v.*) on gestational day 17.5 affected fetal brain on cytokine responses in SR mutant mice, which represent late-stage maternal immune activation. A prior investigation demonstrated that the administration of poly(I:C) leads to elevated levels of various cytokines, including interleukin-6 (IL-6), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interleukin-10 (IL-10). Yet, it is crucial to acknowledge that the outcomes may differ based on the injection technique and the duration following the injection (Möller et al., 2015; Meyer, Feldon, et al., 2006; Meyer, Nyffeler, et al., 2006a). Thus, three of the most frequently seen pro-inflammatory cytokines were chosen and examined in the current study, namely IL-6, IL-1β, and TNF-α.

After successfully establishing the poly(I:C)-induced maternal immune activation paradigm in Experiment 1, we conducted a comprehensive battery of behavioral assessments from the neonatal stage to adolescence and into adulthood in

doi:10.6342/NTU202304492

Experiment 2. The major goal was to characterize the long-term phenotypic effects and potential convergence that resulted from poly(I:C)-induced immune activation in SR mutant mice. Based on previous research, poly(I:C) administration during the later stages of gestation (from gestational days 15–17; 5 mg/kg, *i.v.*) significantly delays the growth of sensorimotor and physical functions in neonatal mice (Arsenault et al., 2014). Accordingly, in Experiment 2.1, we employed three milestone tasks to assess the neurodevelopmental functions of neonatal mice, including the righting reflex (on postnatal days 3-7), the geotaxis reflex (on postnatal days 3-7), and the grasping reflex (on postnatal days 5-7). Besides, on postnatal day 26, we also performed a locomotor activity task to compare locomotor activity between adolescence and adulthood. Next, in Experiment 2.2, we conducted a series of behavioral tasks associated with the negative and cognitive symptoms seen in schizophrenic patients in adulthood (starting from postnatal day 80) to examine the phenotypes between different treatments (vehicle control or poly(I:C)) and genotypes (WT, HET, and HOM). These tasks included open field task, spontaneous alteration task, novel object recognition task, object-based attention task, three-chamber social interaction task, and trace fear conditioning task.

Extending the prior research findings from Experiment 2.1, we aimed to evaluate the possible rescue effect on neuromotor functions using an innovative d-amino acid

doi:10.6342/NTU202304492

oxidase inhibitor, RS-D7, in Experiment 3. Two of the milestone tasks were included to assess the neurodevelopmental functions of neonatal mice, including the righting reflex and the geotaxis reflex, both carried out on postnatal days 3-5. On each testing day, RS-D7 (40 mg/kg, subcutaneously) or saline in an equivalent volume was administered to the poly(I:C)-treated group (E17.5), while the saline group (E17.5) received no further interventions. Referring to prior research conducted in our laboratory (Luo, 2022), the administered dosage of 40 mg/kg has been demonstrated to be both effective and safe when administered via intraperitoneal injection in adult mice. However, there is a lack of previous studies probing the use of subcutaneous injections in neonatal mice.

doi:10.6342/NTU202304492

Chapter 2. Materials and Methods

2.1 Animals

SR mutant mice in the C57BL/6J background were acquired and generated as previously disclosed (Basu et al., 2009). All SR mutant mice, including heterozygous (HET) and homozygous (HOM), and their wild-type (WT) littermates were derived from SR HET breeding pairs and backcrossed onto the C57BL/6J background for at least ten generations. All mice were 80 days old at the start of all behavioral tests. Animals were housed in the animal rooms of National Taiwan University's Psychology Department, with food and water available *ad libitum*. Animal rooms were kept at 22 2°C and on a 12:12 light/dark cycle (lights turned on at 05:00). During the light cycle, all behavioral activities and pharmacological treatments were carried out. All animal procedures were carried out in accordance with protocols approved by National Taiwan University's Animal Care and Use Committee. All attempts were made to reduce the number of mice and their potential pain or suffering to meet the 3Rs principle of animal use (Replacement, Reduction and Refinement). All mice were euthanized at the end of the experiment.

doi:10.6342/NTU202304492

2.2 Timed mating strategy

SR HET mice were mated following the timed mating strategy to determine the specific day which female mice got pregnant. Female and male mice were stayed in a cage overnight, on a one-on-one manner, and on the next day the male mice would be taken out and the body weight of the female mice was recorded and maintained single housing. This day was set as gestational day 0.5 (GD 0.5). On GD 14.5 the body weight of the female mice would be recorded again and compare with the body weight of GD 0.5, the female mice will be labeled as pregnant if the weight gain is more than 5 grams. Pregnant dams were recruited in this study.

2.3 Maternal immune activation induced by poly(I:C) injection

Six pregnant dams were given a single poly(I:C) injection (polyriboinosinic-polyribocytidilic acid potassium salt; Sigma-Aldrich, Saint Louis, USA) and nine pregnant dams received a single injection of saline (sterile pyrogen free 0.9% NaCl) via intravenous (*i.v.*) route at GD 17.5 at a dosage of 5 mg/kg (Meyer, Nyffeler, et al., 2006b). Poly(I:C) was dissolved at a concentration of 10 mg/ml in sterile saline solution

doi:10.6342/NTU202304492

and heated at 50°C for half an hour to make stocks and stored in -20°C following suggestions of the manufacturer. Stock solution of poly I:C was separated into small volume packages to avoid repeated freezing and thawing. Equivalent volumes of sterile saline solution were used as control treatment. In order to minimize sufferings of the mice, mice were receiving gas anesthesia with isoflurane prior the injection. The pregnant dams were injected in the lateral tail vein and kept separately in an enriched environment until their offspring were born.

Exp.1: Examination of pro-inflammatory cytokines to validate the acute effects of poly(I:C)

A batch of SR HET female mice was timed mating and sacrificed on E17.5 to examine the acute effect of poly(I:C) as a manipulation check.

Preparation of fetal tissue

Samples of fetal brain were taken 6 hours after poly(I:C) (5 mg/kg, *i.v.*) or saline injection (equivalent volume, *i.v.*) on E 17.5, which has been shown previously to induce cytokine responses including interleukin 6 (IL-6) and tumor necrosisfactor-α (TNF-α) (Meyer, Nyffeler, et al., 2006a). Following the sacrifice of female dams,

doi:10.6342/NTU202304492

the pups were removed, and their whole brains were freshly taken and immediately frozen at–80°C for storage before use. The tails of pups were kept for genotyping. Fetal brain tissues (n = 5 per group) was prepared for further cytokine assay by grinding with tissue homogenizer on ice in lysis buffer containing Complete Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktail. Tissue homogenate was later centrifuged for 30 minutes at 14,000 rpm at 4 °C, and the supernatant was transferred to a new tube and immediately frozen at – 80°C for storage before use.

Cytokines assays

Fetal brain levels of three cytokines (IL-6, IL-1β and TNF-α) were surveyed using enzyme-linked immunosorbent assay (ELISA). Commercial kits for IL-6 (Mouse IL-6 Uncoated ELISA, Invitrogen, Thermo Fisher Scientific, Inc., USA), IL-1β (Mouse IL-1β Uncoated ELISA, Invitrogen, Thermo Fisher Scientific, Inc., USA) and TNF-α (Mouse TNF-α Uncoated ELISA, Invitrogen, Thermo Fisher Scientific, Inc., USA) were performed closely to the protocols provided by the manufacturer. All standards and samples underwent duplicate runs to minimize experiment bias by calculating the averages of optical density (OD) values. OD values were read utilized microplate reader (Multiskan™ FC Microplate Photometer, Thermo Fisher Scientific, Inc., USA) under 450 nm as the manual suggests. The OD values obtained were further transformed using

doi:10.6342/NTU202304492

Thermo Scientific SkanIt® Software four-parameter logistic (4PL) fit type. All the R-squared values are greater than or equal to 0.99.

Exp.2: Behavior phenotyping of the offspring from poly(I:C)- or saline-treated mothers

After poly I:C-induced maternal immune activation on GD 17.5, pregnant dams were checked twice every day to determine the day of birth which was set as postnatal day 0 (PnD 0). In total, six pregnant dams were recruited in the poly(I:C) group, and nine pregnant dams were recruited in the saline group. The average number of pups we collected in the poly(I:C) group from each dam was 6.5, and the average number of pups in saline group from each dam was 6.3. The detailed information about the subjects we collected in experiment 2 is listed in Table 1.

Development of sensorimotor functions were evaluated using 3 milestones tasks which performed on pups from PnD 3 to PnD 7. On postnatal day 21, offspring was weaned and separated by sex. 2 to 5 mice were maintained in a cage. Spontaneous locomotor activity was evaluated on PnD 26.

Five behavioral tasks related to the cognitive and the negative symptoms of

doi:10.6342/NTU202304492

schizophrenia were started from PnD 80 for phenotyping of adult behavior. The behavioral tasks in sequence are: open field test, spontaneous alteration test, novel object recognition test, object-based attention test, trace fear conditioning test. The order is designed depends on potential sufferings made to the animals, which from mild to severe stress.

Exp. 2.1: Evaluation of sensorimotor developmental milestones in pups

Milestones tasks including righting reflex, geotaxis reflex and grasping reflex along with measurements of their body weights. 73 pups of both sexes and three genotypes (WT, SR HET, SR HOM) were recruited in the study. Before testing, the pup was weighed and tattoo was marked on the tails of each pups for identification. During the tests, pups were separated from the dam less than 10 min.

Righting reflex **(PnD 3-7)**

Righting reflex refers to a response when a pup is put on its back it will immediately turn over to its nature position. During the testing, pups were put upside

doi:10.6342/NTU202304492

down on a plane surface and after released the time it turning over with the four paws on the ground was recorded. In each trial, the maximum time given was 30 seconds. The pups failed to completed the task in 3 consecutive trials were automatically recorded as 30 seconds. Righting reflex was evaluated daily from PnD 3-7.

***Geotaxis reflex* (PnD 3-7)**

Geotaxis reflex refers to a response when a pup is put downwards on a slope, it will turn its head to an upright position. During the testing, pups were put head down on a slope at a 45° angle and after released the time it turned around until facing up was recorded. The pups failed to completed the task in 3 consecutive trials were automatically recorded as 30 seconds. Meanwhile, a geotaxis score was rated depending on the turning angle of a pup. The geotaxis scale is from zero to five which adapted from the Lan et al. (2017), with zero to four refers to the different turning angles (zero: 0°,one : 45°,two : 90°,three : 135°,four : 180°) and five means a pup walking around on the slope. Geotaxis reflex was evaluated daily from PnD 3-7.

***Grasping reflex* (PnD 5-7)**

Grasping reflex refers to the response of a pup holding on a rod when it suspended in the air. The wire with 1 mm diameter is connected its two poles on a cylinder. On the

doi:10.6342/NTU202304492

bottom of the cylinder, multiple layers of tissue were placed to avoid the potential hurt when pups falling. During the testing, a pup was gently hold with its forelimbs on the wire, after released the time it grasping on the wire was recorded. The pups failed to completed the task in 3 consecutive trials were automatically recorded as 0 seconds. Grasping reflex was evaluated daily from PnD 5-7.

Open field test **(PnD 26)**

Spontaneous locomotor activity in adolescence was evaluated in an open-field apparatus (48cm × 24cm × 25cm, Coulbourn Instruments, White hall, PA, USA) under dim-light (60 lx) in a 60-minutes trial. During the testing, mice was put into the chamber individually and the total travel distance was recorded using SMART video tracking system (San Diego Instruments, San Diego, CA).

Exp. 2.2: To evaluate the adult behavioral phenotypes in SR mutant mice with prenatal immune activation.

The order of the behavioral tests was not randomized on purpose. Instead, the sequence of behavioral experiments was intentionally organized to prevent sudden and

doi:10.6342/NTU202304492

overt stress for the mice, with the strategic aim of avoiding more stressful tasks preceding less stressful ones. Each behavioral testing was separated by at least seven days. The following sections cover the specifics of each test.

***Open field test* (PnD 80)**

Spontaneous locomotor activity in adulthood was evaluated using open field test underwent the same procedure as previously described.

Spontaneous alteration test

Spatial working memory of the mouse was assessed in a Y-maze. The natural tendency to explore novelty in which recently visited arm is not visit again immediately was evaluated using the continuous version of the spontaneous alternation procedure, as previously described (O'Tuathaigh et al., 2007). The Y-maze consists of three equally spaced arms placed at an angle of 120°. After habituation in the experiment room for 30 min, the mice were put into the maze directly and allowed to run freely for an 8 min trial. The maze was carefully cleaned with 70 % alcohol between each mouse. All the trials were video-recorded and the order of each arms visited was recorded manually. The total number of arm entries and the rate of alternation were calculated as previously

doi:10.6342/NTU202304492

described (Hughes, 2004; Olmos-Serrano et al., 2016). Spontaneous alteration was identified as continuous visits into the three arms of the Y-maze. Number of alterations was derived from the order of arm entries in window-moving triplet sets. The following formula is used to calculate an alteration rate: [total number of arm entries / number of alternations] × 100 (chance level = 44.4%, by Lennartz (2008)).

Novel object recognition test

Long-term recognitional memory was evaluated in novel object recognition test with an hour inter-testing interval. Three sessions were included in the test and the duration of each session was 10 min. After habituation in the experiment room for 30 min, the mouse was placed into an open-field chamber (48cm × 24cm × 25cm, Coulbourn Instruments, White hall, PA, USA) and allowed to move freely in a 10-min habituation session. Following a 10-minute interval, two identical objects (the beaker) were positioned in the previous chamber with even distance and the mouse was allowed to explore freely in a 10-min training session. After the interval of an hour, one of the beakers was replaced by a novel object (the bottle cap) and the mouse was allowed to explore freely in a 10-min testing session. The replacement was randomized to avoid possible place preference. In the nature of mice to explore novelty, more time spent sniffing the novel object compare with the familiar one was expected. All the sessions

doi:10.6342/NTU202304492

were video-recorded and the total sniffing time spent on each object was calculated utilized Behavioral Observation Research Interactive Software (BORIS) (Friard & Gamba, 2016). A discrimination index was calculated using the formula: (time exploring the novel object – time exploring the familiar object)/time exploring objects.

Object-based attention test (OBAT)

Attentional function in mice was evaluated using an modified version of OBAT as previous described (Alkam et al., 2011). Traditionally, attentional function has been assessed by conducting 5-choice serial reaction time (5-CSRT) task. Compare with 5-CSRT, the OBAT enables rapid assessment of attention deficits within a shorter timeframe, circumventing the need for learning processes and minimizing stress on the animals. The arena consists of two rectangular acrylic chambers, one is exploring chamber (20 cm long, 20 cm wide, 22 cm high) and the other is testing chamber (20 cm long, 10 wide, 22 cm high), separated by an opaque movable wall. The testing included three sessions, after 30-min habituation to the experiment room, the mouse was put into the empty arena and allowed to move freely for 10 min. During the habituation session, the wall was manually moved up every 3 minutes to ensure that the mouse became familiar with the two chambers. In the second session, the mouse was allowed to interact with 5 different objects evenly distributed in the exploring chamber for 3

doi:10.6342/NTU202304492

minutes. All the objects used are made of the same color of Lego and roughly equal in size but vary in shapes. During the exploring session, SMART video tracking system (San Diego Instruments, San Diego, CA) was used to calculate the mouse's total sniffing time of the objects. Immediately followed the end of the second session, the second most sniffing object the mice interacted with was parallelly transferred into the testing chamber and a new object was put into the testing chamber on the other side of the familiar object. The two objects in the testing chamber were evenly distributed. The novel object's material is the same as the original five objects but comes in different shapes. In the third session, the mice were allowed to enter the testing chamber with the wall manually moved up and interacted with the two objects for 3 minutes. The testing session was videotaped and processed with BORIS to determine how much time the mouse spent sniffing the objects. The following method was used to calculate a recognition index: (time exploring the novel object/total time exploring objects) × 100.

Three-chamber social interaction test

Sociability and social recognition were evaluated using a modified version of the three-chamber social interaction test described before (Huang et al., 2015). The benefit of this approach is that the mice can move freely to interact with the stimuli. Three 10-min sessions were included in each trial and the inter-session intervals were 10 min. In

doi:10.6342/NTU202304492

the first session (habituation), the mouse was put into a Plexiglas apparatus (48cm × 24cm × 25cm, Coulbourn Instruments, White hall, PA, USA) with two empty transparent plastic cylindrical container (6 cm diameter × 15 cm height) evenly distributed on each side. After habituation, a stranger was randomly put into one of the cylinders as social stimuli. The stranger was an age- and sex-matched novel WT mouse. In the second session (sociability test), the mice were allowed to interact with the cylindrical containers, one with the stranger inside and the other was leaved empty. Driven by the sociable nature of mouse, more time spent on the stranger would be expected. In the third session (social recognition test), another stranger was put into the empty container in the previous session. The inherent nature of mouse to explore the novelty drives it spend more time sniffing the novel stranger compare with the familiar one. The second and third session were video-recorded and the total sniffing time of both containers the mouse interacted with was analyzed using a combination of python-based open-source toolbox DeepLabCut™(Mathis et al., 2018) and MATLAB®. The tip of the mouse's nose was labeled utilized of DeepLabCut™ and the sniffing behavior was next calculated by MATLAB®. The sniffing behavior was referred to as the mouse's nose enters within a radius of 2 centimeters of the cylindrical container. The following formula yielded a sociability index : sociability (%) = (total sniffing time with the stranger/ total time spent sniffing with the stranger + total time spent sniffing with

doi:10.6342/NTU202304492

the empty one) × 100 and a social recognition index was generated by the formula: social recognition (%) = (total sniffing time with the novel one/total sniffing time with the novel one+ total sniffing time with the familiar one) × 100.

Trace fear conditioning test (TFC)

A trace fear conditioning paradigm derived from Balu et al. (2013) was used to measure hippocampal-based associative learning and memory. The testing includes three trials, the inter-trial interval is one day. All the testing was done with a commercial fear-conditioning system (Coulbourn Instruments, Whitehall, PA, USA) based on TruScan 2.01. In the first trial (the learning day), the mouse was put singly into the conditioning chamber and allowed to move freely for 3 min. A tone-foot shock pairing was presented 7 times with an interval of 4 sec. The tone was exposed as conditional stimuli each lasting for 20 sec and a 0.8 mA foot shock was given 20 sec after the tone each lasting for 2 sec. The chamber was carefully cleaned with 70 % alcohol between every subject. In the second trial (the contextual day), the mouse was put individually into the conditioning chamber to induce fear memory of previous day. In the third trial (the tone-cued day), the mouse was put individually into a chamber with different environmental cues and enabled to move freely for 3 min. The purpose of creating a novel environment is to distinguish it from the conditioning chamber. The

doi:10.6342/NTU202304492

conditioned tone was presented 7 times following the same protocol as the first trial to induce fear memory without the foot shocks. All trials were video-recorded and the freezing behaviors were further analyzed with ezTrack, which is an open-source video analysis system built in Python (Pennington et al., 2019). ezTrack provides an automatic way to score a mouse's motion and freezing while in a conditioning chamber therefore reduces the bias of manual recordings.

