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Establishment and Characterization of an Environmental Two-Hit Mouse Model Involving Prenatal Immune Activation and Stress in Puberty

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Contents

List of Abbreviations

5-HT	serotonin
AA	active avoidance
aCG	anterior cingulate
AMPH	amphetamine
ANOVA	analysis of variance
AKT	protein kinase B
AU	arbitrary units
CA	cornu ammonis
cAPM	cyclic adenosine monophosphate
CD	cluster of differentiation
CNS	central nervous system
CON	prenatal control animals
CORT	corticosterone
CPu	caudate putamen
CS	condition stimulus
CR	conditioned response
D2R	dopamine D2-like receptor
DA	dopamine
dCA	dorsal cornu ammonis
dDG	dorsal dentate gyrus
DF	degree of freedom
DG	dentate gyrus
DISC1	disrupted-in-schizophrenia-1
dsRNA	double stranded RNA
EDTA	ethylenediaminetetraacetic acid
EPM	elevated plus maze
GABA	gamma-amino-butyric acid
GD	gestation day
GFAP	glial fibrillary acidic protein
GSK β	glycogen synthase kinase 3 beta
HPA	hypothalamic-pituitary-adrenal axis
HPC	hippocampus
Iba1	ionized calcium-binding adapter molecule
IL	infralimbic
i.p.	intraperitoneal
i.v.	intravenous
LI	latent inhibition
LSD	least significant difference
MC	motor cortex
MIA	maternal immune activation
MINO	minocycline
mPFC	medial prefrontal cortex
MK-801	dizocilpine
NAc	nuccleus accumbens
NaCl	natrium cloride
NMDA	N-metyl-D-aspartate
NPE	Non-pre-exposed

PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFC	prefrontal cortex
PGE2	Prostaglandin E2
PND	postnatal day
POL	prenatal poly(I:C) treated animals
Poly(I:C)	polyriboinosinic-polyribocytidilic acid
PPI	prepulse inhibition
PrL	prelimbic
PV	parvalbumin
RELN	reelin
RNA	ribonucleic acid
S100B	S100 calcium binding protein B
S+	sub-chronic stress exposure
S-	no stress exposure
Sal	Saline
STR	striatum
TNF	tumor necrosis factor
US	unconditioned stimulus
vCA	ventral cornu ammonis
vDG	ventral dentate gyrus
VEH	vehicle

Summary

The etiology of chronic neuropsychiatric disorders with neurodevelopmental components likely involves multi-factorial pathological processes. Despite the advances in human epidemiological research, however, the extent to which specific genetic and environmental factors can interact with each other and cause chronic brain pathology remains largely elusive. Creating a model system that allows the comparison of single versus combined exposures to environmental (or genetic) factors is therefore highly warranted. In the present thesis, we aimed at developing such a model system by combining a model of prenatal immune challenge with a model of sub-chronic peri-pubertal stress exposure in mice. Each of these environmental factors has been implicated in the etiology of developmental psychiatric disorders, including schizophrenia, bipolar disorder, and autism, but the cumulative impact of combined exposure to these insults remain unexplored thus far.

The experimental series presented in this thesis were designed to examine the behavioral, neurochemical and immunological consequences of combined prenatal immune challenge and exposure to peri-pubertal sub-chronic stress in mice. For this purpose, a low dose of poly(I:C) (1 mg/kg, i.v.) was used to mimic physiologically relevant cytokine elevations during mid-pregnancy (gestation day 9), and a model of sub-chronic variable stress was applied to induce psychological stress across peri-pubertal development (see supplementary figure1, chapter 2).

We first compared the effects of single versus combined exposure to prenatal immune challenge and peri-pubertal stress on adult brain and behavioral pathology (chapter 2 and 3). Multiple phenotypic assays were used in the characterization of the long-term behavioral consequences of the two environmental hits. This first series of experiments revealed for the first time that the two environmental factors synergistically interact in the development of adult behavioral and neurochemical abnormalities, some of which are relevant especially for psychotic disorders such as schizophrenia (chapter 2). We further examined whether the precise timing, during which the second environmental hit (stress exposure) is applied, would have an impact on the nature of interactions between prenatal immune activation and postnatal stress.

To address this, we exposed prenatally immune-challenged and control offspring to sub-chronic stress at a later maturational period, namely late adolescence, and then again compared the single and combined effects of prenatal immune activation and adolescent stress on behavioral functions in adulthood. We found that exposure to stress during such later maturational periods failed to interact with prenatal immune activation with respect to the induction of key behavioral phenotypes, suggesting that the postnatal timing of stress exposure is crucial for such interactions to form (chapter 2). To study the onset of behavioral changes in our two-hit model, we conducted additional behavioral tests in peri-pubertal offspring shortly after exposure to the last stressor. The findings from these analyses suggest that many of the functional abnormalities show a delayed onset and thus only appear in adulthood (chapter 2).

The identification of key mechanisms that mediate the pathological interactions between prenatal immune challenge and peri-pubertal stress seems of high relevance as this would open ways for exploring and developing preventive therapeutic strategies. Thereby, we focused on possible convergence points between the immune- and stress-related manipulations. First, we assessed the consequences of the developmental exposures on basal and stress-induced secretion of corticosterone, which is one of the main effector hormones secreted by the adrenal cortex in response to activation of the hypothalamus-pituitary-adrenal (HPA) - axis. These endocrine investigations suggested that the synergistic interactions on brain and behavioral functions are unlikely to be explained by prenatally acquired changes in stress-induced corticosterone release (Chapter 2). On the other hand, our analyses of microglia and inflammatory markers revealed that prenatal immune activation strongly enhances the offspring's vulnerability to neuroinflammatory changes in response to peri-pubertal stress (chapter 2). Intriguingly, such neuroinflammatory abnormalities in stressed offspring of immune-challenged mothers appeared to be transient and only emerged during and shortly after exposure to stress.

Based on these latter findings, we went on to explore possible preventive interventions that would preferentially target the stress-induced activation of microglia and associated inflammatory processes in the maturing brain (chapter 4). For this purpose, the animals were treated with minocycline, a tetracycline with known

neuroprotective and immunosuppressive functions, during the course of stress exposure (chapter 4). We found that minocycline was sufficient to block stress-induced microglia over-activation and inflammatory cytokine production in prenatally immune challenged animals. Moreover, the pharmacological intervention was found to be effective in preventing the emergence of behavioral abnormalities typically seen in adult offspring with the double-hit exposure (chapter 4).

Taken together, the studies performed in the present thesis highlight that exposure to two environmental insults, each of which has been implicated in the etiology of multi-factorial psychiatric disease, can synergistically interact in the development of adult brain pathology. The presented work also indicates a potentially important role of peri-pubertal neuroinflammation in mediating these pathological interactions, opening avenues for future studies aiming at establishing preventive interventions that target abnormal inflammatory processes during critical stages of brain maturation.

Zusammenfassung

Die Ursachen von chronischen neuropsychiatrischen Erkrankungen mit einer vorangehender Entwicklungsstörung scheinen von verschiedenen pathologischen Prozessen abhängig zu sein. Trotz des Fortschrittes durch epidemiologische Studien ist es unklar, in welchem Ausmass solche spezifischen genetischen oder Umweltfaktoren interagieren und eine chronische Erkrankung des Gehirns auslösen können. Aus diesem Grund ist es wichtig, Modellsysteme zu entwickeln, die es zulassen, Umweltfaktoren oder genetische Einflüsse sowohl im Einzelnen als auch im Zusammenhang erforschen zu können. Die vorliegende Dissertation hatte zum Ziel, ein solches Modell zu entwickeln und zu etablieren. Dazu verbinden wir ein Modell für pränatale immunologische Stimulation mit einem Modell für postnatalem Stress. Beide Umwelteinflüsse wurden mit den Ursachen von psychiatrischen Erkrankungen wie Schizophrenie, Bipolare Störung oder Autismus assoziiert, der kumulative Einfluss solcher Umwelteinflüsse ist jedoch noch weitgehend unerforscht.

Die experimentellen Studien in der vorliegende Dissertation wurden für die Untersuchung der Auswirkungen einer pränataler Infektion und späterem Stress auf mögliche Verhaltensstörungen und auf neurochemische oder immunologische Veränderungen im Gehirn, entworfen.

Um während der Schwangerschaft des Muttertiers eine physiologisch relevante Zytokin-Erhöhung, ähnlich wie bei einer Viruserkrankung, auszulösen, wurde eine niedrige Dosierung von poly(I:C) (1 mg/kg i.v.) appliziert. Die Nachkommen dieser kurzzeitig erkrankten Muttertiere wurden dann während der Pubertät einem sub-chronischen variablen Stress, bestehend aus verschiedene psychologische Stressoren, ausgesetzt (siehe „supplementary figure 1“, Kapitel 2).

Als Erstes verglichen wir die Langzeit Folgen der einzelnen Belastungen mit den Folgen einer kombinierten Belastung auf die Gehirnentwicklung und Verhaltensveränderungen (Kapitel 2 und 3). Dabei wendeten wir verschiedenste Tests an, die eine breite Phänotypisierung der erwachsenen Nachkommen erlauben. Die ersten Testserien zeigten zum ersten Mal, dass verschiedene Umweltfaktoren

interagieren können und dabei eine Verhaltensveränderungen auslösen, die durchaus vergleichbar sind mit Aspekten psychischer Erkrankungen wie Schizophrenie (Kapitel 2). In weiteren Untersuchungen testeten wir, ob die präzise Zeitspanne, während der der Stress erfolgte, einen Einfluss auf die Entstehung der pathologischen Veränderungen hat. Dazu setzten wir die Nachkommen der immun-stimulierten Mütter zu einem späteren Zeitpunkt dem gleichen Stress aus und erforschten wiederum die Langzeit Folgen auf das Verhalten. Diese Tests ergaben, dass die Zeit während der Pubertät tatsächlich relevant war um in einer synergistischen Weise mit der pränatalen Vorgeschichte zusammen zu wirken (Kapitel 2). Um herauszufinden, ob die Verhaltensveränderungen bereits kurz nach dem Stress hervortreten, haben wir die Nachkommen direkt nach dem Stress auf Verhaltensveränderungen getestet. Diese Ergebnisse zeigten, dass die funktionellen Störungen erst im Erwachsenenalter, also verzögert, hervortreten (Kapitel 2).

Die Identifizierung von Schlüsselmechanismen, welche diese pathologischen Interaktionen verursachen, ist hochrelevant, da dieses Wissen neue Wege für präventive Massnahmen eröffnen. Dazu untersuchten wir als Erstes die Auswirkungen der beiden Manipulationen auf die basalen und stress-induzierten Ausschüttungen von Kortikosteron, dem Haupteffektor der Hypothalamus-Hypophyse-Nebennieren-Achse. Aufgrund dieser endokrinen Untersuchungen, sind die Langzeit Folgen nicht erklärbar durch eine direkte pränatal induzierte Veränderung der Stress-Antwort-Achse (Kapitel 2). Im Gegensatz dazu haben weitere Analysen von Mikrogliazellen und immunologischen Mediatoren gezeigt, dass eine pränatale Immunstimulation die Stressverwundbarkeit stark erhöht und es dabei zu einer neuroinflammatorischen Stressantwort kommt. Wichtig dabei ist, dass diese neuroinflammatorische Stressantwort nur vorübergehend, das heisst während und kurz nach dem Stress, erscheint (Kapitel 2).

Aufgrund dieser Ergebnisse haben wir mögliche präventive Massnahmen getestet, welche die stress-induzierte Aktivierung von Mikrogliazellen und die damit verbundene Erhöhung des Zytokinspiegels im zentralen Nervensystem anvisieren (Kapitel 4). Dazu behandelten wir die Mäuse während des Stresses mit Minozyklin, einem Tetrazyklin mit neuroprotektiven und immunsupressiven Wirkungen (Kapitel 4).

Diese Tests deuten darauf hin, dass Minozyklin ausreichte, um die stress-induzierte Immunaktivierung zu verhindern. Weiter hat sich gezeigt, dass diese präventive Massnahme auch gegen die Langzeitfolgen der beiden Umwelteinflüssen wirkte und die Verhaltensstörungen verhinderte (Kapitel 4).

Zusammenfassend zeigen die Studien der vorliegenden Dissertation, dass zwei Umwelteinflüsse, welche bei der Entstehung von komplexen psychiatrischen Krankheiten eine Rolle spielen, zusammenwirken können und dabei zur Entwicklung von Neuropathologien bei Erwachsenen beitragen. Die Arbeit zeigt weiter auf, dass dabei neuroinflammatorische Prozesse während der Pubertät eine wichtige Rolle spielen können. Dies eröffnet neue Wege für präventive Massnahmen durch Verhinderung von pathologischen inflammatorischen Prozessen während kritischen Stadien der Entwicklung.