Exp. 3: To evaluate the potential rescue effect on neuromotor functions with a novel d-amino acid oxidase inhibitor, RS-D7

Drugs preparation

RS-D7 is a new chemical entity in powder and was freshly prepared 30 minutes before subcutaneous injections on each testing day (PnD 3-5).

***Righting reflex* (PnD 3-5)**

Righting reflex refers to a response when a pup is put on its back it will

doi:10.6342/NTU202304492

immediately turn over to its nature position. During the testing, pups were put upside down on a plane surface and after released the time it turning over with the four paws on the ground was recorded. In each trial, the maximum time given was 30 seconds. The pups failed to completed the task in 3 consecutive trials were automatically recorded as 30 seconds. Righting reflex was evaluated daily from PnD 3-5.

***Geotaxis reflex* (PnD 3-5)**

Geotaxis reflex refers to a response when a pup is put downwards on a slope, it will turn its head to an upright position. During the testing, pups were put head down on a slope at a 45° angle and after released the time it turned around until facing up was recorded. The pups failed to completed the task in 3 consecutive trials were automatically recorded as 30 seconds. Meanwhile, a geotaxis score was rated depending on the turning angle of a pup. The geotaxis scale is from zero to five which adapted from the Lan et al. (2017), with zero to four refers to the different turning angles (zero: 0°,one : 45°,two : 90°,three : 135°,four : 180°) and five means a pup walking around on the slope. Geotaxis reflex was evaluated daily from PnD 3-5.

doi:10.6342/NTU202304492

2.4 Statistics and data analyses

All statistical analyses and figure drawings were carried out using GraphPad Prism version 8.0.1 for Windows (GraphPad Software, Boston, Massachusetts, USA). Two-tailed t tests were used for the comparison between different treatment groups in the analysis of ELISA data and behavioral quantitative analyses. Two-way and one-way ANOVA, followed by Holm-Sidak ‘s multiple comparisons test, were used for ELISA and all behavioral quantitative analyses. The level of significance was set at 0.05. All the data was presented as mean ± SEM.

doi:10.6342/NTU202304492

Chapter 3. Results

3.1 Experiment 1: Fetal brain cytokine responses 6 hours after poly(I:C)-induced immune challenge on E17.5

To test the effect of poly(I:C)-induced immune challenge in the fetal brain on E17.5, dams were sacrificed 6 hours after being injected intravenously with poly(I:C) or saline.

Poly(I:C)-induced immune challenge successfully triggered a pro-inflammatory response in the fetal brain

Figure 2 shows the fetal brain cytokine responses 6 hours after immune challenge by administration of poly(I:C) (5 mg/kg) on embryonic day 17.5 via the i.v. route. Interleukin-6 (IL-6) levels were significantly elevated in the fetal brains of the poly(I:C)-treated group compared to the saline-treated group (Figure 2A, $t_{(28)} = 7.734$, $p < 0.0001$). Tumor necrosis factor-alpha (TNF-α) levels were also significantly elevated in the fetal brains of the poly(I:C)-treated group (Figure 2B, $t_{(28)} = 2.675$, $p = 0.0123$). Interleukin-1 beta (IL-1β) levels showed a trend towards elevation in the fetal brains of the poly(I:C)-treated group, although this effect did not reach statistical significance (Figure 2C, $t_{(28)} = 1.926$, $p = 0.0643$). These findings suggest that

doi:10.6342/NTU202304492

poly(I:C)-induced immune challenge triggers a pro-inflammatory response in the fetal brain, characterized by elevated levels of IL-6, TNF-α, and IL-1β.

Absence of genotype-specific effects in response to poly(I:C) challenge

To understand the role of genotypes in response to cytokine elevation and the potential interaction between genetics and treatment, three different genotypes in the SR background (WT, HET, and HOM) in different treatment groups (saline or poly(I:C)) were analyzed using a two-way ANOVA.

Figure 3 shows the fetal brain cytokine responses in different genotype groups 6 hours after immune challenge by administration of poly(I:C) (5 mg/kg) on embryonic day 17.5 via the i.v. route. Interleukin-6 (IL-6) levels were significantly elevated in the fetal brains of the poly(I:C)-treated group, with main effects of treatment (Figure 3A, $F_{\text{treatment (1, 24)}} = 72.61$, $p < 0.0001$) and genotype (Figure 3A, $F_{\text{genotype (2, 24)}} = 4.115$, $p = 0.0291$), but no interaction effect was found between the treatment and the genotype ($F_{\text{interaction (2, 24)}} = 0.8805$, $p = 0.4275$). Tumor necrosis factor-alpha (TNF-α) levels were also significantly elevated in the fetal brains of the poly(I:C)-treated group, with a significant main effect of treatment ($F_{\text{treatment (1, 24)}} = 8.617$, $p = 0.0072$) and a significant interaction between treatment and genotype ($F_{\text{interaction (2, 24)}} = 4.385$, $p = 0.0238$), but

doi:10.6342/NTU202304492

no genotype effect was found ($F_{\text{genotype (2, 24)}} = 0.4730$, $p = 0.6288$). Interleukin-1 beta (IL-1β) levels were also significantly elevated in the fetal brains of the poly(I:C)-treated group, with main effects of treatment ($F_{\text{treatment (1, 24)}} = 4.681$, $p = 0.0407$) and genotype (Figure 3C, $F_{\text{genotype (2, 24)}} = 5.038$, $p = 0.0149$), no interaction was found between treatment and genotype ($F_{\text{interaction (2, 24)}} = 0.6255$, $p = 0.5435$).

These findings suggest that poly(I:C)-induced immune challenge triggers a pro-inflammatory response in the fetal brain, characterized by elevated levels of IL-6, TNF-α, and IL-1β. The main effects of genotype on IL-6 and IL-1β levels suggest that genetic factors may influence the fetal brain's response to poly(I:C) challenge. The significant interaction between treatment and genotype for TNF-α levels suggests that the effect of poly(I:C) challenge on TNF-α levels may be different depending on the genotype of the individual.

3.2 Experiment 2.1: Evaluation of sensorimotor developmental milestones in pups

To understand the role of genotype and treatment on developmental processes, as well as to investigate potential interactions between genetics and treatment, we

doi:10.6342/NTU202304492

conducted two-way ANOVA analysis on three distinct genotypes within the SR background (WT, HET, and HOM) across two treatment groups (saline and poly(I:C)) in three milestones tests.

Body weight of neonatal mice

An unpaired t-test was initially conducted to verify that there were no significant differences between the poly(I:C) and saline groups (Figure 4A, $t_{(8)} = 0.1691$, $p = 0.8699$). Subsequently, a two-way ANOVA was performed to assess potential distinctions among different genotypes and treatment groups, with the results also indicating no significant differences (Figure 4B). These findings suggest that poly(I:C) challenge does not affect neonatal mouse body weight, regardless of genotype.

Righting reflex

Figure 5 shows the time taken for neonatal mice to achieve the righting reflex on postnatal days 3–7 in Experiment 2.1. There were no significant differences in the performance of the righting reflex among different genotypes and treatment groups on postnatal days 3, 4, 6, and 7 (Figure 5A, postnatal day 3: $F_{\text{interaction}\,(2,\,72)} = 1.949$, $p = 0.1498$, $F_{\text{treatment}\,(1,\,72)} = 0.1792$, $p = 0.6734$, $F_{\text{genotype}\,(2,\,72)} = 0.3992$, $p = 0.6723$; postnatal

doi:10.6342/NTU202304492

day 4: $F_{\text{interaction (2, 71)}} = 0.1702$, $p = 0.8438$, $F_{\text{treatment (1, 71)}} = 0.08058$, $p = 0.7773$, $F_{\text{genotype (2, 71)}} = 0.04784$, $p = 0.9533$; postnatal day 6: $F_{\text{interaction (2, 72)}} = 0.1312$, $p = 0.8772$, $F_{\text{treatment (1, 72)}} = 0.7114$, $p = 0.4018$, $F_{\text{genotype (2, 72)}} = 0.1211$, $p = 0.8861$; postnatal day 7: $F_{\text{interaction (2, 56)}} = 0.08296$, $p = 0.9205$, $F_{\text{treatment (1, 56)}} = 0.5240$, $p = 0.4722$, $F_{\text{genotype (2, 56)}} = 0.1873$, $p = 0.8297$). However, on postnatal day 5, prenatal poly(I:C) injection significantly delayed the development of the righting reflex, regardless of the genotype of the neonatal mice (Figure 5A, $F_{\text{interaction (2, 72)}} = 0.5835$, $p = 0.5606$, $F_{\text{treatment (1, 72)}} = 4.501$, $p = 0.0373$, $F_{\text{genotype (2, 72)}} = 0.3978$, $p = 0.6733$). The median time to righting reflex in the poly(I:C)-treated groups was significantly longer than in the saline-treated groups (Figure 5B). The data distribution of the different groups on postnatal day 5 is shown in Figure 5C. It's worth noting that the poly(I:C)-HOM group performed the worst in the geotaxis reflex test on postnatal day 5. This finding implies that the SR HOM genotype may be more vulnerable to the impacts of prenatal poly(I:C) challenges. These findings suggest that prenatal poly(I:C) challenge delays the development of the righting reflex in neonatal mice.

Geotaxis reflex

Two assessments were applied to measure geotaxis reflex performance. The first assessment recorded the time it took neonatal mice to achieve the geotaxis reflex.

doi:10.6342/NTU202304492

However, we encountered several problems with this approach that make it challenging to accurately assess performance. Firstly, under this method, we only recorded the time taken by those mice that successfully completed the task. During testing, some neonates only partially turned rather than completing a full rotation. Secondly, it is difficult to claim that faster completion indicates better development. To address these limitations, we incorporated an additional assessment to provide a more comprehensive quantification of geotaxis reflex performance. The second assessment involved a five-point scoring system, which provided a more delicate evaluation of neonatal performance by determining scores on a five-point scale. The subsequent section will provide a detailed analysis of the results.

Figure 6 shows the time taken for neonatal mice to achieve the geotaxis reflex on postnatal days 3–7 in Experiment 2.1. There were no significant differences in the performance of the geotaxis reflex among different genotypes and treatment groups on any of the days tested (Figure 6A, Figure 6B, postnatal day 3: $F_{\text{interaction (2, 72)}} = 2.294$, $p = 0.1082$, $F_{\text{treatment (1, 72)}} = 1.230$, $p = 0.2711$, $F_{\text{genotype (2, 72)}} = 0.03491$, $p = 0.9657$; postnatal day 4: $F_{\text{interaction (2, 72)}} = 1.219$, $p = 0.3015$, $F_{\text{treatment (1, 72)}} = 0.02939$, $p = 0.8644$, $F_{\text{genotype (2, 72)}} = 1.623$, $p = 0.2045$; postnatal day 5: $F_{\text{interaction (2, 72)}} = 2.966$, $p = 0.0578$, $F_{\text{treatment (1, 72)}} = 0.9377$, $p = 0.3361$, $F_{\text{genotype (2, 72)}} = 0.2644$, $p = 0.7684$; postnatal day 6: $F_{\text{interaction (2, 72)}} = 1.018$, $p = 0.3663$, $F_{\text{treatment (1, 72)}} = 0.4409$, $p = 0.5088$, $F_{\text{genotype (2, 72)}}$

doi:10.6342/NTU202304492

= 0.4490, p = 0.6401; postnatal day 7: $F_{\text{interaction (2, 56)}}$ = 1.538, p = 0.2238, $F_{\text{treatment (1, 56)}}$ = 0.04201, p = 0.8383, $F_{\text{genotype (2, 56)}}$ = 0.8049, p = 0.4522).

Figure 7 shows the five-point scoring assessment for neonatal mice to achieve the geotaxis reflex on postnatal days 3–7 in Experiment 2.1. There were no significant differences in the performance of the geotaxis reflex among different genotypes and treatment groups on postnatal days 3, 4, 6, and 7 (Figure 7A, postnatal day 3: $F_{\text{interaction (2, 70)}}$ = 1.954, p = 0.1493, $F_{\text{treatment (1, 70)}}$ = 0.2253, p = 0.6365, $F_{\text{genotype (2, 70)}}$ = 0.6341, p = 0.5334; postnatal day 4: $F_{\text{interaction (2, 70)}}$ = 0.8935, p = 0.4138, $F_{\text{treatment (1, 70)}}$ = 0.007871, p = 0.9296, $F_{\text{genotype (2, 70)}}$ = 0.7523, p = 0.4751; postnatal day 6: $F_{\text{interaction (2, 70)}}$ = 1.340, p = 0.2685, $F_{\text{treatment (1, 70)}}$ = 0.1647, p = 0.6861, $F_{\text{genotype (2, 70)}}$ = 0.002144, p = 0.9979; postnatal day 7: $F_{\text{interaction (2, 54)}}$ = 2.994, p = 0.0585, $F_{\text{treatment (1, 54)}}$ = 1.790, p = 0.1866, $F_{\text{genotype (2, 54)}}$ = 0.06418, p = 0.9379). However, on postnatal day 5, the median score for the geotaxis reflex in the poly(I:C)-treated groups was significantly lower than in the saline-treated groups (Figure 7B), indicating that prenatal poly(I:C) injection significantly delayed the development of the geotaxis reflex, regardless of the genotype of the neonatal mice ($F_{\text{interaction (2, 70)}}$ = 2.651, p = 0.0777, $F_{\text{treatment (1, 70)}}$ = 4.140, p = 0.0457, $F_{\text{genotype (2, 70)}}$ = 2.132, p = 0.1262). The data distribution of the different groups on postnatal day 5 is shown in Figure 7C.

These findings suggest that prenatal poly(I:C) challenge delays the development

doi:10.6342/NTU202304492

of the geotaxis reflex in neonatal mice. This effect is independent of genotype, suggesting that poly(I:C) challenge may have a global impact on sensorimotor development. Furthermore, it is interesting to note that the poly(I:C)-HOM group performed the worst in the geotaxis reflex test on postnatal day 5 in both assessments. This finding suggests that the SR HOM genotype may be more sensitive to the effects of prenatal poly(I:C) challenge.

Grasping reflex

Figure 8 shows the time accumulated for neonatal mice to hold the bar in the grasping reflex test on postnatal days 5–7 in Experiment 2.1. There were no significant differences in the performance of the grasping reflex among different genotypes and treatment groups on postnatal days 5 and 6 (Figure 8A, postnatal day 5: $F_{\text{interaction (2, 72)}} = 0.04742$, $p = 0.9537$, $F_{\text{treatment (1, 72)}} = 0.5531$, $p = 0.4595$, $F_{\text{genotype (2, 72)}} = 0.1288$, $p = 0.8794$; postnatal day 6: $F_{\text{interaction (2, 72)}} = 0.1431$, $p = 0.8669$, $F_{\text{treatment (1, 72)}} = 3.102$, $p = 0.0825$, $F_{\text{genotype (2, 72)}} = 0.8047$, $p = 0.4512$). However, on postnatal day 7, prenatal poly(I:C) injection significantly delayed the development of the grasping reflex, regardless of the genotypes in neonatal mice (Figure 8B, $F_{\text{interaction (2, 56)}} = 0.5327$, $p = 0.5900$, $F_{\text{treatment (1, 56)}} = 6.201$, $p = 0.0158$, $F_{\text{genotype (2, 56)}} = 1.210$, $p = 0.3060$). The data distribution of the different groups on postnatal days 6 and 7 is shown in Figures 8C

doi:10.6342/NTU202304492

and 8D, respectively. The median time to hold the bar in the grasping reflex test was significantly shorter in the poly(I:C)-treated groups compared to the saline-treated groups on postnatal days 6 and 7 (Figures 8C and 8D).

These results showed that prenatal poly(I:C) challenge delays the development of the righting, geotaxis, and grasping reflexes in neonatal mice. This effect is independent of genotype, suggesting that poly(I:C) challenge may have a global impact on sensorimotor development. More research is needed to investigate the mechanisms underlying the effects of prenatal poly(I:C) challenge on sensorimotor development. It is also important to understand whether the delay in the development of these reflexes in poly(I:C)-treated mice persists into adulthood.

Locomotor activity in adolescent mice

Besides milestone tasks, in Experiment 2.1, we also assessed locomotor activity in adolescent mice (postnatal day 26) using an open-field test (OFT). We found no significant differences in total traveled distance or traveled distance in 5-min time bins between saline- and poly(I:C)-treated mice, but a main effect of genotype was that SR HET mice exhibited significantly lower locomotor activity during an hour of testing regardless of treatment (Figure 9, $F_{\text{interaction (2, 43)}} = 0.1118$, $p = 0.8945$, $F_{\text{treatment (1, 43)}} =$

doi:10.6342/NTU202304492

1.185, $p = 0.2825$, $F_{\text{genotype (2, 43)}} = 3.349$, $p = 0.0445$). These findings suggest that prenatal poly(I:C) challenge does not have much impact on locomotor activity in adolescent mice. Yet, a genotype effect was found, which is meaningful to compare with locomotor activity in the follow-up experiment in adulthood.

3.3 Experiment 2.2: Behavioral phenotyping in adult mice

To understand the role of SR genotypes, prenatal poly(I:C)-induced immune challenge and the potential interaction between genetics and treatment in adult cognitive functions, three different genotypes in the SR background (WT, HET, and HOM) in different treatment groups (saline or poly(I:C)) were analyzed using a two-way ANOVA.

Prenatal poly(I:C) challenge and genotype separately affect locomotor activity in adult mice

To start with Experiment 2.2, we assessed locomotor activity in adult mice (postnatal day 80) using an open-field test (OFT). We found that prenatal poly(I:C) challenge and genotype significantly affect locomotor activity in adult mice with no

doi:10.6342/NTU202304492

interaction effect (Figure 10, $F_{\text{interaction (2, 90)}} = 0.4470$, $p = 0.6409$, $F_{\text{treatment (1, 90)}} = 4.109$, $p = 0.0456$, $F_{\text{genotype (2, 90)}} = 6.478$, $p = 0.0024$). The elevation in locomotor activity was more pronounced in homozygous (HOM) mice compared to heterozygous (HET) mice, as indicated by Holm-Sidak's multiple comparisons (Mean $_{\text{HET}} = 21491$, Mean $_{\text{HOM}} = 24906$, $p = 0.0018$).

Consistent with prior research, prenatal poly(I:C) challenge was associated with increased locomotor activity in adult mice, regardless of genotype (da Silveira et al., 2017). Additionally, SR knockout mice exhibited a hyperactive locomotor phenotype, as previously described (Basu et al., 2009).