Chapter 1

General Introduction

Chronic psychiatric diseases such as schizophrenia, bipolar disorder, and major depression typically emerge in late adolescence or early adulthood (reviewed in Paus et al., 2008), even though subtle preclinical symptoms can appear long before the full-blown onset (Hafner et al., 1999). According to one prevalent hypothesis (Weinberger 1987; Murray and Lewis, 1987), the etiology of such psychiatric disorders involves altered neurodevelopmental processes induced by the combination of genetic abnormalities and exposure to environmental adversities in early life. An important refinement of this neurodevelopmental hypothesis emphasis the contribution of altered brain maturation and the progressive brain changes associated with the emergence of major mental illnesses (Rapoport et al., 2005; Fatemi and Folsom, 2009).

Human genetic linkage studies and epidemiological research has identified a plethora of genetic and environmental factors that are associated with increased risk of developing chronic mental illnesses. In support of the neurodevelopmental hypothesis, many of these risk factors can influence neurodevelopmental and/or brain maturational processes (Cannon et al., 2003; Tandon et al., 2008). For example, a higher risk for schizophrenia was found in individuals with aberrant expression of *neuregulin-1* (NRG-1) (Stefansson et al., 2003) or *disrupted-in-schizophrenia-1* (DISC1) (Chubb et al., 2008), both of which are involved in fundamental molecular processes of neural development and plasticity (Harrison and Weinberger, 2005; Ross et al., 2006). Besides the genetic predisposition to mental illnesses, the influence of environmental factors has attained increasing interest in this context. With respect to the latter, various forms of environmental adversities have been associated with increased risk for chronic neuropsychiatric disorders, many of which operate at early stages of brain development or critical periods of brain maturation. These include maternal gestational exposure to infection and associated inflammatory processes, obstetric complications, traumatizing experiences in childhood, and chronic intake of drugs of abuse in adolescent life (Susser et al., 1996, reviewed in McDonald and Murray, 2000; Bresnahan et al., 2005; Dean and Murray, 2005; Brown and Derkits, 2010).

The Two-Hit Hypothesis of Psychiatric Disease: A Role for Infection and Stress During Development?

None of the individual genetic or environmental risk factors seem to be sufficient to cause the development of multi-symptomatic mental illnesses such as schizophrenia or bipolar disorder. Indeed, single risk factors of these brain disorders have rather modest effect sizes across large populations (Tsuang et al., 2004; Harrison and Weinberger, 2005; Ross et al., 2006). It has therefore been hypothesized that the combination of multiple risk factors is required for such mental illnesses to develop (Tandon et al., 2008; van Os and Kapur, 2009; van Os et al., 2010). One influential theory that accounts for the pathological actions of multiple risk factors is known as the "two-hit hypothesis" of chronic psychiatric disease (Keshavan, 1999; Keshavan and Hogarty, 1999, Bayer et al., 1999). Conceptually, this hypothesis suggests that a first hit in early prenatal life renders the brain more vulnerable to a second hit later in life (Keshavan, 1999; Keshavan and Hogarty, 1999, Bayer et al., 1999). Hence, a genetic or environmental factor affecting early prenatal brain development might lead to latent neuropathology, and its clinical manifestation would dependent on the presence of a second (environmental) hit in postnatal life (Keshavan, 1999; Keshavan and Hogarty, 1999, Bayer et al., 1999; Tsuang et al., 2004). Several human studies have already provided support for this theory by showing that the presence of certain genetic factors can modulate the effects of environmental insults in the development of complex brain disorders such as schizophrenia or bipolar disorder (reviewed in Tsuang et al., 2004; van Os et al., 2008). For example, individuals with a family history of schizophrenia are more likely to experience marked psychotic symptoms after stressful life events than individuals without a family history of psychosis (Miller et al., 2001).

One of the noticeable environmental factors operating during fetal development is maternal infection during pregnancy. Initial retrospective and subsequent prospective epidemiological studies have repeatedly found a significant association between maternal infection during pregnancy and increased risk of psychiatric disorders in the offspring, including schizophrenia, autism, and bipolar disorder (reviewed in Brown and Susser, 2002; Brown, 2006; Fatemi et al., 2005; Brown and Derkits 2010). The precise mechanisms by which such prenatal infections can increase neuropsychiatric disease

risk are not yet fully understood. Interestingly, this association does not seem to be restricted to a single viral or bacterial pathogen. Rather, it is seen following maternal exposure to various different infectious agents such as viral (Torrey et al., 1988; Brown et al., 2001; Brown et al., 2004) or bacterial pathogens (Sorensen et al., 2009), as well as the parasite toxoplasma gondii (Brown et al., 2005; Mortensen et al., 2007). It is therefore believed that the maternal immune responses to infection, rather than direct infectious processes in the fetus, are critical in mediating the link between prenatal infection and subsequent development of neuropsychiatric disease. The induction of pro-inflammatory cytokines and other mediators of inflammation may be particular relevant because they are involved in the immunological reaction to virtually all infections (Gilmore and Jarskog, 1997; Meyer et al., 2009b). Cytokines are low-molecular weight proteins with numerous functions in the peripheral immune system, where they help recruiting innate immune cells and modulate their activity (Curfs et al., 1997). Besides their typical immunological functions, cytokines play important roles in neural cell migration, differentiation and survival (Deverman and Patterson, 2009). Upon maternal immune challenge, certain cytokines such as interleukin (IL)-6 can also cross the placenta and enter the fetal system, where they may interfere with fetal brain development (Dahlgren et al., 2006; Deverman and Patterson, 2009).

Despite the biological plausibility of cytokine actions in response to maternal infection, there are also several alternative mechanisms whereby the maternal infectious processes could negatively affect fetal brain development (reviewed in Meyer et al., 2009b). Such alternative mechanisms include maternal and fetal nutritional deprivation (Brown and Susser, 2008; Susser et al., 2008) or infection-induced stimulation of maternal/fetal stress response systems (Koenig et al., 2002). Some viral pathogens or antibodies are also able to penetrate placenta and thereby harm fetal brain development directly (Aronsson et al., 2002). All of these pathological processes may operate at the same time, and therefore, the eventual impact on fetal brain development in the event of maternal infection may depend on their combined actions (Meyer et al., 2009b).

Like most other individual risk factors of complex neuropsychiatric disorders, maternal infection during pregnancy appears has only modest effect sizes on disease

risk in large populations (Tsuchiya et al., 2003; Varese et al., 2012; Selten et al., 2010). For example, the global incidence of schizophrenia following influenza pandemics typically increases only marginally even though 20-50 % of the general population is usually infected during influenza pandemics (Selten et al., 2010; Mednick et al., 1988). This again suggest that if maternal infection plays a role in the development of chronic brain disorders, then it likely does so by interacting with other genetic or environmental factors.

Another environmental risk factor that is frequently associated with neuropsychiatric disease is exposure to psychological stress (Brown and Birley, 1968; van Os et al., 2010). Stressful life events often precede psychotic episodes and are thought to trigger the onset or relapse of psychotic attacks (Brown and Birley, 1968; Day et al., 1987). A variety of clinical investigations have also demonstrated that exposure to early traumatizing insults such as sexual, physical and emotional abuse is associated with an increased risk of developing psychiatric disease later in life (Morgan and Fischer, 2007; van Winkel et al., 2008; van Os et al., 2010). Exposure to such stressors in critical periods of postnatal life, namely during childhood and/or early adolescence, is believed to most detrimental because stress can critically interact with ongoing brain maturational processes (de Kloet et al., 2005; Schreier et al., 2009; Dahl and Gunnar, 2009; Sideli et al., 2012).

Despite the existing evidence that maternal infection during pregnancy and exposure to traumatizing experiences in childhood play a role in the etiology of major neuropsychiatric disorders, it remains unknown whether and to what extent these two environmental factors can interact with each other to shape the vulnerability to long-term brain dysfunctions. As outlined in more detail below, it was the main impetus of the present thesis to seek evidence for such interactive effects in translational mouse model system. Hence, the work included in this thesis is a direct test of an environmental ''two-hit hypothesis" of neurodevelopmental disorders, in which prenatal infection represents the first environmental hit and exposure to postnatal stress the second environmental hit.

Objectives and Outline of the Thesis

Modeling an Environmental Two-Hit Hypothesis in Mice

Despite the widely appreciated role of prenatal infection and postnatal stress in the developmental psychiatric disorders, the consequences of postnatal stress exposure in individuals with prenatal infectious histories remain unknown. Ethical and technical reasons readily prevent a direct exploration of causal effects of such combined exposure in humans. One powerful approach to circumvent these limitations is the use of animal models, which allow a direct comparison between the effects of single versus double exposure to environmental insults such as infection and stress. The establishment and use of appropriate experimental model systems is thus essential in order to advance our understanding of how specific environmental (or genetic) factors can interact with each other to increase the risk for long-term brain dysfunctions.

Extensive experimental research over the last years has led to the establishment of various rodent models of prenatal infection that are relevant for human neuropsychiatric disorders (reviewed in Meyer et al., 2009a). One of the most widely used and well-characterized models is based on maternal gestational exposure to poly(I:C) [= p*olyriboinosinic-polyribocytidilic acid*] model (reviewed in Meyer et al., 2009a,b; Meyer, 2014). Poly(I:C) is a synthetic analogue of virus-specific double-stranded RNA, which induces a cytokine-associated acute phase response typically seen following viral infections (Kimura et al., 1994; Fortier et al., 2004). Poly(I:C) acts on toll like receptor 3 (Alexopoulou et al., 2001) and stimulates the production and release of several pro-inflammatory cytokines, leading to an intense but time-limited inflammatory response (Meyer et al., 2005). One important aspect of the model is that the efficacy of prenatal poly(I:C)-induced immune activation to induce schizophrenia-related brain and behavioral abnormalities is dependent on the strength of immune challenge (Shi et al., 2003, Meyer et al., 2005). Hence, by modifying the dosing of poly(I:C), the model allows to control the strength of the postnatal phenotype so that it facilitates the identification of interactions with other environmental insults (Meyer et al., 2009a; Meyer and Feldon, 2010).

For the work presented in this thesis, a low (sub-threshold) dose of poly(I:C) was chosen in order to transiently activate the maternal immune system during specific

stages of pregnancy. The main working hypothesis of the presented work was that an immune-mediated first hit in prenatal life might render the organism more vulnerable to postnatal stress-induced disruption of subsequent brain maturation. Therefore, we selected a dose of poly(I:C) that was expected to induce only restricted brain pathology in the offspring, unless it was combined with the second environmental hit that was applied in the form of postnatal stress exposure.

Postnatal stress exposure in rodent models is also known to induce long-lasting changes in normal brain functioning. The nature and/or severity of such pathological outcomes, however, seem to be critically dependent on the duration of stress exposure (days vs. weeks), the precise developmental timing (adolescence vs. adulthood), and the type of stressors (unpredictable vs. predictable or social vs. physical) (reviewed in Holder and Blaustein, 2013). The pubertal/adolescent period of development is believed to be particularly vulnerable to physical and psychological stress (Romeo and McEwen, 2006; Holder and Blaustein, 2013). Therefore, we selected the peri-pubertal period as the critical maturational stage, during which prenatally immune-challenged and control offspring with subjected to psychological stress. We used a sub-chronic stress model consisting of five relatively mild stressors applied during ten days (Koenig et al., 2005), which was expected to be associated with significant but restricted long-term outcomes when applied to prenatal control offspring. Five distinct and unpredictable stressors were used in order to avoid habituation to a repeated stressor (Helmreich et al., 1997).

Behavioral, Neuroanatomical, and Neurochemical Characterization of the Environmental Two-Hit Model

It is arguably impossible to mimic a complex human brain disorder such as schizophrenia in animals. However, one fruitful experimental approach is to focus on individual behavioral, physiological, and neuroanatomical phenotypes of the disorder, rather than to model the entire syndrome (Lipska and Weinberger, 2000; Arguello and Gogos, 2006). Such phenotypes can be assessed by the use of cross-species translational paradigms, which have been developed for the identification and characterization of neuropsychological, cognitive, and psychopharmacological core dysfunctions implicated in human neuropsychiatric disorders. Thus, in a first step we characterized the long-term behavioral effects of single and combined exposure to prenatal immune challenge

and peri-pubertal stress using such translational behavioral paradigms (chapter 2). We then explored possible neurochemical, neuroanatomical, and neuroinflammatory abnormalities in offspring with single or double exposure to the environmental insults (chapter 2 and 3). These investigations aimed at identifying possible mechanisms that could mediate the anticipated pathological interactions between prenatal immune challenge and peri-pubertal stress. Based on these investigations, a final series of experiments was conducted to explore the preventive potential of the broad-spectrum antibiotic minocycline in the two-hit model (chapter 4).

The present thesis consists of cumulative research articles originally written for separate peer-reviewed publications in scientific journals. A more detailed description of the objectives of the separate experiments can be found in the corresponding research articles (chapter 2-4).