Lack of impact on working memory in adult mice following prenatal poly(I:C) exposure and SR mutations

Next, we assessed working memory in adult mice using a spontaneous alteration test in the Y-maze. We found no significant differences in total entries ($F_{\text{interaction (2, 90)}} = 0.3007$, $p = 0.7411$, $F_{\text{treatment (1, 90)}} = 0.0001239$, $p = 0.9911$, $F_{\text{genotype (2, 90)}} = 0.6290$, $p = 0.5355$) or spontaneous alteration rates ($F_{\text{interaction (2, 90)}} = 1.719$, $p = 0.1852$, $F_{\text{treatment (1, 90)}} = 1.063$, $p = 0.3054$, $F_{\text{genotype (2, 90)}} = 1.631$, $p = 0.2015$) between treatment and genotype (Figure 11).

doi:10.6342/NTU202304492

These findings suggest that prenatal poly(I:C) challenge does not have a lasting impact on working memory in adult mice. However, it is important to note that this study only examined working memory in a single context. It is possible that prenatal poly(I:C) challenge could have a subtle impact on working memory in other contexts, such as in response to a stressor or in a more challenging task.

Prenatal poly(I:C) challenge affects long-term memory in adult WT mice

We tested long-term memory of adult mice using a novel object recognition test (NORT) with an hour intertrial interval. In terms of total sniffing time during the testing session (which includes time spent on both the familiar and novel objects), we observed no significant differences among different genotypes, treatments, or their interactions (Figure 12A, $F_{\text{interaction (2, 88)}} = 0.3784$, $p = 0.6861$, $F_{\text{treatment (1, 88)}} = 0.01352$, $p = 0.9077$, $F_{\text{genotype (2, 88)}} = 0.5794$, $p = 0.5624$). This result indicates that, in the context of this task, exploratory behaviors did not differ significantly across the various groups. However, when we examined the discrimination index using a priori comparison, we found that poly(I:C)-treated WT mice discriminated against the novel object worse compared to saline-treated WT mice (Figure 12B, $t_{(18)} = 2.180$, $p = 0.0428$). This finding suggests that prenatal poly(I:C) challenge impaired long-term memory in adult WT mice but had no such effect on other genotypes. A two-way ANOVA revealed no significant effects

doi:10.6342/NTU202304492

of genotype or treatment, nor their interaction, although the treatment main effect approached marginal significance ($F_{\text{interaction (2, 88)}}$ =1.572, p = 0.2134, $F_{\text{treatment (1, 88)}}$ = 3.690, p = 0.0580, $F_{\text{genotype (2, 88)}}$ = 0.5337 p = 0.5883). These findings indicate that prenatal poly(I:C) challenge may specifically affect long-term memory in WT mice.

Attention unaffected by prenatal poly(I:C) and SR mutations in adult mice

We evaluated attention in adult mice using an object-based attention task (OBAT). When considering the total sniffing time during the testing session, which includes time spent on both the familiar and novel objects, we observed no significant differences among various genotypes, treatments, or their interactions (Figure 13A, $F_{\text{interaction (2, 90)}}$ = 2.714, p = 0.0717, $F_{\text{treatment (1, 90)}}$ = 0.08501, p = 0.7713, $F_{\text{genotype (2, 90)}}$ = 0.1606, p = 0.8519). This finding implies that exploratory behaviors did not differ significantly across different experimental groups in this task. Subsequently, we found no significant differences in recognition index among different genotypes, treatments, or their interactions (Figure 13B, $F_{\text{interaction (2, 90)}}$ = 0.2491, p = 0.7800, $F_{\text{treatment (1, 90)}}$ = 0.2412, p = 0.6246, $F_{\text{genotype (2, 90)}}$ = 0.2779, p = 0.7580). These findings suggest that prenatal exposure to poly(I:C) and SR mutations does not have an impact on attention in adult mice.

doi:10.6342/NTU202304492

Sociability and social recognition unaffected by prenatal poly(I:C) and SR mutations in adult mice

A three-chamber social interaction test was used to assess sociability and social recognition in adult mice. In the sociability test, we discovered no significant differences in social preference rate between different genotypes, treatments, or their interactions (Figure 14A, $F_{\text{interaction (2, 90)}} = 0.3039$, $p = 0.7387$, $F_{\text{treatment (1, 90)}} = 0.06992$, $p = 0.7921$, $F_{\text{genotype (2, 90)}} = 0.03670$, $p = 0.9640$). Likewise, in the assessment of social recognition, we also found no significant differences in social recognition rate across different genotypes, treatments, or their interactions (Figure 14B, $F_{\text{interaction (2, 89)}} = 1.185$, $p = 0.3107$, $F_{\text{treatment (1, 89)}} = 0.03776$, $p = 0.8464$, $F_{\text{genotype (2, 89)}} = 2.576$, $p = 0.0817$). These findings suggest that prenatal poly(I:C) challenge and SR mutations does not have a lasting impact on sociability and social recognition in adult mice.

Prenatal poly(I:C) challenge impairs contextual fear memory in adult SR HET and HOM mice

A trace fear conditioning test was applied to assess associative learning and memory in adult mice. To determine whether there were any differences in baseline learning across experimental groups, we initially compared the total freezing time on the first

doi:10.6342/NTU202304492

day (day 1), during which mice were first exposed to the conditioned stimuli and began associating them with the auditory cues. A two-way ANOVA revealed no significant differences across different genotypes, treatments, or their interactions ($F_{\text{interaction (2, 89)}}$ =1.674, p = 0.1933, $F_{\text{treatment (1, 89)}}$ = 1.456, p = 0.2308, $F_{\text{genotype (2, 89)}}$ = 1.569, p = 0.2140). This result suggests that baseline learning was consistent among groups of different genotypes and treatments. Regarding the phases of contextual and tone-cued fear memory retrieval on days 2 and 3, respectively, there were also no significant differences across different genotypes, treatments, or their interactions (contextual: $F_{\text{interaction (2, 89)}}$ =1.674, p = 0.1933, $F_{\text{treatment (1, 89)}}$ = 1.456, p = 0.2308, $F_{\text{genotype (2, 89)}}$ = 1.569, p = 0.2140; tone-cued: $F_{\text{interaction (2, 89)}}$ = 1.674, p = 0.1933, $F_{\text{treatment (1, 89)}}$ = 1.456, p = 0.2308, $F_{\text{genotype (2, 89)}}$ = 1.569, p = 0.2140).

Subsequently, we conducted an analysis of freezing time in various treatment groups on each day using one-way ANOVA. In the saline-treated group (Figure 15A), we found a marginal but statistically significant difference during the learning phase on day 1 ($F_{(2, 52)}$ = 3.224, p = 0.0479). However, there were no significant differences observed in freezing time among the three genotypes in contextual fear memory on day 2 ($F_{(2, 53)}$ = 1.381, p = 0.2601) and tone-cue fear memory on day 3 ($F_{(2, 53)}$ = 1.401, p = 0.2553).

doi:10.6342/NTU202304492

By contrast, in the poly(I:C)-treated group, one-way ANOVA revealed significant differences on day 2 among different genotypes (Figure 15B, $F_{(2, 37)} = 3.457$, $p = 0.0420$). Further multiple comparisons using Holm-Sidak's method demonstrated notable deficits in contextual fear memory retrieval on day 2 for SR HOM and HET mice, as evidenced by decreased freezing time compared to SR WT mice (Mean $_{WT} = 216.6$, Mean $_{HET} = 145.1$, Mean $_{HOM} = 132.0$; WT v.s. HET, WT v.s. HOM, $p = 0.0390$). While, no significant differences observed in freezing time during the learning phase and tone-cue fear memory (learning: $F_{(2, 37)} = 0.6397$, $p = 0.5332$; tone-cued: $F_{(2, 37)} = 1.467$, $p = 0.2436$).

These findings suggest that prenatal poly(I:C) challenge specifically impairs contextual fear memory in adult SR HET and HOM mice. The lack of effect of prenatal poly(I:C) challenge on contextual fear memory in WT mice suggests that genetic factors may influence the susceptibility of mice to the effects of prenatal poly(I:C) challenge on this type of memory. More research is needed to identify the specific mechanisms by which prenatal poly(I:C) challenge impairs contextual fear memory in SR mutant mice.

doi:10.6342/NTU202304492

3.4 Experiment 3: The potential rescue effect on neuromotor development delay with RS-D7

Building upon the earlier findings from Experiment 2.1, which indicated that prenatal poly(I:C) challenge leads to a delayed development of the righting reflex and geotaxis reflex in neonatal mice, regardless of their genotypes. It seems that within the context of milestone tests conducted on neonatal mice, prenatal poly(I:C) challenge exerts a more salient impact compared to the SR genetic mutations. In Experiment 3, we sought to evaluate the effects of prenatal poly(I:C) challenge and explore the potential rescue effect of RS-D7, a novel d-amino acid oxidase inhibitor, on the development of the righting reflex and geotaxis reflex in neonatal mice. We combined data from three different genotypes in the SR background (WT, HET, and HOM) and conducted separate one-way ANOVA tests for each postnatal day (P3-P5) to see if there were any differences between these groups under various treatment conditions (saline, poly(I:C) + saline, and poly(I:C) + RS-D7). The results are detailed in the section that follows.

doi:10.6342/NTU202304492

Body weight of neonatal mice

Figure 16 shows the body weight of neonatal mice in Experiment 3, which was recorded on each day prior to the subcutaneous (s.c.) treatments and behavioral testing. The results of one-way ANOVA conducted for postnatal day 3 ($F_{(2, 27)} = 0.114$, $p = 0.0607$), postnatal day 4 ($F_{(2, 27)} = 0.604$, $p = 0.2197$), and postnatal day 5 ($F_{(2, 27)} = 0.250$, $p = 0.1248$) revealed no statistically significant differences. These findings indicate that the body weight of neonatal mice remains unaffected by the prenatal poly(I:C) challenge, regardless of the specific treatments applied.

RS-D7 treatment restored the development delay of the righting reflex in neonatal mice exposed to prenatal poly(I:C)

Figure 17 illustrates the time taken for neonatal mice to achieve the righting reflex on postnatal days 3–5 in Experiment 3. There were no significant differences in the performance of the righting reflex among different treatment groups on postnatal days 3 ($F_{(2, 27)} = 0.03645$, $p = 0.9643$) and 5 ($F_{(2, 27)} = 0.250$, $p = 0.1248$). Interestingly, significant differences were observed on day 4 ($F_{(2, 27)} = 4.552$, $p = 0.0198$), where prenatal poly(I:C) challenge caused a noticeable delay in the development of the righting reflex on postnatal day 4, as evidenced by post hoc comparisons using Holm-

doi:10.6342/NTU202304492

Sidak' s method (Mean $_{saline}$ = 11.82, Mean $_{poly(I:C) + saline}$ = 17.71; p = 0.0374). However, daily subcutaneous injections of RS-D7 (40 mg/kg) significantly ameliorated the development delay of the righting reflex on postnatal day 4 when compared to the poly(I:C) + saline group (Mean $_{poly(I:C) + saline}$ = 17.71, Mean $_{poly(I:C) + RS-D7}$ = 8.360; p = 0.0133). These findings suggest that prenatal poly(I:C) challenge delayed the development of the righting reflex, and that RS-D7 treatment had the potential to restore the delay in neonatal mice exposed to prenatal poly(I:C).

Trends in geotaxis reflex development delay in neonatal mice exposed to prenatal poly(I:C) and RS-D7 treatment

In Experiment 3, we assessed the effects of prenatal poly(I:C) challenge and RS-D7 treatment on the development of the geotaxis reflex in neonatal mice on postnatal days 3–5 (Figure 18). Figure 18A shows the five-point scoring assessment for neonatal mice to achieve the geotaxis reflex on postnatal days 3–7 in Experiment 3. Prenatal poly(I:C) challenge showed a tendency to delay the development of the geotaxis reflex on postnatal day 3, although it did not reach statistical significance ($F_{(2, 27)}$ = 3.133, p = 0.0598). Conversely, daily subcutaneous administrations of RS-D7 (40 mg/kg) appeared to exhibit a trend in restoring the geotaxis reflex to levels comparable to those observed in the saline group. Furthermore, there were no statistically significant

doi:10.6342/NTU202304492

differences in the performance of the geotaxis reflex on postnatal days 4 ($F_{(2, 27)}$ = 0.7000, p = 0.5054) and 5 ($F_{(2, 27)}$ = 0.3684, p = 0.6953) among the various treatment groups.

Figure 18B shows the time taken for neonatal mice to achieve the geotaxis reflex on postnatal days 3–7 in Experiment 3. The results of one-way ANOVA conducted for postnatal day 3 ($F_{(2, 27)}$ = 0.5531, p = 0.5815), postnatal day 4 ($F_{(2, 27)}$ = 0.2603, p = 0.7728), and postnatal day 5 ($F_{(2, 27)}$ = 0.6551, p = 0.5275) revealed no statistically significant differences among distinct treatment groups. These findings suggest that prenatal poly(I:C) exposure might influence the development of the geotaxis reflex, and that treatment with RS-D7 has the ability to restore it in neonatal mice exposed to prenatal poly(I:C).

doi:10.6342/NTU202304492

Chapter 4. Discussion

4.1 Summary of results

In the present study, we conducted three experiments to investigate potential gene-environment interactions between SR mutations and poly(I:C)-induced neurodevelopmental deficits in a mouse model based on the two-hit hypothesis. In Experiment 1, we observed elevated levels of three pro-inflammatory cytokines (IL-6, IL-1β, and TNF-α) following prenatal poly(I:C) challenge, in comparison to the saline-treated group, regardless of genotype. These results affirmed the successful induction of immune activation in fetal brains through systematic poly(I:C) injection.

In Experiment 2.1, we found that prenatal poly(I:C) challenge significantly delayed the development of the righting reflex and geotaxis reflex in neonatal mice. Notably, this effect only observed on postnatal day 5 in both tests and it was independent of genotype, suggesting that poly(I:C) challenge may have a global impact on sensorimotor development. Furthermore, it is interesting to note that the poly(I:C)-HOM group performed the worst in both assessments, indicating that the SR HOM genotype may be more sensitive to the effects of prenatal poly(I:C) exposure.

In Experiment 2.2, we discovered that prenatal poly(I:C) challenges affect locomotor

doi:10.6342/NTU202304492

activity, long-term memory, and contextual fear memory in adult SR mice in a manner specific to the genotype. Conversely, attention, working memory, sociability, and social recognition remained unaffected by different treatments or genotypes. All of the behavioral tasks showed no interaction between treatment and genotype.

In Experiment 3, we found that prenatal poly(I:C) challenge significantly delayed the development of the righting reflex on postnatal day 4. However, daily subcutaneous injection of RS-D7 successfully restore the delay in neonatal mice exposed to prenatal poly(I:C). In addition, the prenatal poly(I:C) challenge showed a tendency to delay the development of the geotaxis reflex on postnatal day 3, although it did not reach statistical significance. Furthermore, daily subcutaneous administrations of RS-D7 appeared to exhibit a trend in restoring the geotaxis reflex to levels comparable to those observed in the saline group.

4.2 Elevation of cytokines induced by poly(I:C) injection

Consistent with previous studies of late-gestation poly(I:C) challenges, which also reported elevated levels of pro-inflammatory cytokine such as IL-6 (Meyer, Nyffeler, et al., 2006a), and TNF-α, (Arsenault et al., 2014), our results confirm immune activation within fetal brains following acute poly(I:C) exposure. Indeed, several

doi:10.6342/NTU202304492

studies have pointed out cytokines as key mediators of immune response, and there is substantial evidence of dysregulated cytokine profiles in peripheral blood and cerebrospinal fluid (CSF) of individuals with schizophrenia (Goldsmith et al., 2016; Hartwig et al., 2017; Upthegrove & Khandaker, 2020).

Notably, Upthegrove and colleagues (2014) found that medication-naive first episode psychosis patients exhibited elevated levels of IL-1β, TNF-α, IL-6 and sIL-2r compared to control subjects (Upthegrove & Barnes, 2014). These cytokine profiles are closely similar to the findings of our present study, suggesting a resemblance to schizophrenia. Additionally, another study further demonstrated that maternal administration of IL-6 alone is sufficient to induce the behavioral abnormalities observed in other models of inflammatory (Smith et al., 2007). Based on these associations, the elevation of pro-inflammatory cytokines, particularly IL-6, may serve as potential biomarkers linking inflammation to schizophrenia.

4.3 A comparison of behavioral phenotyping results between our current findings and previous studies

The adult behavioral phenotypes compared to previous studies are summarized in

doi:10.6342/NTU202304492

Table 3. In line with previous study, our SR HOM mice exhibited hyperlocomotion activity in the open field test on PND 80 (Basu et al., 2009). Additionally, mice treated with poly(I:C) displayed a similar hyperlocomotion pattern, consistent with findings in a previous study (da Silveira et al., 2017). However, it is worth noting that there exists a conflict, as other studies, such as Meyer et al. (2008), found that basal locomotion activity remained unaffected by poly(I:C) challenge.

We assessed the working memory in adult mice using spontaneous alteration test in a Y-maze. However, our results revealed no significant main effects in treatment, genotype, or their interactions. In previous studies, poly(I:C) challenge on E17.5 was shown to bring deficits in working memory examined by Morris water maze (Meyer et al., 2008), T-maze (Connor et al., 2012), and matching-to-position dry maze paradigm (Richetto et al., 2013). The observed inconsistencies may be due to sensitivity of the tasks or variations in experimental circumstances and procedures, including differences in mouse ages, genders, and housing conditions. To explain the observed discrepancies, future studies should conduct a more comprehensive study of these aspects.

In a NORT to examine long-term memory, we found that prenatal poly(I:C) challenge specifically affects long-term memory in adult WT mice. Notably, this effect was not observed in SR mutations (HET and HOM). Consistent with the previous study, which also indicated that long-term memory assessed by NORT remained normal in

doi:10.6342/NTU202304492

SR HOM mice (Matveeva et al., 2019). It is important to note that this impact is a unique discovery, and more research is needed to test its resilience and clarify the underlying mechanisms.

In our study, a three-chamber social interaction test also revealed a lack of significant effects in treatment, genotype, or their interactions. This finding contradicts prior research where poly(I:C) challenge during late gestation led to significant impairments in social interaction (Bitanihirwe et al., 2010), as well as deficits in social recognition among male SR HOM mice in our laboratory (Luo, 2022). This inconsistency in results may be attributed to the inclusion of both male and female mice in our study, as well as the potential influence of specific experimental conditions and procedures on the sensitive nature of social behavior.

A trace fear conditioning test was applied to assess associative learning and memory functions in adult mice. Our findings revealed that prenatal poly(I:C) challenge impairs contextual fear memory in adult SR HET and HOM mice, but this effect was not seen in the saline group. This result is inconsistent with a previous study in our laboratory indicated that single-housed male SR HOM mice exhibited deficits in the retrieval of contextual and cue fear memory (Luo, 2022). The inclusion of both male and female mice in our study, as well as the potential influence of housing conditions, may explain the inconsistency in these results. Furthermore, it is

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noteworthy that a previous study by Chess et al. (2009) reported that treatment with L-kynurenine influenced contextual fear conditioning and context discrimination, but had no significant impact on cue-specific fear conditioning, aligning with our own findings. This finding is particularly intriguing in light of the established association between kynurenic acid (KYNA) and inflammatory processes. The relationship between poly(I:C)-induced inflammation and trace fear conditioning deserve further studying for advancing our understanding of associative learning and memory functions.