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Chapter 2

Stress in Puberty Unmasks Latent Neuropathological Consequences of Prenatal Immune Activation in Mice

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ABSTRACT

Prenatal infection and exposure to traumatizing experiences during peri-puberty have each been associated with increased risk for neuropsychiatric disorders. Evidence for the cumulative impact of such prenatal and postnatal environmental challenges on brain functions and vulnerability to psychiatric disease is lacking. Here we show in a translational mouse model that combined exposure to prenatal immune challenge and peri-pubertal stress induces synergistic pathological effects on adult behavioral functions and neurochemistry. We further demonstrate that the prenatal insult markedly increases the vulnerability of the pubescent offspring to brain immune changes in response to stress. Our findings reveal interactions between two adverse environmental factors that have individually been associated with neuropsychiatric disease and support multiple environmental hit theories of mental illnesses with delayed onsets.

MAIN TEXT

Prenatal maternal infection and postnatal exposure to psychological trauma are two environmental risk factors for developmental psychiatric disorders, including autism, schizophrenia, and bipolar disorder (Brown, 2001; Pedersen and Mortensen, 2001; Herbert 2010; Tsuchiya et al., 2003). In spite of their relatively frequent occurrence (Green et al, 2010; Holmes and Slap, 1998, Brown and Patterson, 2011), both factors seem to have rather modest effect sizes in large populations (Tsuchiya et al., 2003, Varese et al 20012, Selten et al., 2010). For example, the global incidence of schizophrenia after influenza pandemics only increases marginally (relative risk ratios of 1 to 2.5) even though 20 to 50% of the general population is typically infected during influenza pandemics (Selten et al., 2010; Mednick et al., 1988). It has therefore been proposed that developmental stressors, such as infection or traumatizing experiences, may unfold their neuropathological impact primarily in genetically predisposed subjects (Clarke et al., 2009). Another feasible scenario is that initial exposure to a prenatal environmental insult, such as infection, can render the offspring more vulnerable to the pathological effects of a second postnatal stimulus, such as stress (Bayer et al., 1999; Maynard et al., 2001). However, this hypothesis still awaits direct verification. We therefore tested if stress in puberty has the potential to unmask latent psychopathology in neurodevelopmentally vulnerable subjects with prenatal infectious histories.

We compared the consequences of prenatal immune activation with or without additional postnatal stress challenge in mice (fig. S1 and supplementary methods). Prenatal immune activation was induced by the viral mimetic polyriboinosinic-polyribocytidilic acid [poly(I:C)], a synthetic analog of double-stranded RNA that induces a cytokine-associated, viral-like acute-phase response (Meyer et al., 2009). We used a low dose of poly(I:C) [1 mg/kg, administered intravenously on gestation day 9 (GD9)] to mimic physiologically relevant and transient cytokine elevations (fig. S2) (Meyer et al., 2009). Offspring born to poly(I:C)-exposed or control mothers were then left undisturbed or exposed to variable and unpredictable stress during peri-pubertal development, a maturational period known to be highly sensitive to the disrupting effects of traumatizing events relevant to psychosis-related disease (Spear, 2009; Fisher et al. 2010). The stress protocol included five distinct stressors: (i) electric foot shock,

(ii) restraint stress, (iii) swimming stress, (iv) water deprivation, or (v) repeated home age changes, applied on alternate days between postnatal days (PNDs) 30 and 40 (fig. S1 and supplementary methods).

We assessed the effects of the double-hit protocol on adult (PND 70 to 100) brain functions using behavioral tests relevant to translational models of neuropsychiatric disease (Meyer et al., 2009) (supplementary methods and tables S1 to S19). Stress exposure increased anxiety-like behavior in the elevated plus maze test independently of the prenatal immunological manipulation (Fig. 1A), which suggests that peri-pubertal offspring with a prenatal infectious history do not differ from prenatal controls in the development of stress-induced anxiety-like abnormalities. We further revealed independent effects of immune challenge and stress in the disruption of selective associative learning as measured by the paradigm of latent inhibition (LI): Nonstressed control offspring displayed a robust LI effect in the conditioned active avoidance paradigm (Fig. 1B). This LI effect arising from repeated preexposures to the conditioned stimulus before conditioning was fully abolished in all other groups (Fig. 1B). Prenatal immune activation and peri-pubertal stress caused synergistic effects in the development of sensorimotor gating deficiency, as assessed by the paradigm of prepulse inhibition (PPI) of the acoustic startle reflex (Fig. 1C), as well as in the precipitation of behavioral hypersensitivity to the psychotomimetic drugs amphetamine (AMPH) (Fig. 1D and fig. S3A) and dizocilpine (MK-801) (Fig. 1E and fig. S3B). Neither immune activation alone nor stress alone affected sensorimotor gating and psychotomimetic drug sensitivity. Abnormalities in these domains became evident only after combined exposure to the two environmental factors.

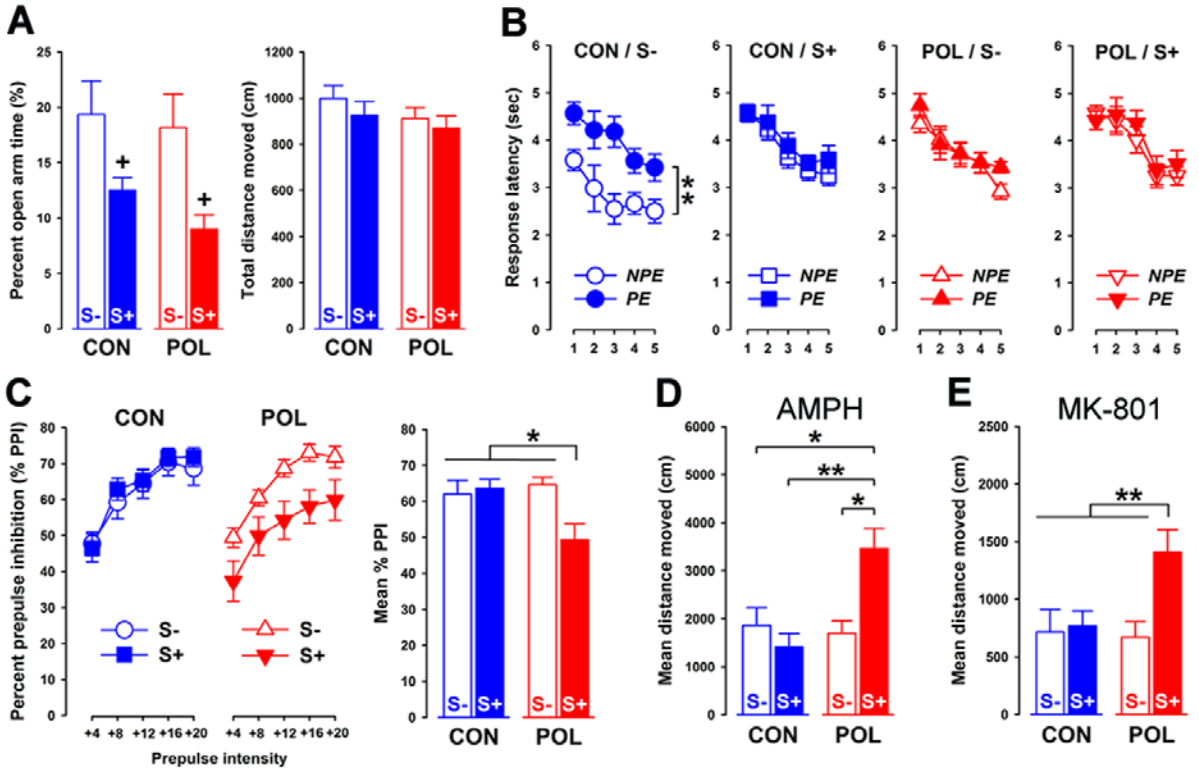


Figure 1. Prenatal immune activation and peri-pubertal stress cause independent and synergistic pathological effects on adult behavioral functions. (A) Adult mice subjected to peri-pubertal stress (S+) display enhanced anxiety-like behavior in the elevated plus maze test (as indexed by the reduced time spent on the open arms) compared with nonstressed (S-) offspring regardless of the prenatal conditions [CON, vehicle control; POL, poly(I:C)]; +P < 0.05, main effect of peri-pubertal stress. N = 16 to 19 per group. (B) Response latencies in nonpreexposed (NPE) and CS-preexposed (PE) subjects as a function of successive 10-trial blocks in the LI test with a conditioned active avoidance procedure. The LI effect is present in CON/S- subjects (**P < 0.01) but is completely disrupted by prenatal immune activation alone (POL/S-), peri-pubertal stress alone (CON/S+), or their combination (POL/S+). NPE, N =7 to 9 per group; PE, N =7 to 10 per group. (C) Sensorimotor gating as assessed by PPI of the acoustic startle reflex. (Left) % PPI as a function of increasing prepulse intensities (dB above background of 65 dB); (right) the mean % PPI across all prepulse levels. *P < 0.05, reduction of % PPI in POL/S+ animals relative to all other groups. N = 16 to 19 per group. (D) The mean distance moved in a standard open-field arena during a 90-min period after administration of AMPH [2.5 mg/kg, intraperitoneally (i.p.)]. *P < 0.05 and **P < 0.01, increase in AMPH-induced activity displayed by POL/S+. N = 8 to 10 per group. (E) The mean distance moved in the open field during a 90-min period after administration of MK-801 (0.15 mg/kg, i.p.). **P < 0.01, increase in MK-801–induced activity displayed by POL/S+ compared with all other groups, post hoc group comparisons. N = 8 per group. All data are means ± SEM.

We further evaluated the postnatal onset of the identified behavioral abnormalities in our environmental double-hit model. With the exception of anxiety-related behavior, none of the other behavioral functions were affected at peri-pubertal age (PND 41 to 45) (Fig. 2 and fig. S4). Hence, the emergence of multiple behavioral dysfunctions such as LI deficiency, PPI attention, and psychotomimetic drug hypersensitivity in singly or doubly challenged offspring are dependent on postpubertal maturational processes, which, in turn, is consistent with the clinical course of mental illnesses with delayed onsets, including schizophrenia and bipolar disorder (Paus et al., 2008). We also revealed that a later application of stress in adolescence, at PND 50 to 60, did not elicit the interaction with prenatal immune activation (fig. S5). The findings emphasize that the precise timing of postnatal stress is critical for the interaction with the prenatal immune challenge.

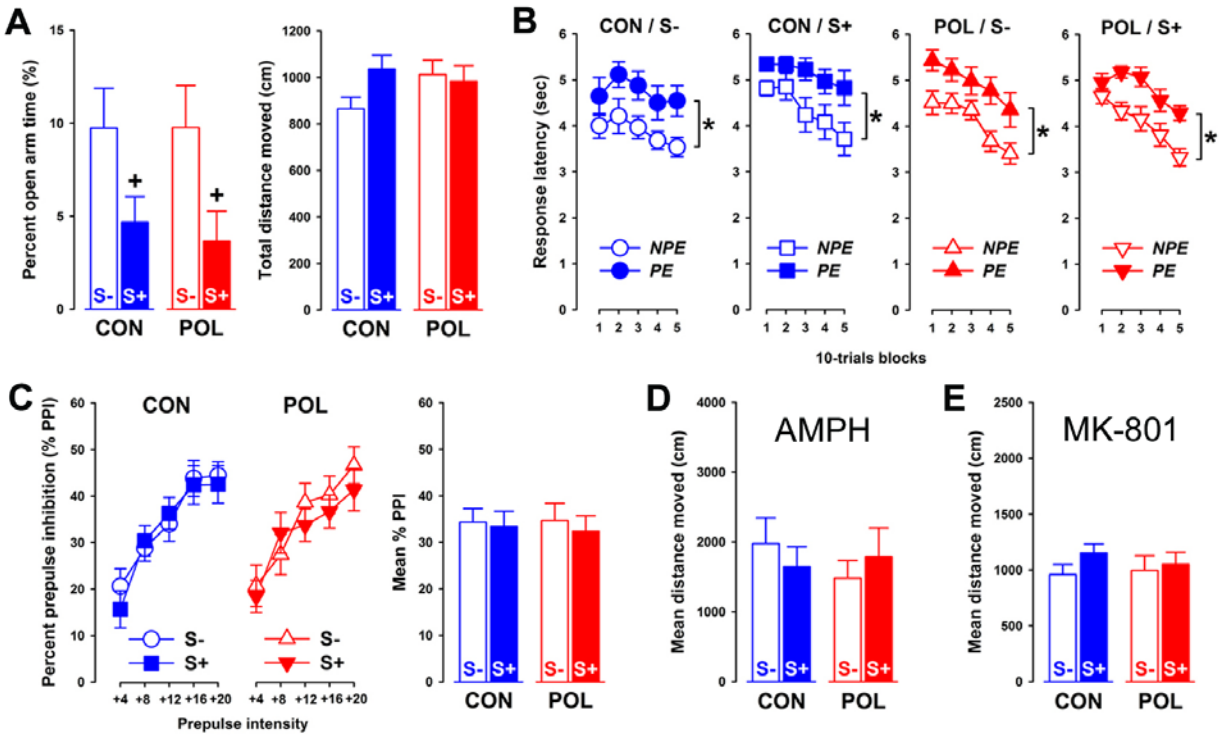


Figure 2. Short-term effects of single or combined exposure to prenatal immune activation and peri-pubertal stress on behavioral functions in pubescence. (A) Peripubertal mice subjected to stress in puberty (S+) display enhanced anxiety-like behavior in the elevated plus maze test (as indexed by the reduced time spent on the open arms) compared with nonstressed (S-) offspring regardless of the prenatal conditions; +P < 0.05, main effect of peri-pubertal stress. *N* = 13 to 15 per group. (B) Response latencies in NPE and PE subjects as a function of successive 10-trial blocks in the LI test with a conditioned active avoidance procedure. *P < 0.05, main effect of CS preexposure (LI) in all groups. NPE, *N* = 9 per