4.4 Possible convergence of SR and poly(I:C) on NMDAR

Previous studies have demonstrated that NMDAR-mediated neurotransmission and protein levels of NMDAR subunits are altered in SR HOM mice (Basu et al., 2009; Mustafa et al., 2010). In particular, NR1 NMDA receptor protein levels in the corpus striatum increased fourfold (Mustafa et al., 2010). This finding suggests that in SR HOM mice, where the SR gene is knocked out throughout their system, may exhibit an overexpression of NMDAR subunits as a compensatory mechanism for their essential functions.

Interestingly, changes in NMDAR subunit protein levels were also seen in prenatal late poly(I:C)-treated offspring, with a specific reduction in NMDA-receptor subunit

doi:10.6342/NTU202304492

NR1 immunoreactivity (NR1-IR) in the dorsal hippocampus (dHIP) after prenatal immune challenge in late gestation (Meyer et al., 2008). Moreover, evidences have shown that pro-inflammatory cytokines released following poly(I:C) challenge may increase KYNA and finally give rise to hypofunction of NMDAR (MacDowell et al., 2021). The KYNA metabolism is known to be regulated by inflammatory processes and acted as endogenous antagonists of the NMDAR in schizophrenia (de Bartolomeis et al., 2022; Jorratt et al., 2021; MÜller & Schwarz, 2006).

In our findings in Experiment 3 we found that a novel DAOI, RS-D7, successfully rescued the development delay in neonates exposed to poly(I:C). These results suggest that the deficits induced by poly(I:C) exposure in this context can be restored to normal through the modulation of NMDAR by RS-D7, thereby signaling a potential interaction or association within the NMDA receptor pathway.

In summary, both genetic and prenatal immune challenge models demonstrate alterations in the NMDAR pathway, suggesting a potential convergence in the modulation of NMDAR function at the biochemical and behavioral level.

doi:10.6342/NTU202304492

4.5 Limitations of current study

While our findings hold importance, it is essential to acknowledge the limitations and constraints within the current study. Firstly, the experiment's limitation stems from practical limits that restrict the simultaneous generation of a large amount of offspring. As a result, data obtained in several batches introduces a number of variables, making it difficult to demonstrate statistical significance convincingly. Secondly, our experiment primarily focuses on the behavioral aspect to examine differences between treatment and genotypes. Although behavior is a crucial aspect in the field of neuroscience, as it represents the ultimate output of our nervous systems and exerts various functions related to cognition and the maintenance of our daily lives, bio-chemical levels are deserve further studying in order to find out the subtle alterations that may not be detected by behavioral observations and for elucidating the underlying mechanisms. Further study is needed to further understand the alterations in bio-chemical and molecular levels. Finally, in terms of adult phenotyping, in order to screen for possible alterations in behavior, we included a broad range of cognitive and negative symptoms related tasks but didn’t do further testing or manipulations pertaining to the observed deficits.

doi:10.6342/NTU202304492

4.6 Contributions and conclusion

To the best of our knowledge, our study represents the pioneering investigation into the combined effects of prenatal poly(I:C) challenge and SR mutations in a two-hit mouse model. Additionally, it is also the first research extends to the examination of SR mutant mice, encompassing both HET and HOM mutants. Additionally, we are the first to introduce the subcutaneous administration of RS-D7 to newborn mice.

In our current thesis, we aim to address the question of whether interactions exist between prenatal poly(I:C) challenges and SR mutations within the framework of a two-hit mouse model. Based on our findings, we conclude that there were no observed interactions in terms of cytokines levels and behavioral phenotypes between different genotypes of SR mutant mice and poly(I:C) immune challenge in both neonates and adulthood. Nevertheless, potential interactions or convergences appear to exist between poly(I:C)-induced immune alterations and the NMDAR pathway, especially at the biochemical level. Furthermore, the "two-hits" concept can refer to both genetic and environmental influences, or they might refer to distinct temporal occasions. Poly(I:C) exposure may act as a priming event, causing subsequent responses to be more apparent upon stimulation. The pharmacological intervention may serve to improve reactions to the second stimulus in advance. In conclusion, our study has shed light on the intricate

doi:10.6342/NTU202304492

interplay of genetic and environmental factors in schizophrenia, offering valuable insights into the role of prenatal immune challenges and SR mutations. While our research did not reveal direct interactions between these elements, the emergence of potential connections with the NMDAR pathway suggests a promising avenue for further investigation and therapeutic exploration in this complex disorder.

doi:10.6342/NTU202304492

Table and Figures

Table 1. Subject information

Mice ID	Cage	Genotype	Group	Dams
E1-1	E1	HET	Poly(I:C)	3-9 (1st)
E1-2	E1	WT		
E1-4	E1	HET		
E1-5	E1	WT		
E1-6	E1	HET		
E1-7	E2	HET		
E1-8	E2	HET		
E1-9	E2	HET		
E1-10	E3	HOM	Saline	3-6 (1st)
E1-11	E3	HET		
E1-12	E3	HET		
E1-13	E3	HOM		
E2-11	E4	HET	Poly(I:C)	7-2 (1st)
E2-12	E4	HOM		
E2-13	E4	HET		
E2-9	E5	HET		
E2-10	E5	HOM		
E2-2	E6	HOM	Saline	3-9 (2nd)
E2-5	E6	HET		
E2-8	E6	HET		
E2-1	E7	HET		
E2-3	E7	WT		
E2-4	E7	HOM		
E2-6	E7	HOM		
E2-7	E7	HOM		
E8-1	E8M	HET	Saline	51-8 (1st)
E8-2	E8M	WT		
E8-3	E8F	HET		
E8-4	E8F	HET		

doi:10.6342/NTU202304492

E9-1	E9M	HOM	Saline	3-5 (1st)
E9-2	E9F	HET		
E9-3	E9F	HOM		
E9-4	E9M	HOM		
E9-5	E9M	HOM		
E9-6	E9F2	HET	Saline	10-2 (1st)
E9-7	E9F2	HET		
E9-8	E9M2	HOM		
E9-9	E9M2	HOM		
E9-10	E9F2	HET		
E9-11	E9F2	HET		
E9-12	E9F2	HOM		
E9-13	E9M2	WT		
E9-14	E9F3	WT	Poly(I:C)	10-6 (1st)
E9-15	E9F3	WT		
E9-16	E9M3	HOM		
E9-17	E9F3	WT		
E9-18	E9M3	WT		
E10-1	E10F	WT		
E10-2	E10F	HET		
E10-3	E10M	HET		
E11-1	E11F	HET	Poly(I:C)	11-1 (1st)
E11-2	E11F	HOM		
E11-3	E11M	HOM		
E11-4	E11M	HOM		
E11-5	E11F2	HOM		
E11-6	E11F2	WT		
E11-7	E11F2	HET		
E 13-1	E13F	HOM	Poly(I:C)	0311-3 (1st)
E 13-2	E13F	HOM		
E 13-3	E13F	HET		
E 13-4	E12M	HET		
E 13-5	E13F	HOM		

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E 14-1	E14M	WT	Saline	11-1 (2nd)
E 14-2	E14M	HOM		
E 14-3	E14M	HET		
E 14-4	E14F	HET		
E 14-6	E14F	HET		
E 12-1	E14F	HOM	Saline	23-1 (1st)
E 12-2	E12M	WT		
E 12-3	E12M	HET		
E 12-4	E12M	WT		
E 12-5	E14F	HOM		
E 12-6	E14F	HET		
E1213-1	E1213-1	HET	Saline	0714-7 (1st)
E1213-2	E1213-1	HET		
E1213-3	E1213-1	HOM		
E1213-4	E1213-2	HET		
E1213-5	E1213-2	WT		
E1213-6	E1213-2	HET		
E1213-7	E1213-3	WT		
E1213-8	E1213-3	HET		
E1213-9	E1213-3	HET		
E 16-1	E0217SM	WT	Saline	0819-5 (1st)
E 16-2	E0217SM	WT		
E 16-3	E0217SM	HET		
E 16-4	E0217SM	HOM		
E 16-5	E0217SF	HOM		
E 16-6	E0217SF	HOM		
E 16-7	E0217SF	HOM		
E 16-8	E0217SF	HOM		
E17-1	E0217PM	HOM	Poly(I:C)	0714-7 (2nd)
E17-2	E0217PM	WT		
E 17-4	E0217PM	HET		
E 17-5	E0217PM	HOM		
E 17-6	E0217PSF	WT		
E 17-7	E0217PSF	HET		

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Table 2 Summary of specific aims

Experiment	Specific aim	Experimental design
Exp. 1	Characterization of poly(I:C)-induced acute cytokines responses	After a six-hour period following the administration of poly(I:C) or saline on E17.5, the levels of three cytokines (IL-6, IL-1β, and TNF-α) were assessed in fetal mouse brains using ELISA.
Exp. 2.1	Characterization of development in neonates	To assess the neuro-sensory motor functions in three genotypes of SR mutant mice (WT, HET, and HOM) prenatally treated with poly(I:C) or saline on E17.5, three behavioral tasks were conducted during postnatal days 3-7 (righting reflex and geotaxis reflex) and postnatal days 5-7 (grasping reflex).
Exp. 2.2	Characterization of adult behavioral phenotypes	To assess behaviors relevant to the negative and cognitive symptoms in schizophrenia, six behavioral tasks were conducted in three genotypes of SR mutant mice (WT, HET, and HOM) prenatally treated with poly(I:C) or saline on E17.5. The tasks included the open-field task, spontaneous alteration task, novel object recognition task, object-based attention task, three-chamber social interaction task, and trace fear conditioning task.
Exp. 3	Evaluation of rescue effect by RS-D7 in neonates	To assess the neuro-sensory motor functions in SR mutant mice prenatally treated with poly(I:C) or saline on E17.5, two behavioral tasks were conducted on postnatal days 3-5 (righting reflex and geotaxis reflex), following daily subcutaneous injections of either saline or RS-D7.

doi:10.6342/NTU202304492

Table 3 Summary of adult phenotypes in the current study compared to previous studies

Behavioral task	SR HOM (previous)	Late poly(I:C) (previous)	Current study
Open Field	Hyperactive (male) (Basu et al., 2009)	Hyperactive (male) (da Silveira et al., 2017)	Hyperactive in SR HOM and poly(I:C)-WT
T-maze	Normal (Basu et al., 2009)	Deficit (male) (Connor et al., 2012)	-
NORT	Normal (Matveeva et al., 2019)	-	Worse in poly(I:C)-WT
OBAT	-	-	Normal
Spontaneous alteration	-	-	Normal
Social interaction	Deficit (male) (Luo, 2022)	Deficit (male) (Bitanihirwe et al., 2010)	Normal
Trace Fear conditioning	Deficit (male) (Luo, 2022)	-	Deficit in poly(I:C)-HET and poly(I:C)-HOM

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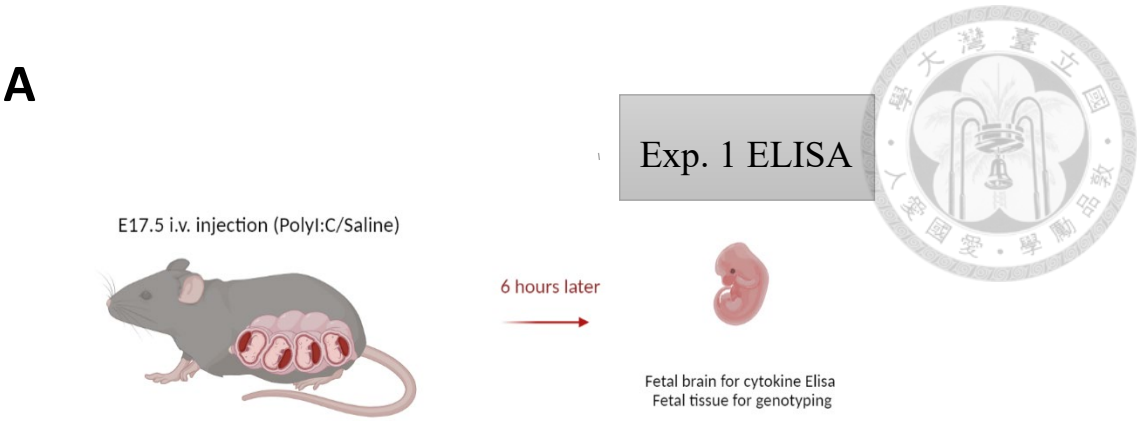

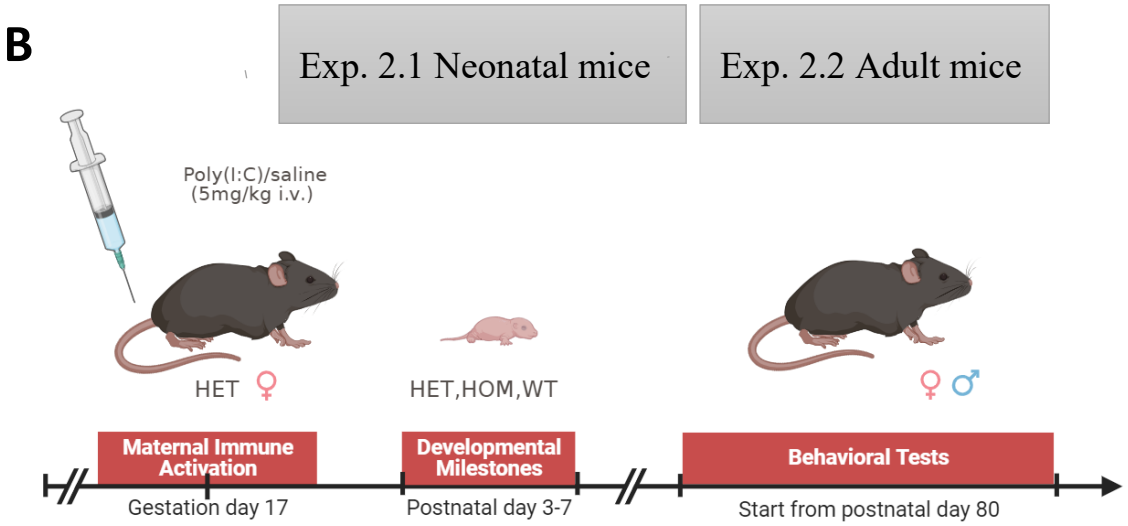

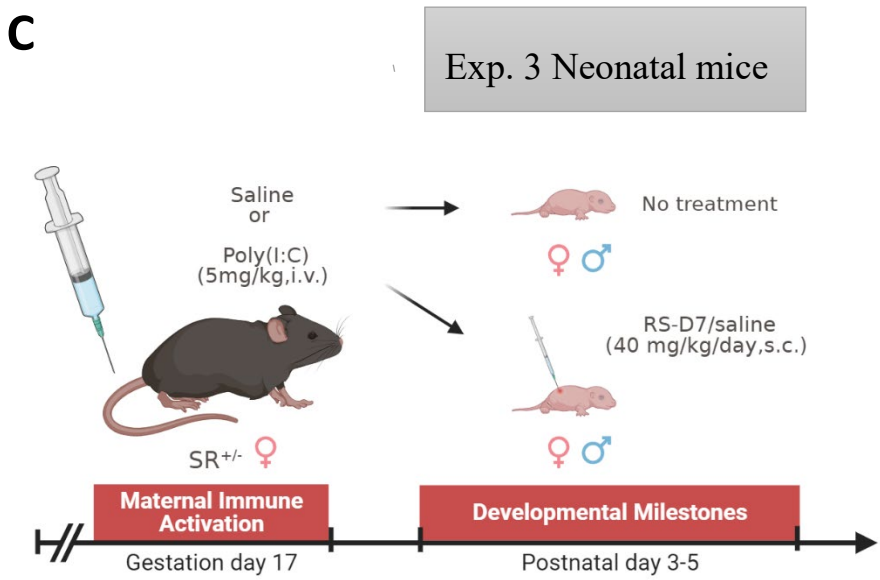

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Figure 1 Experimental timeline in current study.

(A) In Exp. 1, to confirm the prenatal immune activation levels in the fetal brains, after a 6-hour period of the administration of poly(I:C) or saline on E17.5, the fetal brains were dissected freshly for further ELISA testing. (B) After the administration of poly(I:C) or saline on E17.5, the developmental milestones were evaluated during postnatal day 3-7 in neonatal mice (Exp. 2.1). A series of adult behavioral tasks were conducted in the same batch of mice from postnatal 80 (Exp. 2.2). (C) In Exp. 3, after the administration of poly(I:C) or saline on E17.5, the developmental milestones were evaluated during postnatal day 3-5 in neonatal mice. The offspring of poly(I:C)-treated dams were tested following daily subcutaneous injections of either saline or RS-D7, and the offspring of saline-treated dams received no treatments. Figures created with BioRender.com.

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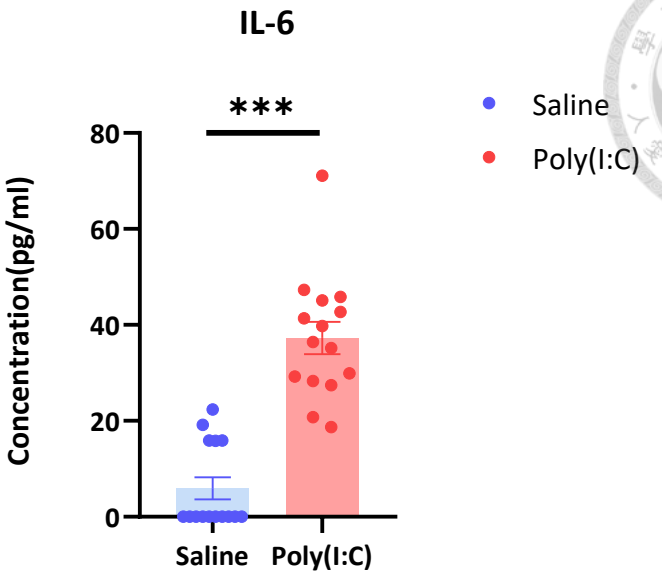
A



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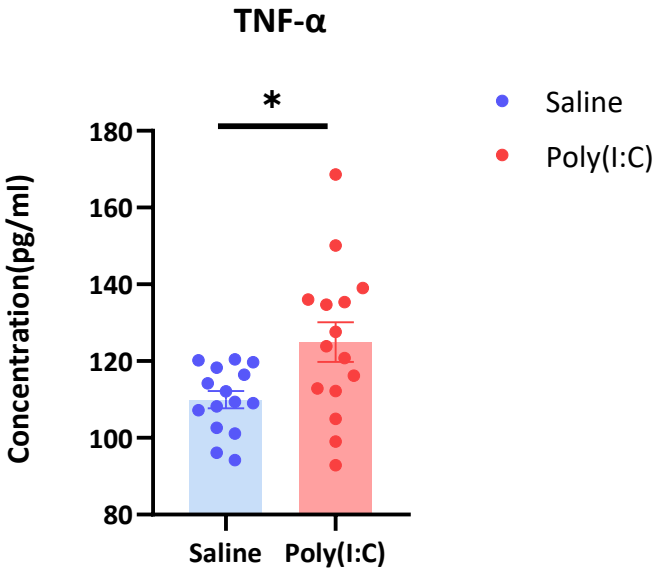

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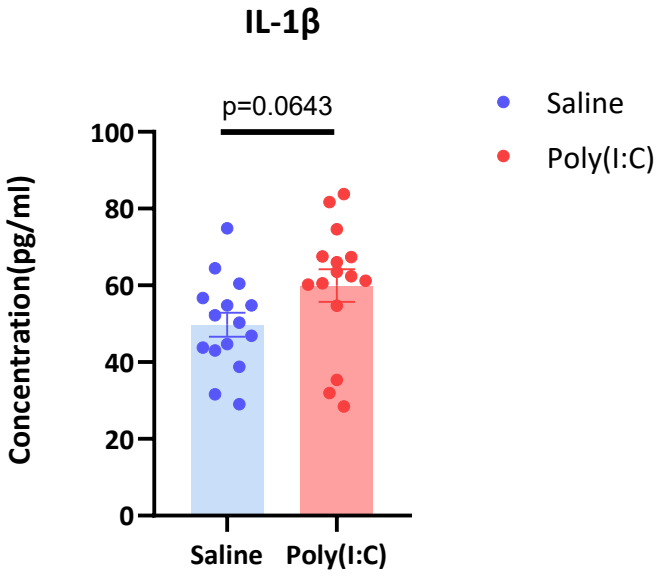

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Figure 2 Fetal brain cytokine responses 6 hours after immune challenge by administration of poly(I:C) (5 mg/kg) on embryotic day 17.5 via the i.v. route

(A) An elevation of IL-6 levels was detected in the fetal brains of the poly(I:C)-treated group using ELISA. (B) Elevated TNF-α levels were observed in the fetal brains of the poly(I:C)-treated group. (C) Elevated IL-1β levels were observed in the fetal brains of the poly(I:C)-treated group, with a trend that approached significance despite the effect not reaching statistical significance. * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$, statistical significance based on unpaired t test. N = 15 in both the saline- and poly(I:C)-treated groups. All values are mean ± SEM.

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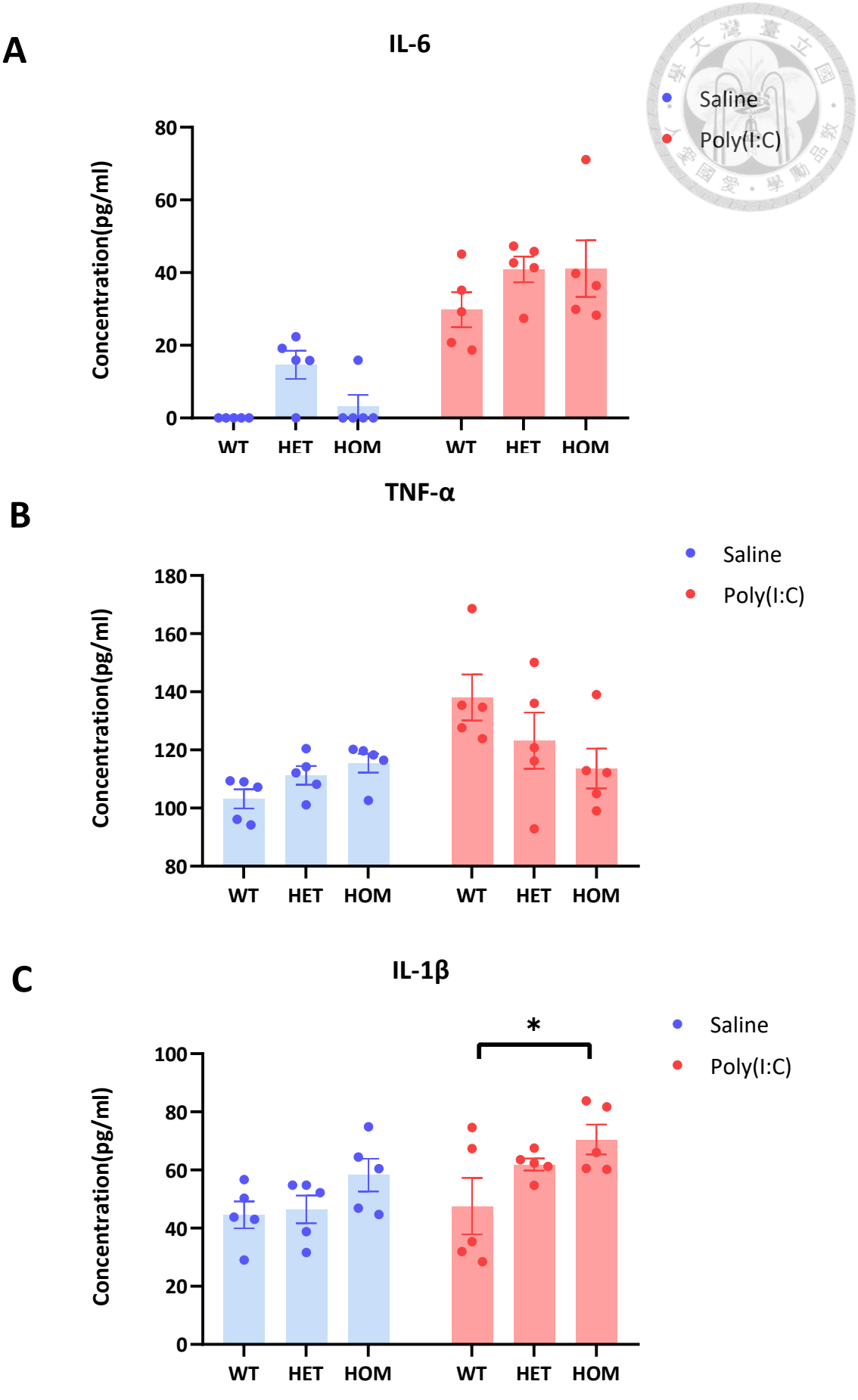

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Figure 3 Fetal brain cytokine responses in different genotype groups 6 hours after immune challenge by administration of poly(I:C) (5 mg/kg) on embryotic day 17.5 via the i.v. route

(A) A significant elevation of IL-6 levels was detected in the fetal brains of the poly(I:C)-treated group, with a main effect in genotypes. (B) A pronounced increase in TNF-α levels was observed in the fetal brains of the poly(I:C)-treated group, with a significant interaction between treatment and genotypes also being noted. (C) Elevated IL-1β levels in the fetal brains of the poly(I:C)-treated group were found to be statistically significant, with a main effect observed in genotypes. * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$, statistical significance based on two-way ANOVA. N = 15 in both the saline- and poly(I:C)-treated groups. All values are mean ± SEM.

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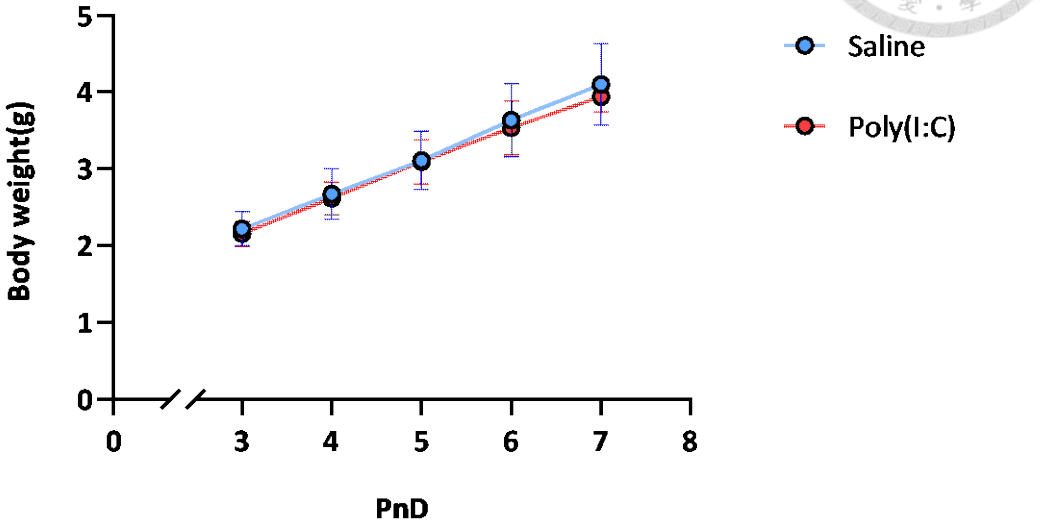
A



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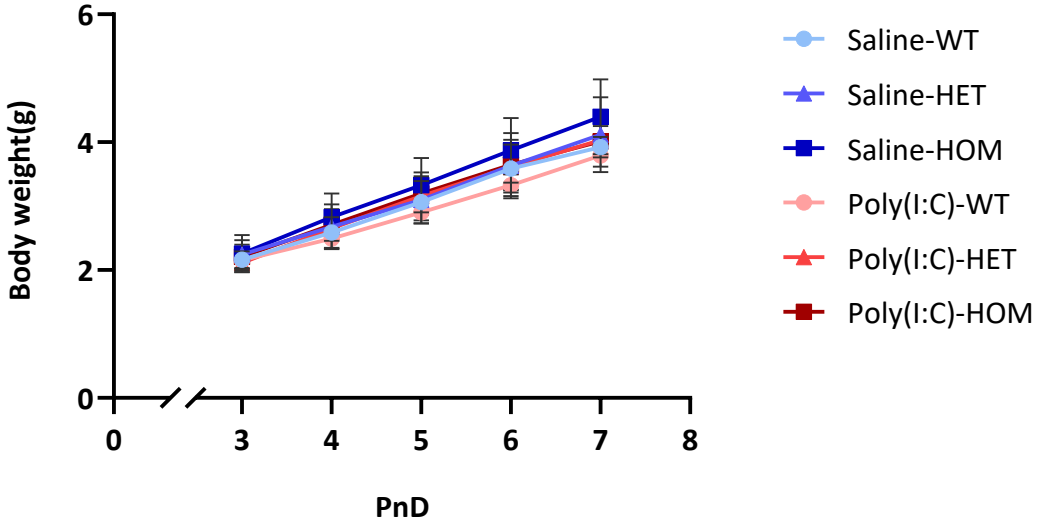

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Figure 4 Body weight of the neonatal mice in Exp.2.1

(A) In Experiment 2.1, there were no significant differences in neonatal mouse body weight observed among different genotypes and treatment groups. (B) There were no significant differences observed in poly(I:C) and saline groups, either. N = 8 in saline-WT group, N = 23 in saline-HET group, N = 15 in saline-HOM group, N = 11 in poly(I:C)-WT group, N = 14 in poly(I:C)-HET group and N = 11 in poly(I:C)-HOM group. N = 55 in saline group and N = 36 in poly(I:C) group. All values are mean ± SD.

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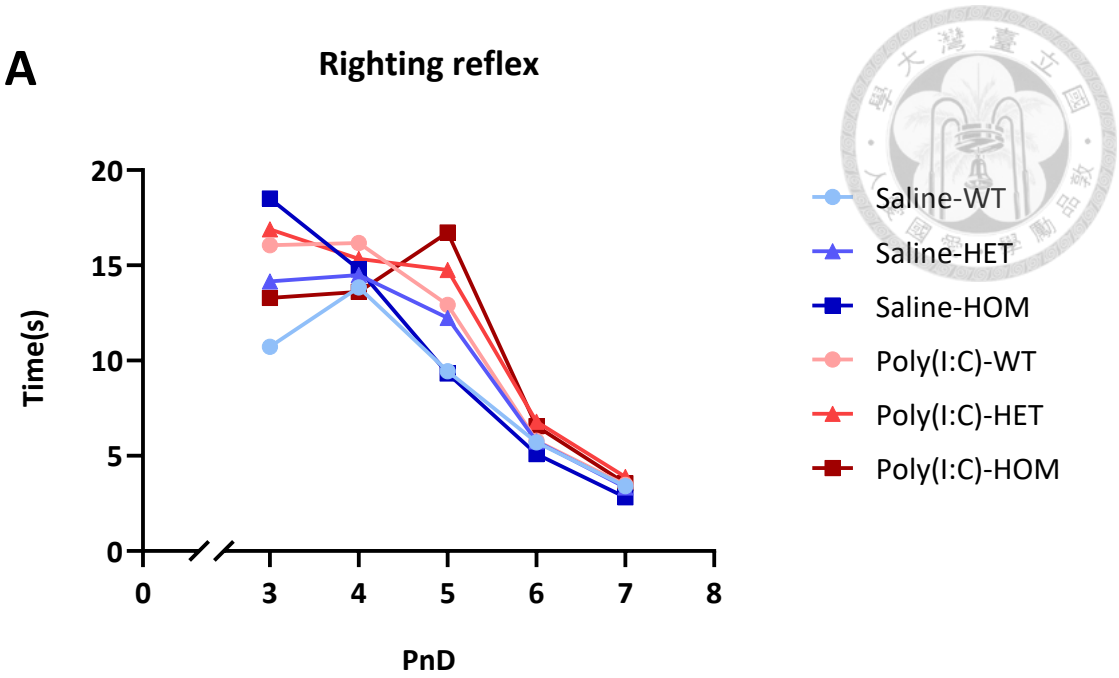

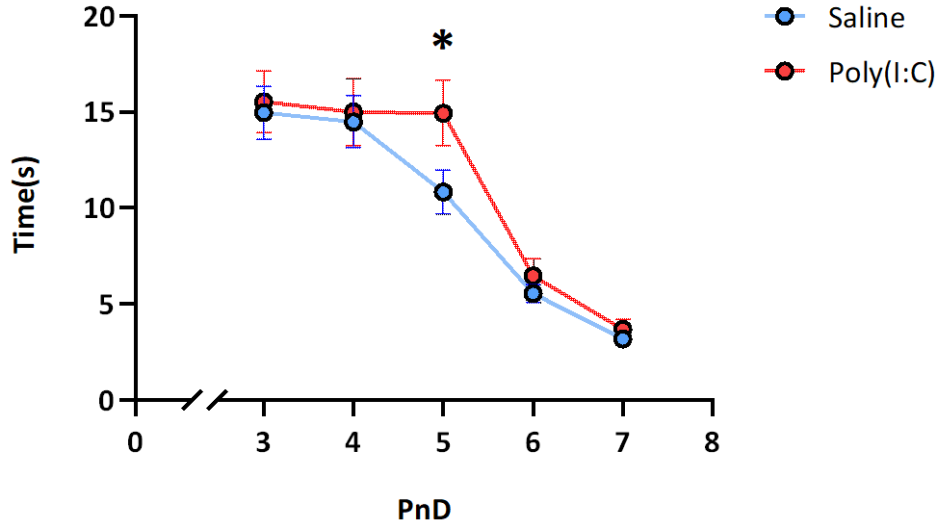

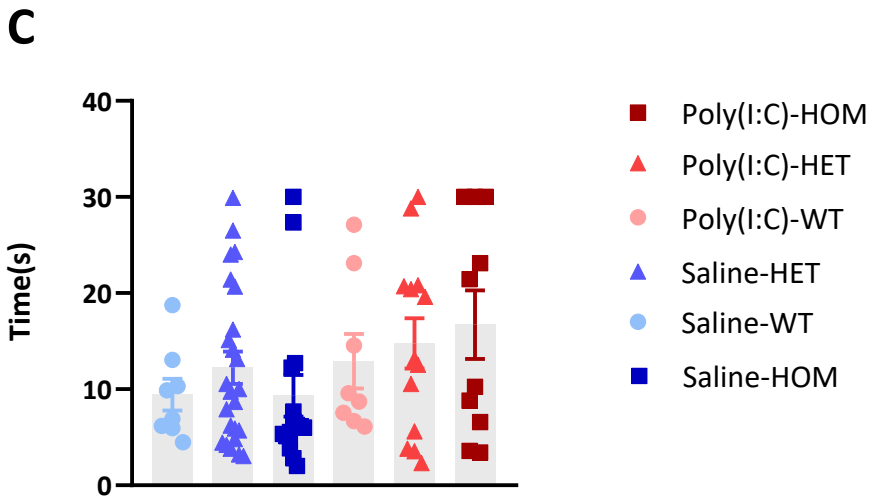

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Figure 5 Time taken for neonatal mice to achieve the righting reflex on postnatal days 3–7 in Exp.2.1

(A) There were no significant differences in the performance of the righting reflex in neonatal mouse observed among different genotypes and treatment groups. (B) Prenatal poly(I:C) injection significantly delayed the development of the righting reflex on postnatal day 5, regardless of the genotypes in neonatal mice. (C) Data distribution of groups in different treatment and genotypes on postnatal day 5. * = $p < 0.05$, statistical significance based on unpaired t test. N = 8 in saline-WT group, N = 23 in saline-HET group, N = 15 in saline-HOM group, N = 11 in poly(I:C)-WT group, N = 14 in poly(I:C)-HET group and N = 11 in poly(I:C)-HOM group. N = 55 in saline group and N = 36 in poly(I:C) group. All values are mean ± SEM.