group; PE, *N* = 9 to 10 per group. (C) Sensorimotor gating as assessed by PPI of the acoustic startle reflex. (Left) % PPI as a function of increasing prepulse intensities (dB above background of 65 dB); (right) the mean % PPI across all prepulse levels. *N* = 13 to 15 per group. (D) The mean distance moved in a standard open-field arena during a 90-min period after administration of AMPH (2.5 mg/kg, i.p.). *N* = 12 to 13 per group. (E) The mean distance moved in the open field during a 90-min period after administration of MK-801 (0.15 mg/kg, i.p.). *N* = 10 to 11 per group. All data are means ± SEM.

The adult behavioral abnormalities emerging after prenatal immune activation and peri-pubertal stress exposure are unlikely to be associated with changes in the hypothalamus-pituitary-adrenal (HPA) stress-response system: Neither single nor combined exposure to the environmental adversities affected basal plasma levels of corticosterone (CORT), the main effector hormone of the HPA axis (fig. S6). The developmental stressors also did not affect CORT secretion after acute stress reexposure in adulthood (fig. S6). However, our high-performance liquid chromatography analyses identified brain region–specific neurochemical changes in adult mice exposed to prenatal immune activation and/or peri-pubertal stress: Prenatal immune activation was sufficient to increase the levels of dopamine (DA) in the nucleus accumbens (NAc) independently of postnatal stress (Fig. 3A), and stress exposure decreased the content of serotonin (5-HT) in the medial prefrontal cortex (PFC) regardless of the prenatal history (Fig. 3B). It intrigued us that enhanced DA levels in the hippocampus (HPC) were only manifest after combined exposure to prenatal immune challenge and peri-pubertal stress (Fig. 3A); this highlighted synergistic effects between the two adverse events in the precipitation of adult hippocampal DA imbalances.

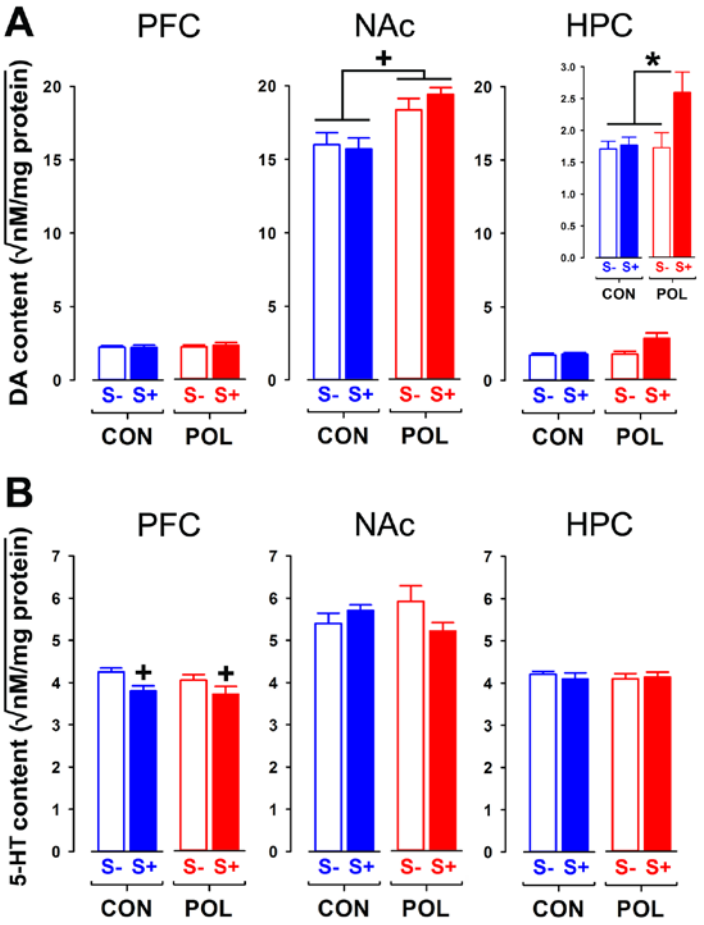


Figure 3. Neurochemical parameters in adult offspring exposed to single or combined prenatal immune activation and peri-pubertal stress. (A) DA contents (nM/mg protein, square root–transformed) in the medial PFC, NAc, and HPC of adult (PND 70) offspring under the conditions described above. +P < 0.05, main effect of prenatal immune activation; *P < 0.05, increase in HPC DA levels in POL/S+ mice relative to all other groups. *N* = 8 to 11 per group. (B) 5-HT contents (nM/mg protein, square root–transformed) in the PFC, NAc, and HPC of groups of adult mice. +P < 0.05, main effect of stress exposure. *N* = 8 to 11 per group. All data are means ± SEM.

Prenatal immune activation (at high intensity) and chronic stress exposure have individually been linked to the development of immune alterations in the brain and periphery (Hsiao et al., 2012; Meyer et al., 2011, Frank et al., 2007). Here, we elucidated whether initial exposure to prenatal immune challenge could change the offspring's neuroimmunological responses to peri-pubertal stress. Two brain areas of primary interest were selected, namely, the HPC (including CA1 to CA3 subregions and dentate gyrus) and the PFC (including anterior cingulate, prelimbic, and infralimbic cortices). These brain regions are highly sensitive to chronic stress exposure (Franklin et al., 2010) and show neuroanatomical abnormalities after intense prenatal immune challenge,

including CNS immune changes (Meyer et al., 2011). We also included a cortical control region (secondary motor cortex, MC) that is largely insensitive to neuronal and immunological adaptations after chronic stress.