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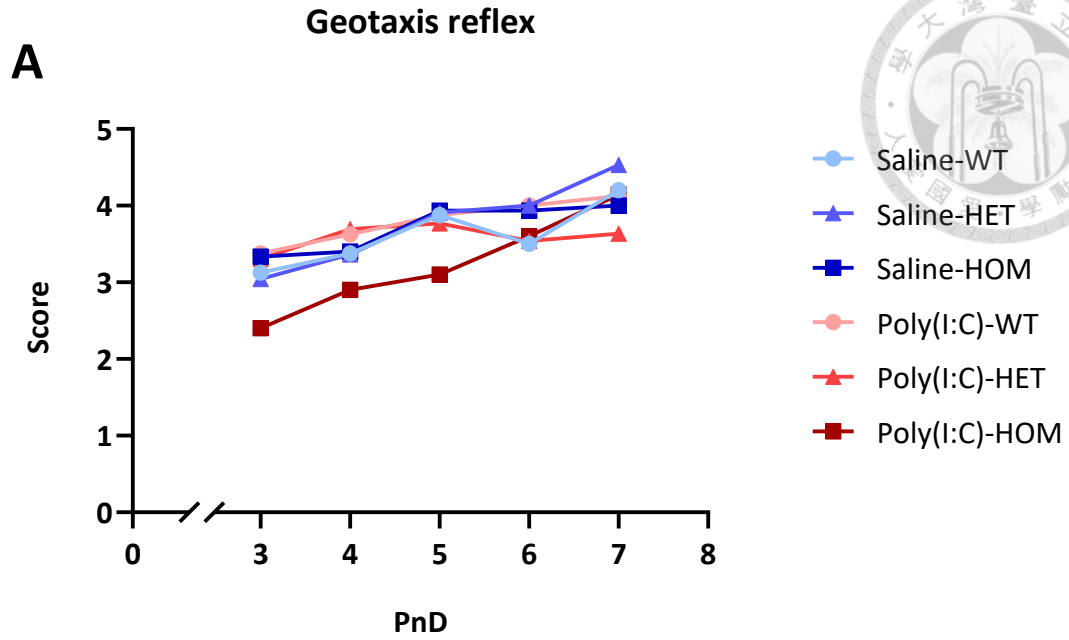

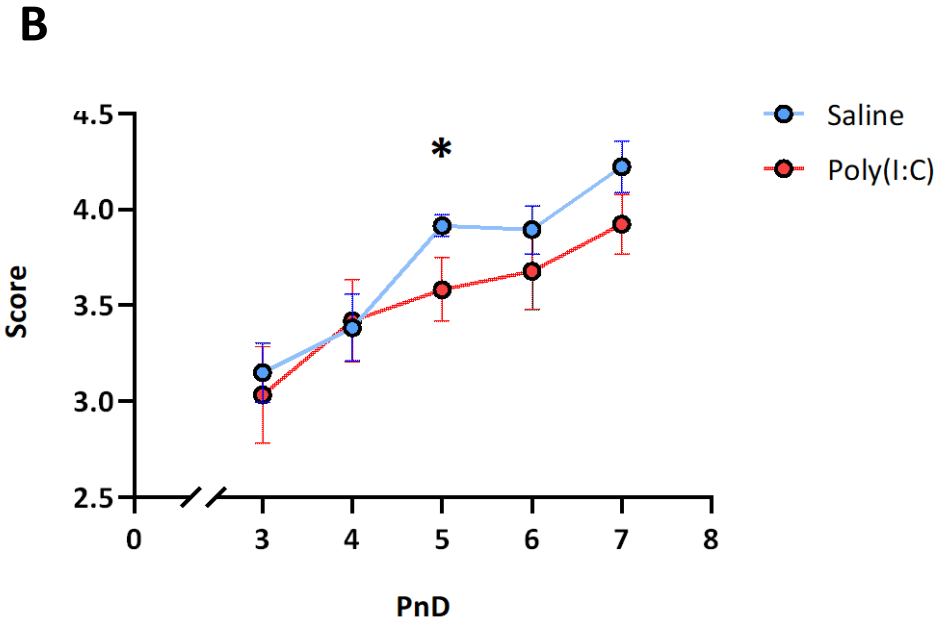

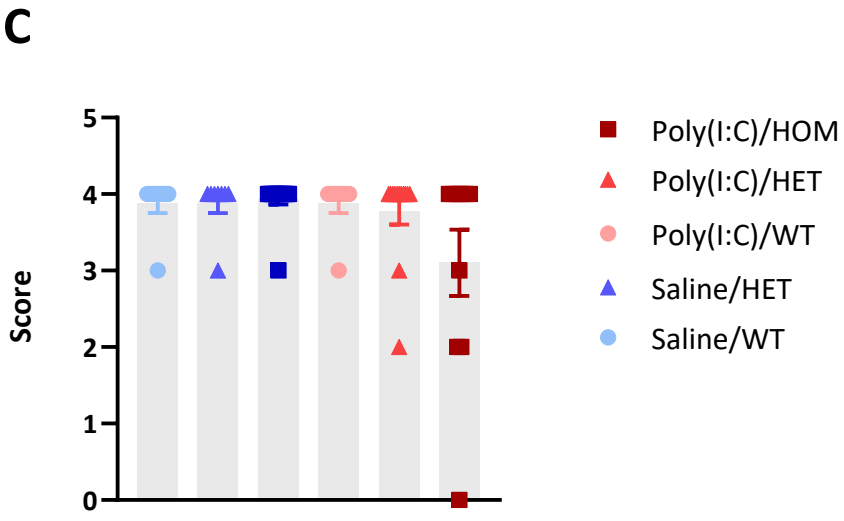

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Figure 6 Five-point scoring assessment for neonatal mice to achieve the geotaxis reflex on postnatal days 3–7 in Exp.2.1

(A) There were no significant differences in the performance of the geotaxis reflex in neonatal mouse observed among different genotypes and treatment groups. (B) Prenatal poly(I:C) injection significantly delayed the development of the geotaxis reflex on postnatal day 5, regardless of the genotypes in neonatal mice. (C) Data distribution of groups in different treatment and genotypes on postnatal day 5. * = $p < 0.05$, statistical significance based on unpaired t test. N = 8 in saline-WT group, N = 23 in saline-HET group, N = 15 in saline-HOM group, N = 11 in poly(I:C)-WT group, N = 14 in poly(I:C)-HET group and N = 11 in poly(I:C)-HOM group. N = 55 in saline group and N = 36 in poly(I:C) group. All values are mean ± SEM.

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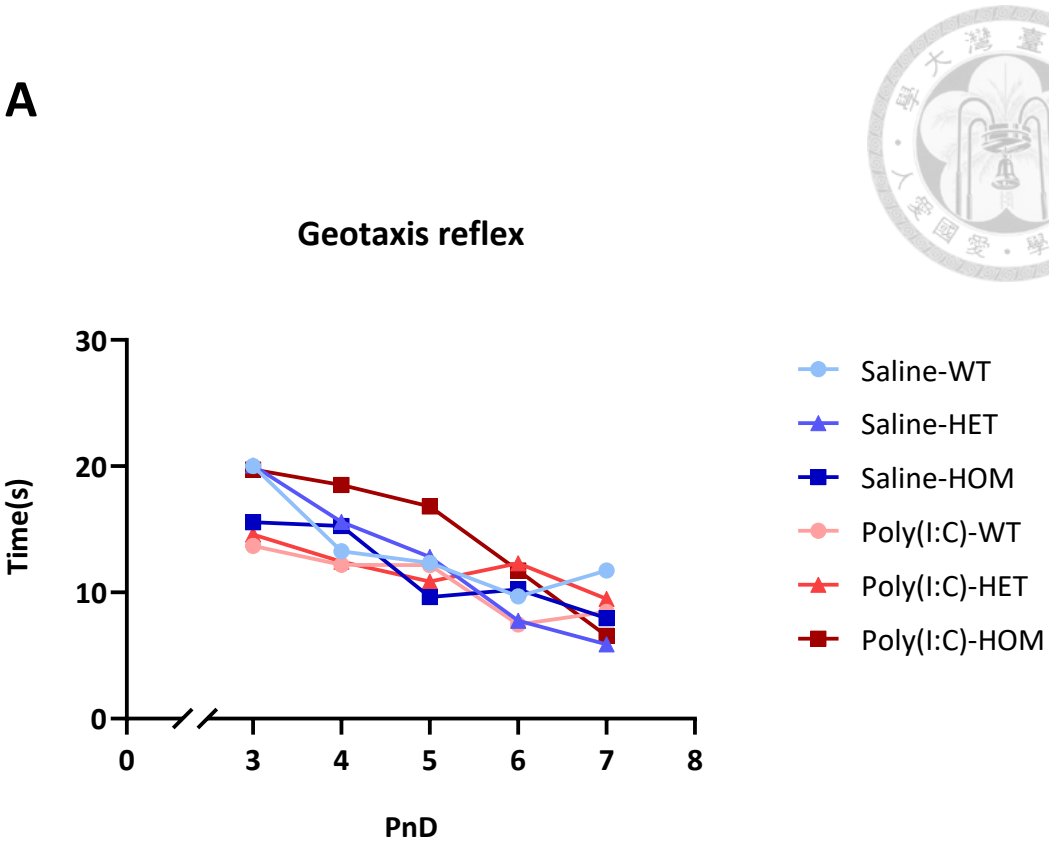

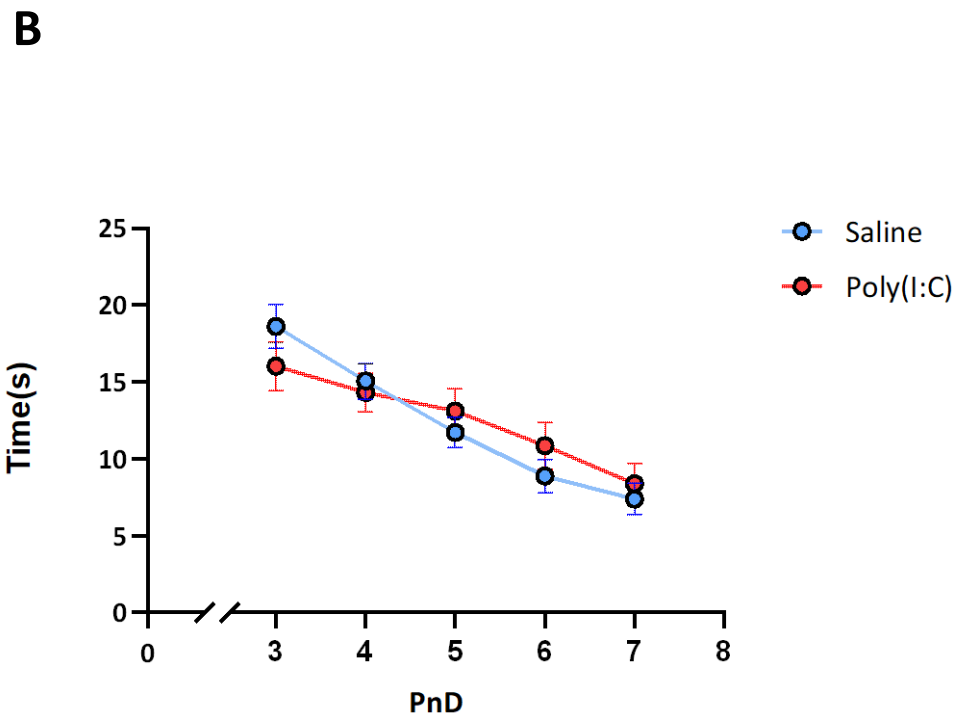

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Figure 7 Time taken for neonatal mice to achieve the geotaxis reflex on postnatal days 3–7 in Exp.2.1.

(A) There were no significant differences in the performance of the geotaxis reflex in neonatal mouse observed among different genotypes and treatment groups. (B) There were no significant differences observed in poly(I:C) and saline groups, either. N = 8 in saline-WT group, N = 23 in saline-HET group, N = 15 in saline-HOM group, N = 11 in poly(I:C)-WT group, N = 14 in poly(I:C)-HET group and N = 11 in poly(I:C)-HOM group. N = 55 in saline group and N = 36 in poly(I:C) group. All values are mean ± SEM.

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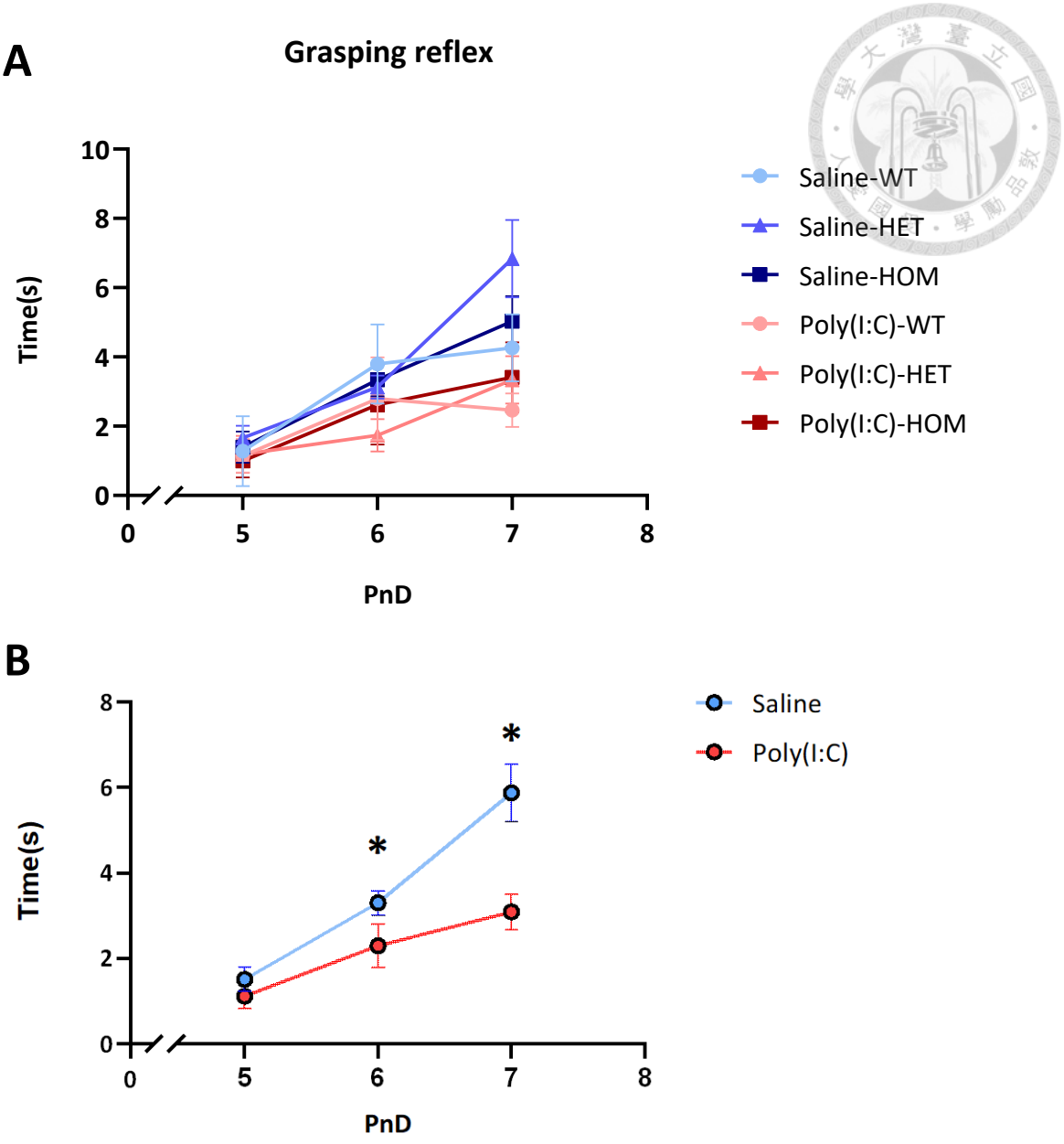

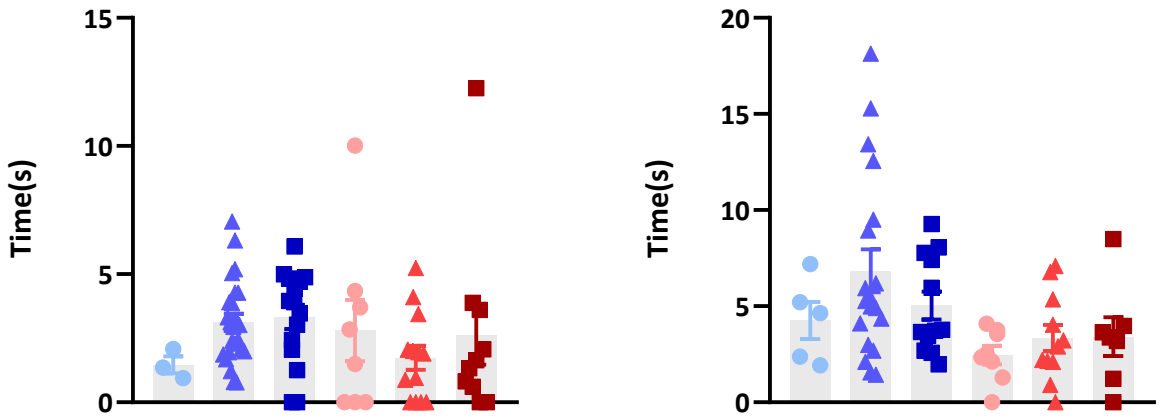

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Figure 8 . Time accumulated for neonatal mice to hold the bar in the grasping reflex test on postnatal days 5–7 in Exp. 2.1.

(A) There were no significant differences in the performance of the grasping reflex in neonatal mouse observed among different genotypes and treatment groups. (B) Prenatal poly(I:C) injection significantly delayed the development of the grasping reflex on postnatal day 6 and 7, regardless of the genotypes in neonatal mice. (C) Data distribution of groups in different treatment and genotypes on postnatal day 6. (D) Data distribution of groups in different treatment and genotypes on postnatal day 7. * = $p < 0.05$, statistical significance based on unpaired t test. N = 8 in saline-WT group, N = 23 in saline-HET group, N = 15 in saline-HOM group, N = 11 in poly(I:C)-WT group, N = 14 in poly(I:C)-HET group and N = 11 in poly(I:C)-HOM group. N = 55 in saline group and N = 36 in poly(I:C) group. All values are mean ± SEM.

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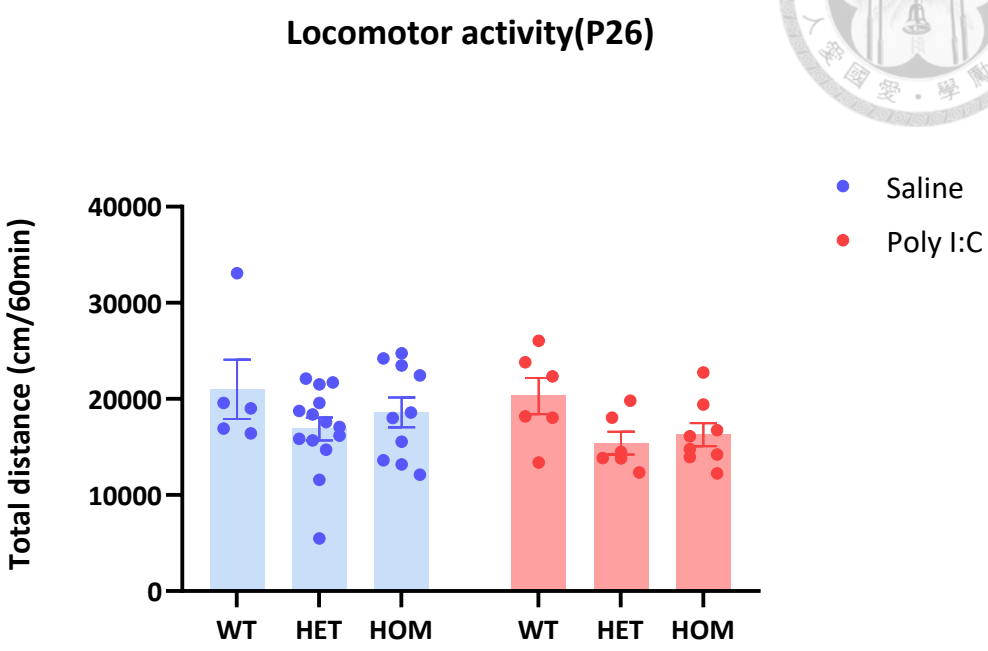

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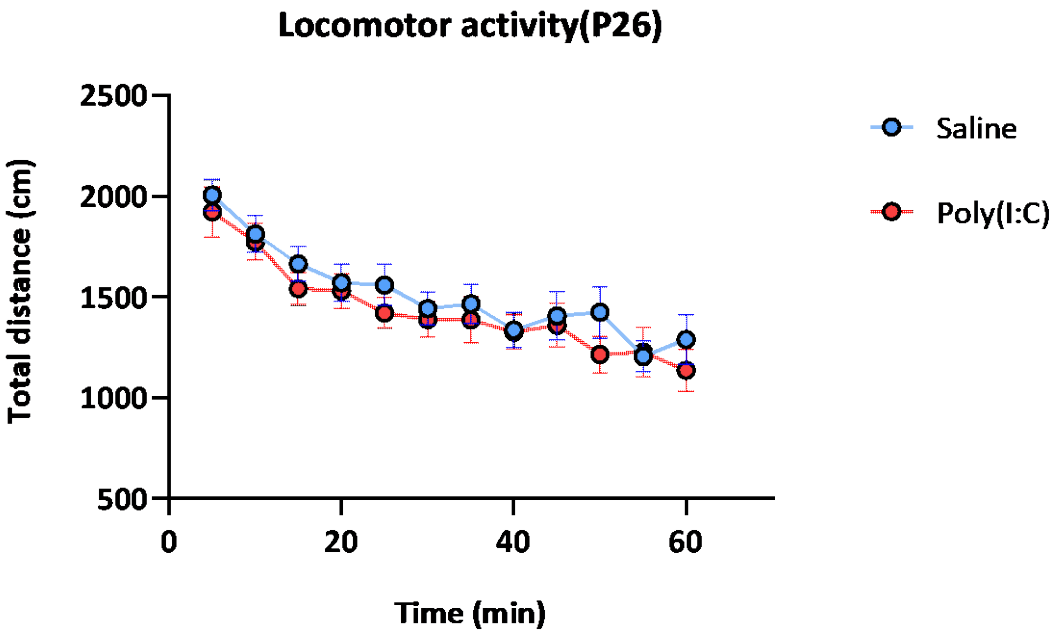

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Figure 9 Evaluation of locomotor activity through an open-field test (OFT) on postnatal day 26 in Exp. 2.1.

(A) There were no significant differences nor interaction in the total traveled distance among different genotypes and treatment groups. (B) Traveled distance in 5-min time bins didn't differ between adolescent mice in saline- or poly(I:C)-treated groups. N = 5 in saline-WT group, N = 14 in saline-HET group, N = 10 in saline-HOM group, N = 6 in poly(I:C)-WT group, N = 6 in poly(I:C)-HET group and N = 8 in poly(I:C)-HOM group. N = 29 in saline group and N = 20 in poly(I:C) group. All values are mean ± SEM.

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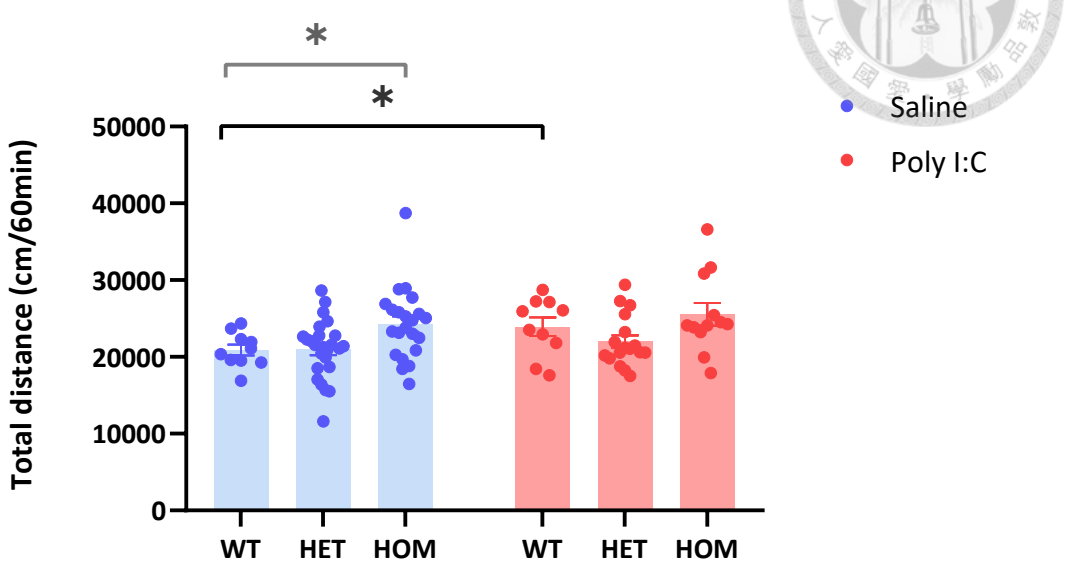

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Figure 10 Evaluation of locomotor activity through an open-field test (OFT) in Exp. 2.2.

Hyper-locomotor activity was found in poly(I:C)-WT and saline-HOM groups. Significant main effects of treatment and genotype (HOM>HET) were found. * = $p <$ 0.05, statistical significance based on unpaired t test, two-way ANOVA and Holm-Sidak 's multiple comparisons test. N = 10 in saline-WT group, N = 25 in saline-HET group, N = 22 in saline-HOM group, N = 10 in poly(I:C)-WT group, N = 17 in poly(I:C)-HET group and N = 12 in poly(I:C)-HOM group. All values are mean ± SEM.

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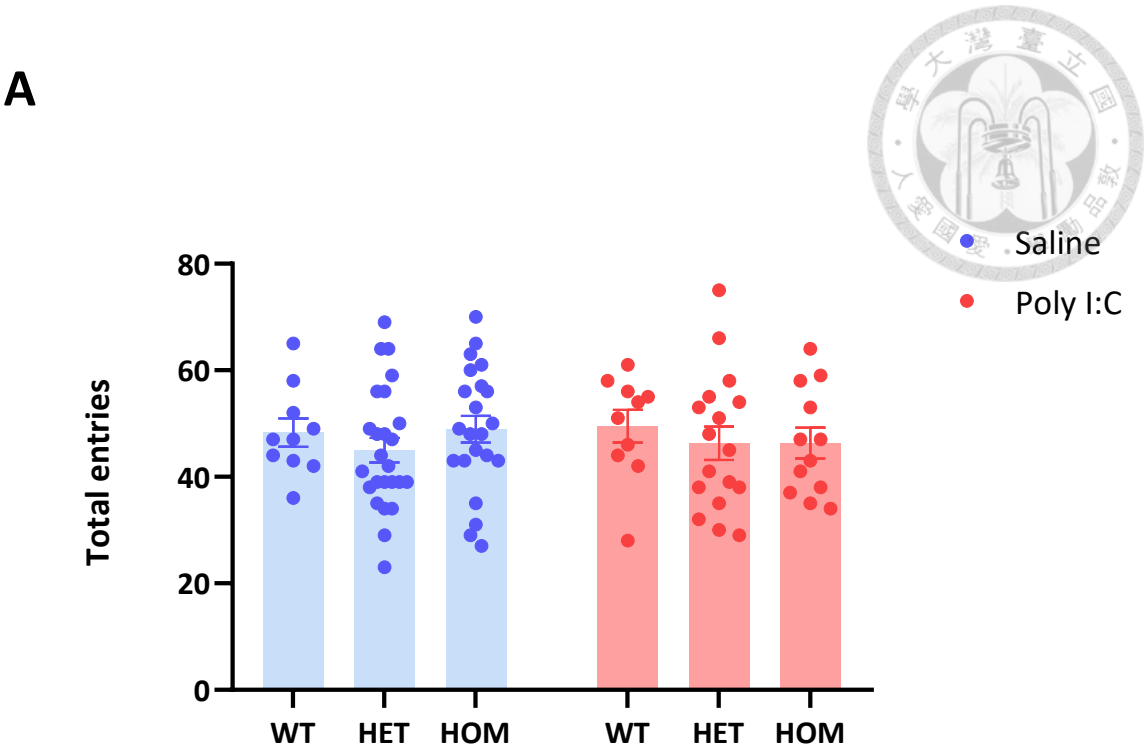

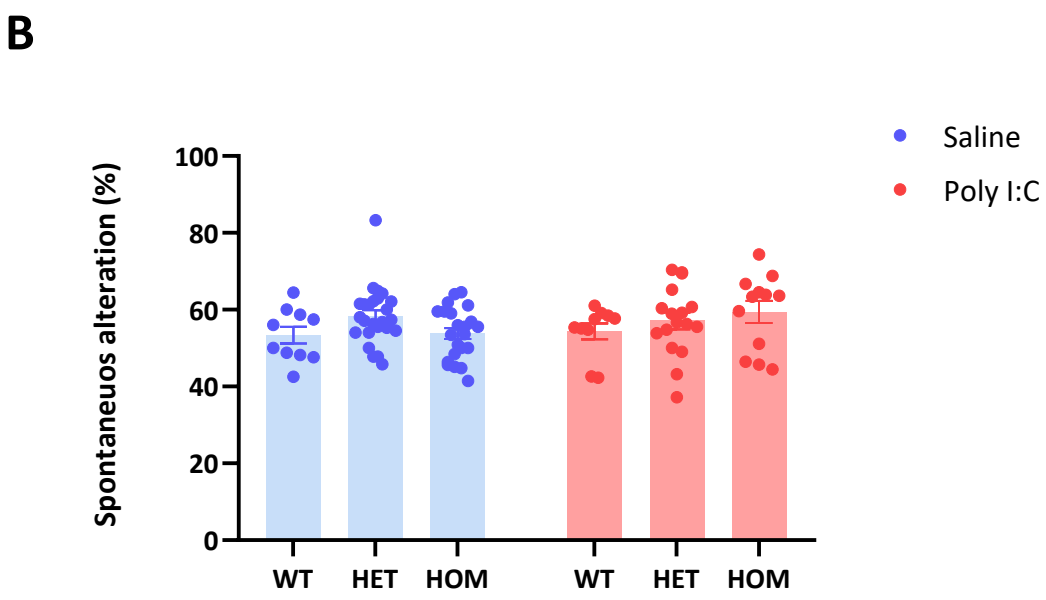

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Figure 11 Evaluation of working memory through a spontaneous alteration test utilizing the Y-maze in Exp. 2.2.

(A) Total entries did not show significant differences or interactions among various genotypes and treatment groups. (B) Spontaneous alteration rate (%) did not show significant differences or interactions among various genotypes and treatment groups. N = 10 in saline-WT group, N = 25 in saline-HET group, N = 22 in saline-HOM group, N = 10 in poly(I:C)-WT group, N = 17 in poly(I:C)-HET group and N = 12 in poly(I:C)-HOM group. All values are mean ± SEM.

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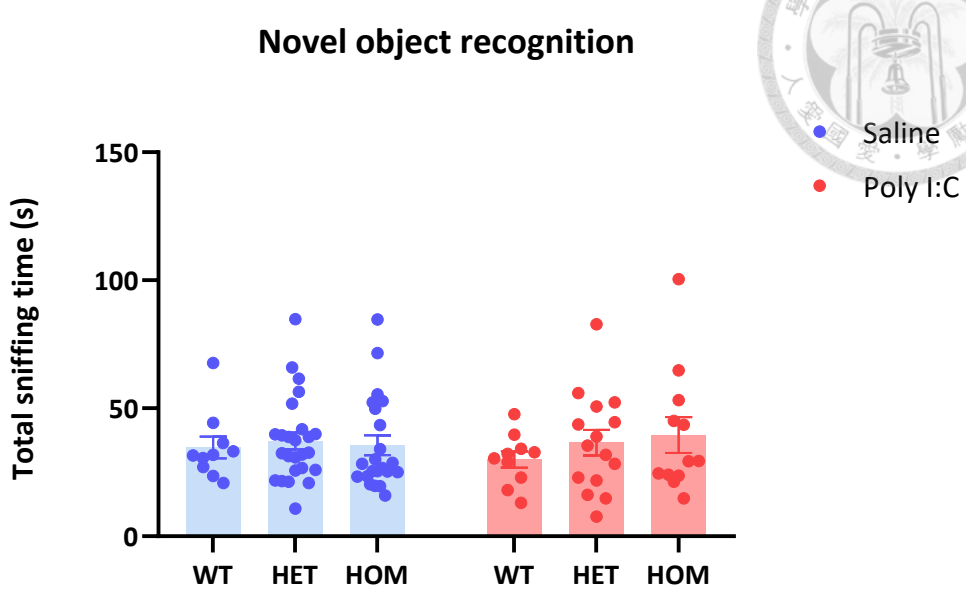

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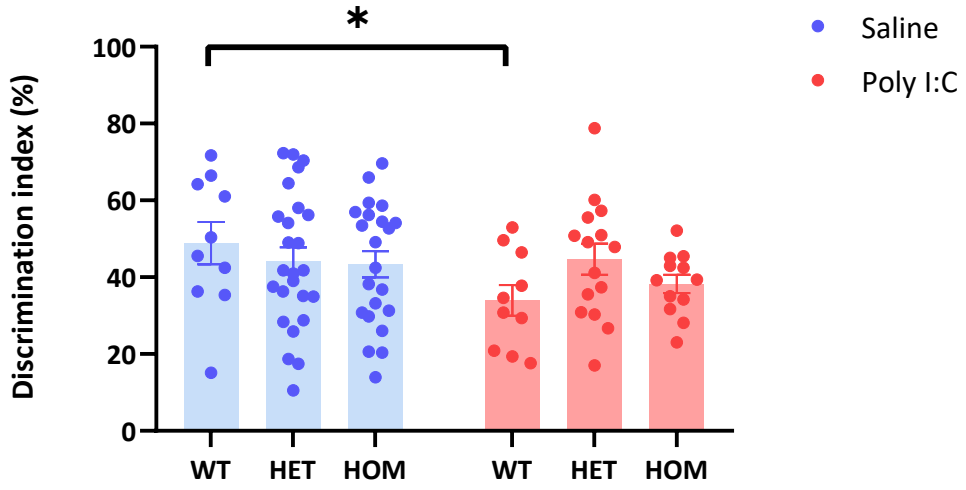

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Figure 12 Evaluation of long-term memory through a novel object recognition test (NORT) in Exp. 2.2.

(A) Total sniffing time calculated in the testing trial did not show significant differences or interactions among various genotypes and treatment groups. (B) Discrimination index (%) in poly(I:C)-WT group showed deficit in long-term memory. * = $p < 0.05$, statistical significance based on unpaired t test. N = 10 in saline-WT group, N = 25 in saline-HET group, N = 22 in saline-HOM group, N = 10 in poly(I:C)-WT group, N = 17 in poly(I:C)-HET group and N = 12 in poly(I:C)-HOM group. All values are mean ± SEM.

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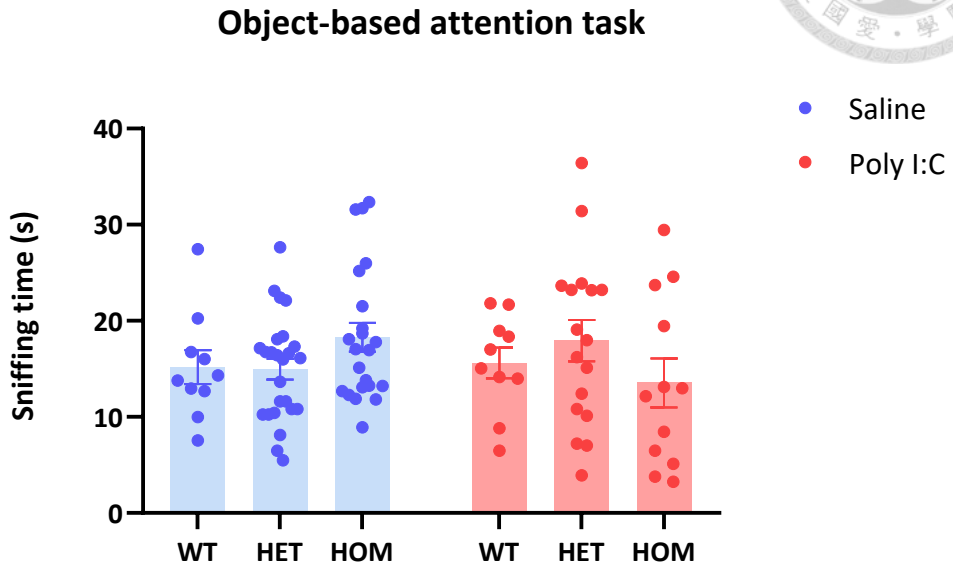

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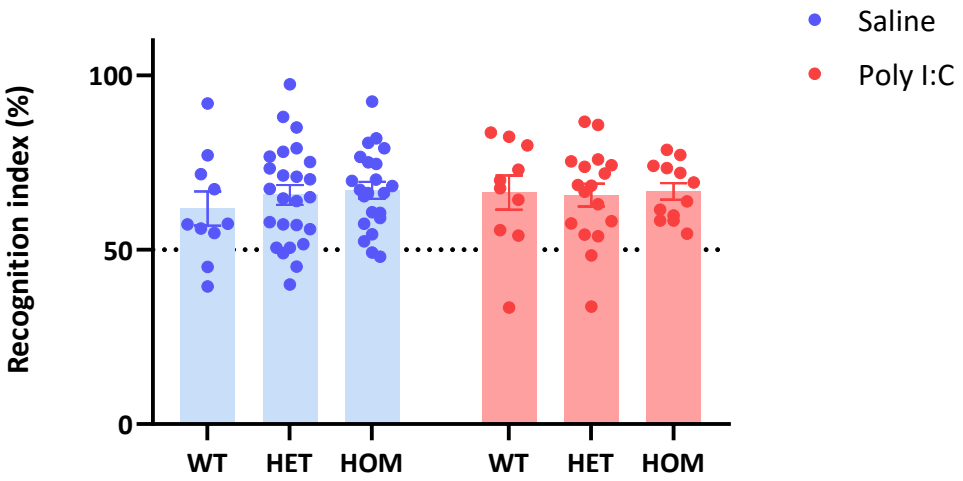

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Figure 13 Evaluation of attention through an object-based attention task (OBAT) in Exp. 2.2.

(A) Total sniffing time calculated in the testing trial did not show significant differences or interactions among various genotypes and treatment groups. (B) Recognition index (%) did not show significant differences or interactions among various genotypes and treatment groups. N = 10 in saline-WT group, N = 25 in saline-HET group, N = 22 in saline-HOM group, N = 10 in poly(I:C)-WT group, N = 17 in poly(I:C)-HET group and N = 12 in poly(I:C)-HOM group. All values are mean ± SEM.

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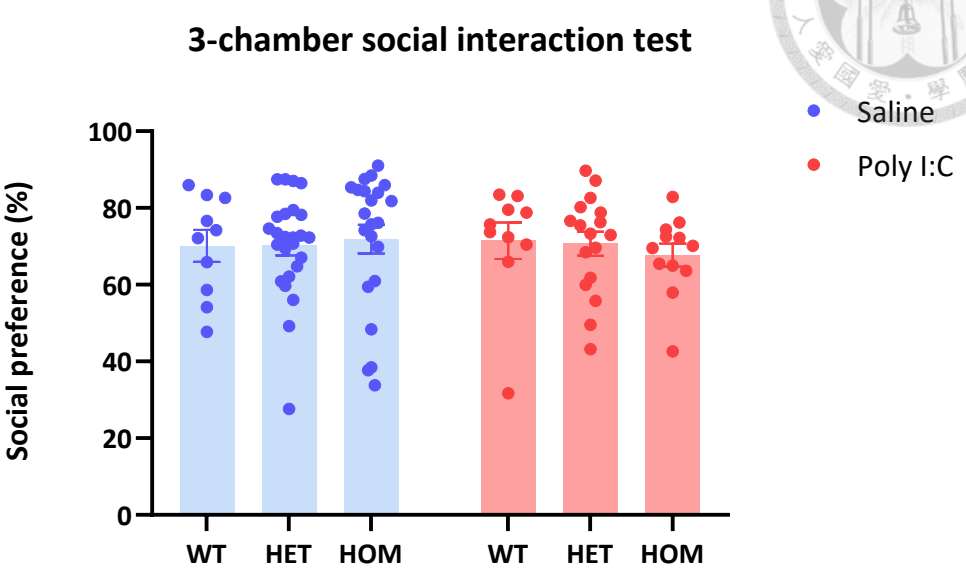

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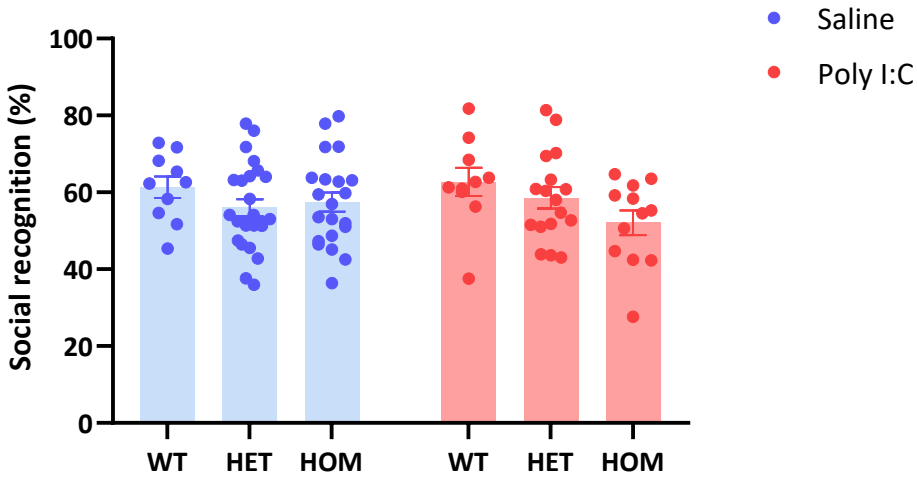

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Figure 14 Evaluation of sociability and social recognition through a three-chamber social interaction test in Exp. 2.2.

(A) Social preference rate (%) in the sociability test did not show significant differences or interactions among various genotypes and treatment groups. (B) Social recognition rate (%) in the social recognition test did not show significant differences or interactions among various genotypes and treatment groups. N = 10 in saline-WT group, N = 25 in saline-HET group, N = 22 in saline-HOM group, N = 10 in poly(I:C)-WT group, N = 17 in poly(I:C)-HET group and N = 12 in poly(I:C)-HOM group. All values are mean ± SEM.

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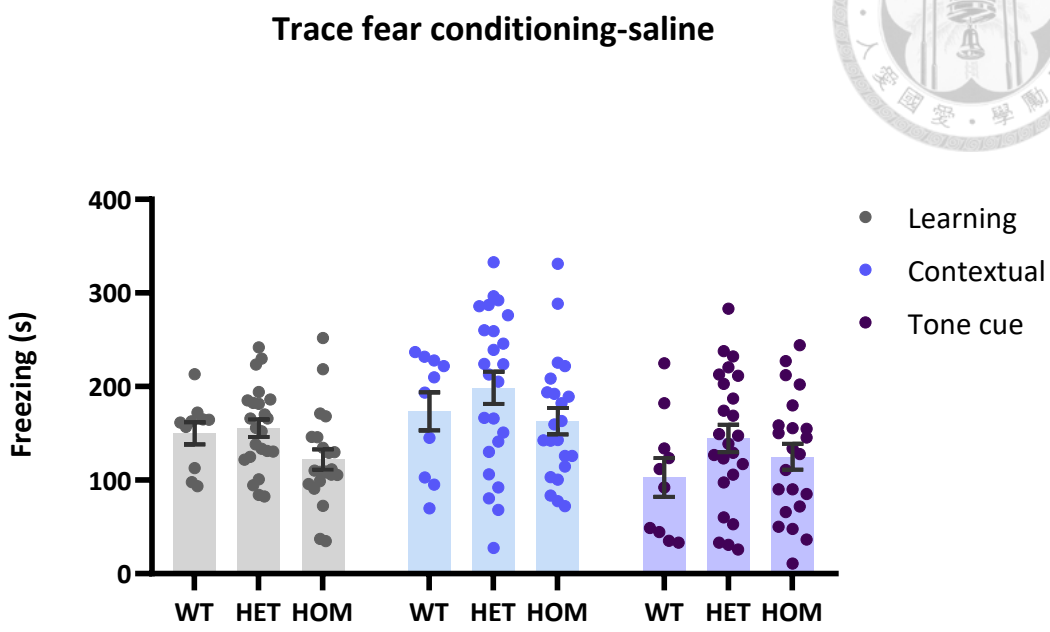

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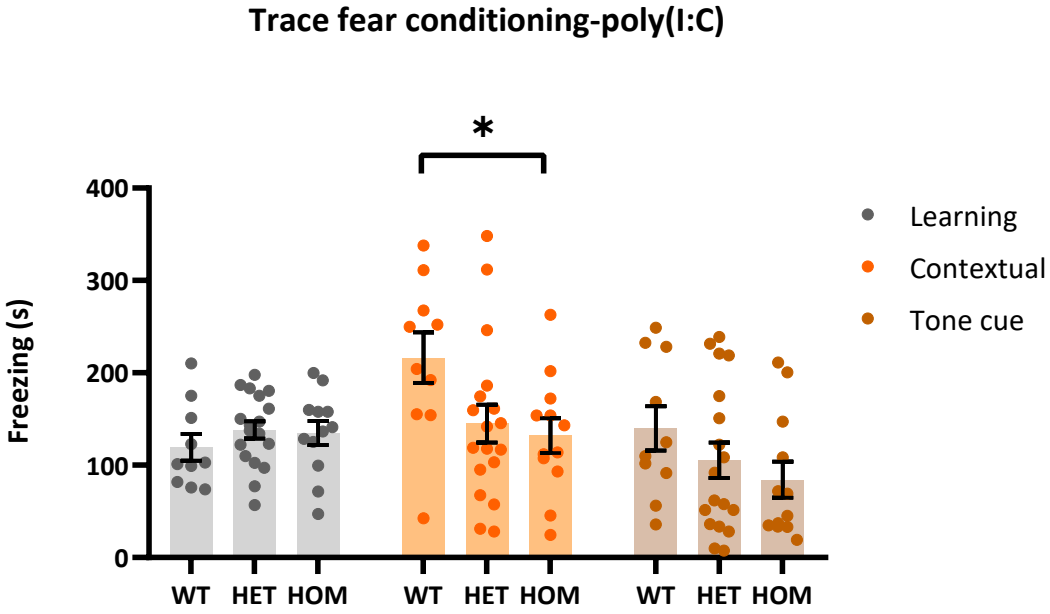

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Figure 15 Evaluation of associative learning and memory through a trace fear conditioning test in Exp. 2.2.

(B) In the saline group, there were no significant differences or interactions observed in freezing time, across the learning phase on day 1, contextual fear memory on day 2, and tone-cue fear memory on day 3. (B) In the poly(I:C)-HOM group, significant deficits in contextual fear memory was observed in decreased freezing time on day 2. There were no significant main effects or interactions observed in freezing time, across the learning phase on day 1, contextual fear memory on day 2, and tone-cue fear memory on day 3. *= $p < 0.05$, statistical significance based on unpaired t test. N = 10 in saline-WT group, N = 25 in saline-HET group, N = 22 in saline-HOM group, N = 10 in poly(I:C)-WT group, N = 17 in poly(I:C)-HET group and N = 12 in poly(I:C)-HOM group. All values are mean ± SEM.

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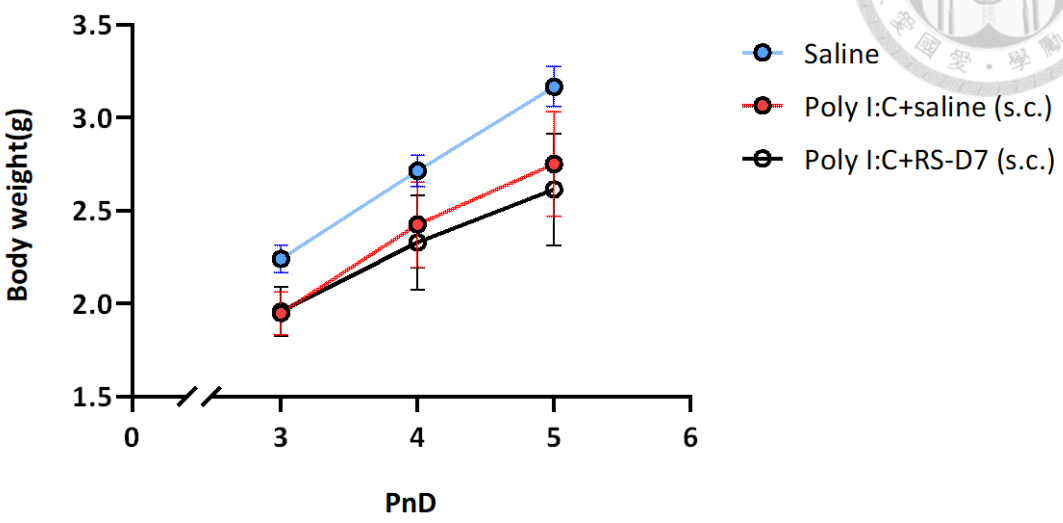

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Figure 16 Body weight of the neonatal mice in Exp. 3

In the poly(I:C) group, neonatal mice received daily subcutaneous injections of either saline or RS-D7 (40 mg/kg) on postnatal days 3 through 5. In the saline group, no treatments were administered to the mice. N = 15 in saline group, N = 8 in poly(I:C) +saline group and N = 7 in poly(I:C) +RS-D7 group. All values are mean ± SEM.

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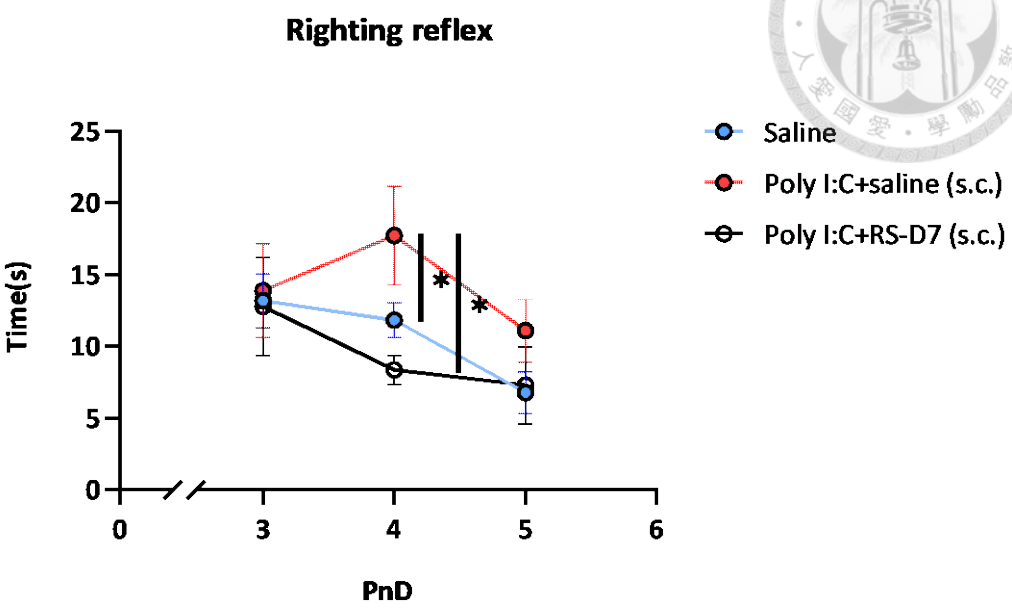

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Figure 17 Time taken for neonatal mice to achieve the righting reflex on postnatal days 3–5 in Exp. 3

The prenatal poly(I:C) injection exhibited a marginally significant effect on the delay of righting reflex development on postnatal day 4. However, daily subcutaneous injections of RS-D7 (40 mg/kg) significantly enhanced performance when compared to the poly(I:C) + saline group on postnatal day 4. * = $p < 0.05$, statistical significance based on unpaired t test. N = 15 in saline group, N = 8 in poly(I:C) + saline group and N = 7 in poly(I:C) + RS-D7 group. All values are mean ± SEM.

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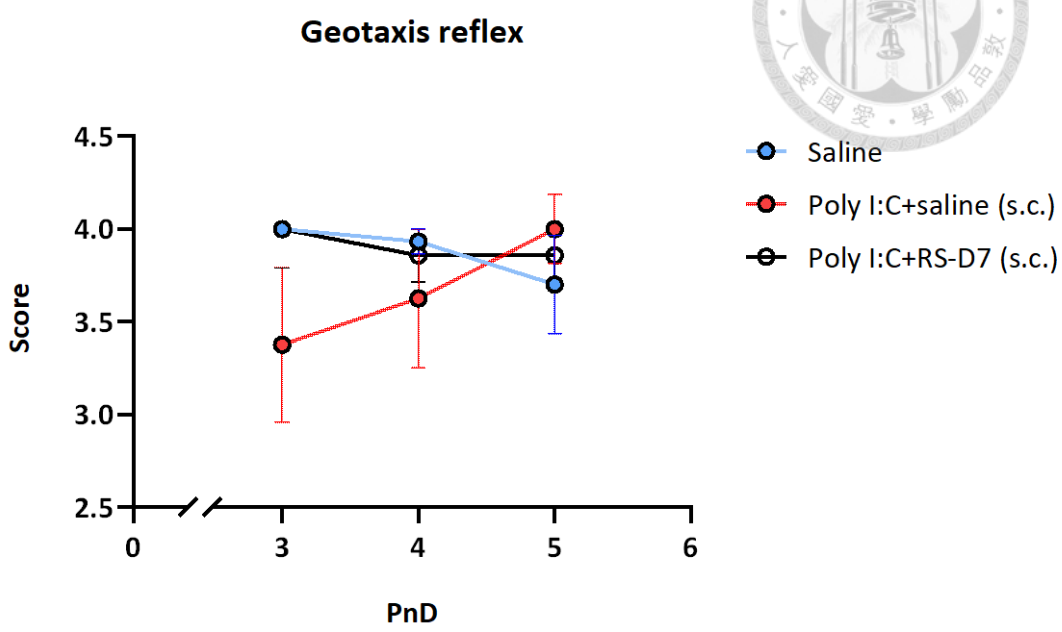

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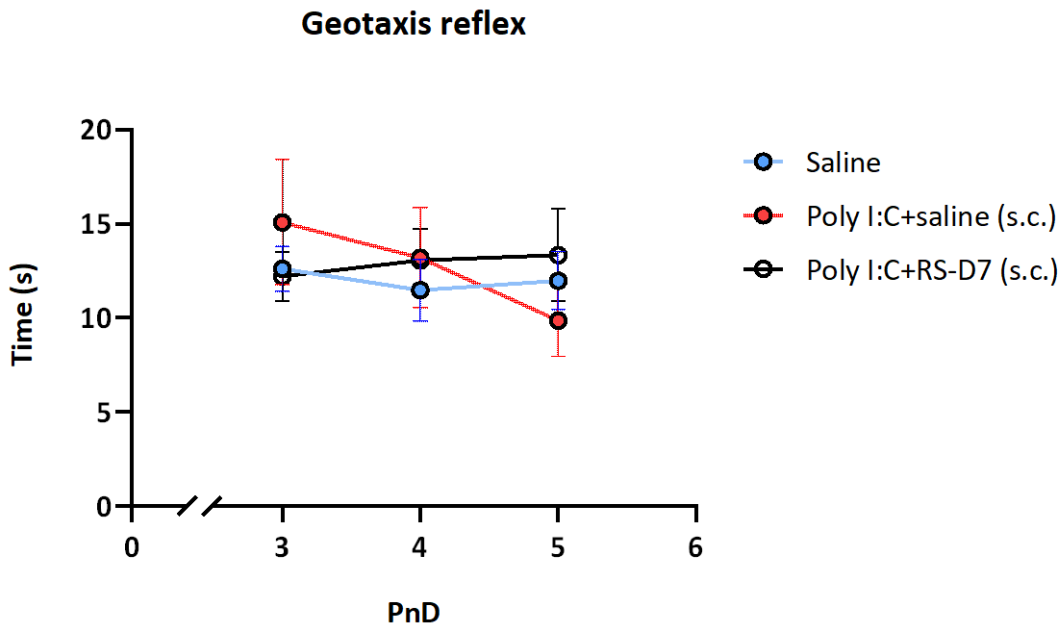

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Figure 18 Performance of the neonatal mice in the geotaxis reflex on postnatal days 3–5 in Exp. 3

(A) Five-point scoring system for neonatal mice assessing geotaxis reflex development on postnatal days 3–5. Prenatal poly(I:C) injection significantly prolonged the emergence of the geotaxis reflex on postnatal day 3 when compared to the saline-injected group. However, daily subcutaneous administrations of RS-D7 (40 mg/kg) significantly restored performance levels comparable to those observed in the saline group. (B) Time taken for neonatal mice to achieve the geotaxis reflex on postnatal days 3–5. There were no significant differences in the performance of the geotaxis reflex in neonatal mouse observed among different treatment groups. * = $p < 0.05$, statistical significance based on unpaired t test. N = 15 in saline group, N = 8 in poly(I:C) + saline group and N = 7 in poly(I:C) + RS-D7 group. All values are mean ± SEM.

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