We first used immunohistochemical techniques to study the activation of microglia, a population of immunocompetent cells in the CNS (Ransohoff and Perry, 2009). Unbiased stereological estimations of microglia immunoreactive for the calcium-binding protein Iba1 revealed that peri-pubertal stress only led to a ~5% increase in total microglia numbers in the HPC at the adult stage (PND 70), without affecting microglia morphology (fig. S7). Neither single nor combined exposure to prenatal immune activation and stress altered the expression of CD68 (fig. S8), a cellular marker typically expressed by activated microglia in the CNS (Ransohoff and Perry, 2009). Likewise, the two environmental factors did not change the hippocampal levels of the inflammatory molecules interleukin-1β (IL-1β), tumor necrosis factor–α (TNF-α), and prostaglandin E2 (PGE2) in adulthood (fig. S8). Thus, single or combined exposure to prenatal immune activation and peri-pubertal stress exert only a minimal long-term impact on microglia cells and induce no overt changes in the central and peripheral secretion of prototypical inflammatory factors.

However, we revealed that the prenatal insult markedly increased the offspring's vulnerability to stress-induced neuroimmunological changes at peri-pubertal age (PND 41): Combined immune activation and stress led to a 2.5- to 3-fold increase in hippocampal (Fig. 4, B to D) and prefrontal (fig. S9, B to D) expression of markers characteristic of activated microglia (CD68 and CD11b) at PND 41. No such changes were found in the MC control region (fig. S10, B and C). The hippocampal microglia response was further accompanied by the presence of elevated levels of the proinflammatory cytokines IL-1β and TNF-α (Fig. 4E) but not with plasma changes in inflammatory markers or the stress hormone CORT (fig. S11). The neuroimmunological effects of combined exposure to the two environmental insults thus appear to be localized in stress-sensitive brain areas, such as the HPC and PFC, and are unlikely to be associated with functional changes in the HPA axis. Single or combined exposure to immune activation and stress also did not affect the number or activation status of

astrocytes in PND 41 mice (fig. S13), which suggests that the two environmental challenges largely spared astroglial functions at peri-pubertal age.

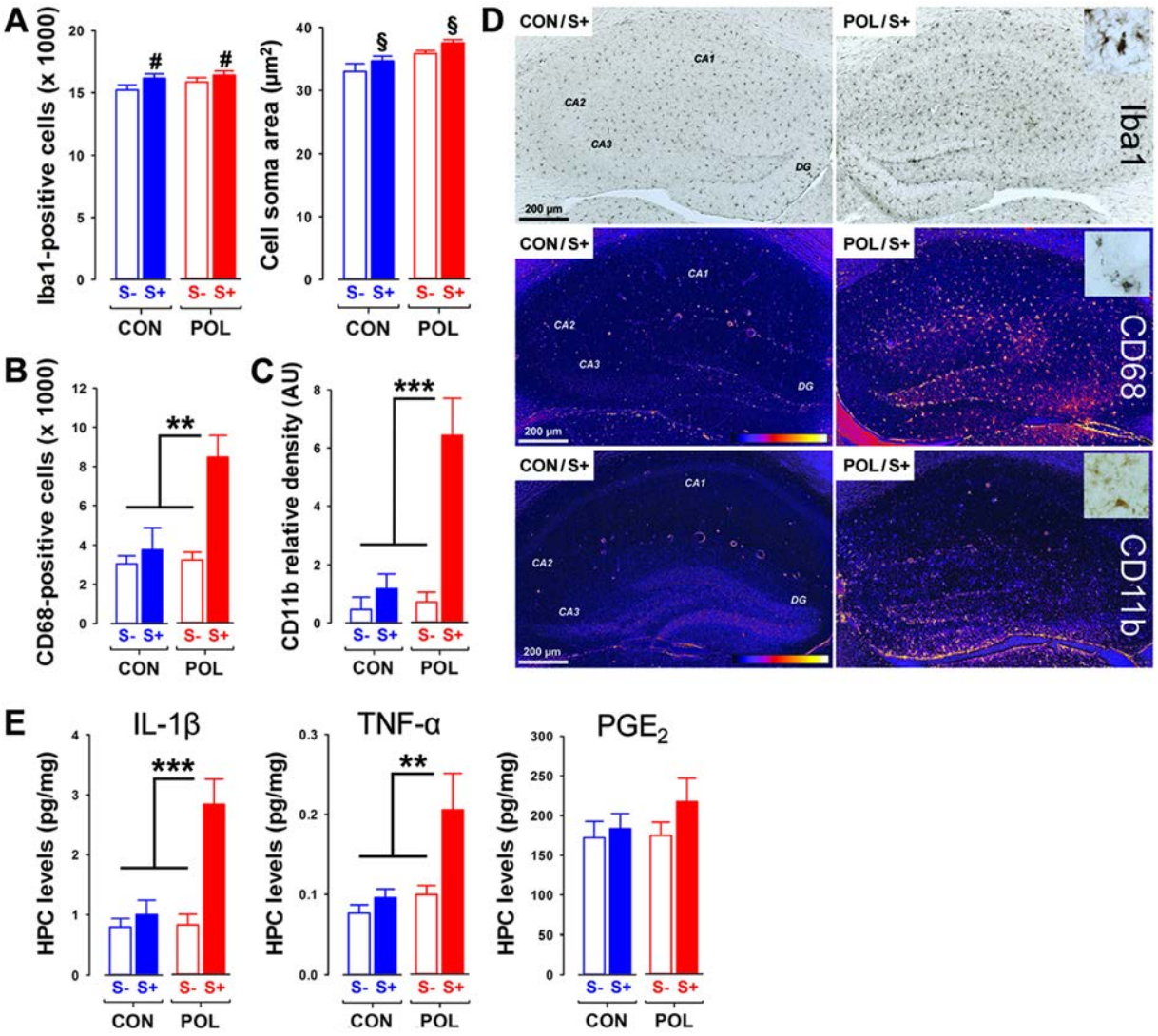


Figure 4. Altered neuroimmune responses in the pubescent brain after combined prenatal immune activation and peri-pubertal stress. All measures were taken on postnatal day 41, i.e., 1 day after exposure to the last peri-pubertal stressor. (A) Stereological estimates and cell soma area of Iba1-positive microglia cells in the HPC of mice treated as described above. #P < 0.05 and §P < 0.01, main effect of peri-pubertal stress. *N* = 11 to 12 per group. The number of primary processes and number of branch points of Iba1-positive microglia cells in the HPC are given in fig. S12. (B) Stereological estimates of CD68-positive cells in the HPC; **P < 0.01, post hoc comparisons. *N* = 11 to 12 per group. (C) The relative optical density [arbitrary units (AU)] of CD11b immunoreactivity in the HPC; ***P < 0.001, post hoc comparisons. *N* = 11 to 12 per group. (D) Color-coded coronal brain sections of representative CON/S+ and POL/S+ offspring at the level of the HPC [CA1 to CA3 regions and dentate gyrus (DG) are highlighted] stained with antibodies against Iba1, CD68, or CD11b. Insets are at higher magnification. In color-coded sections (CD68 and

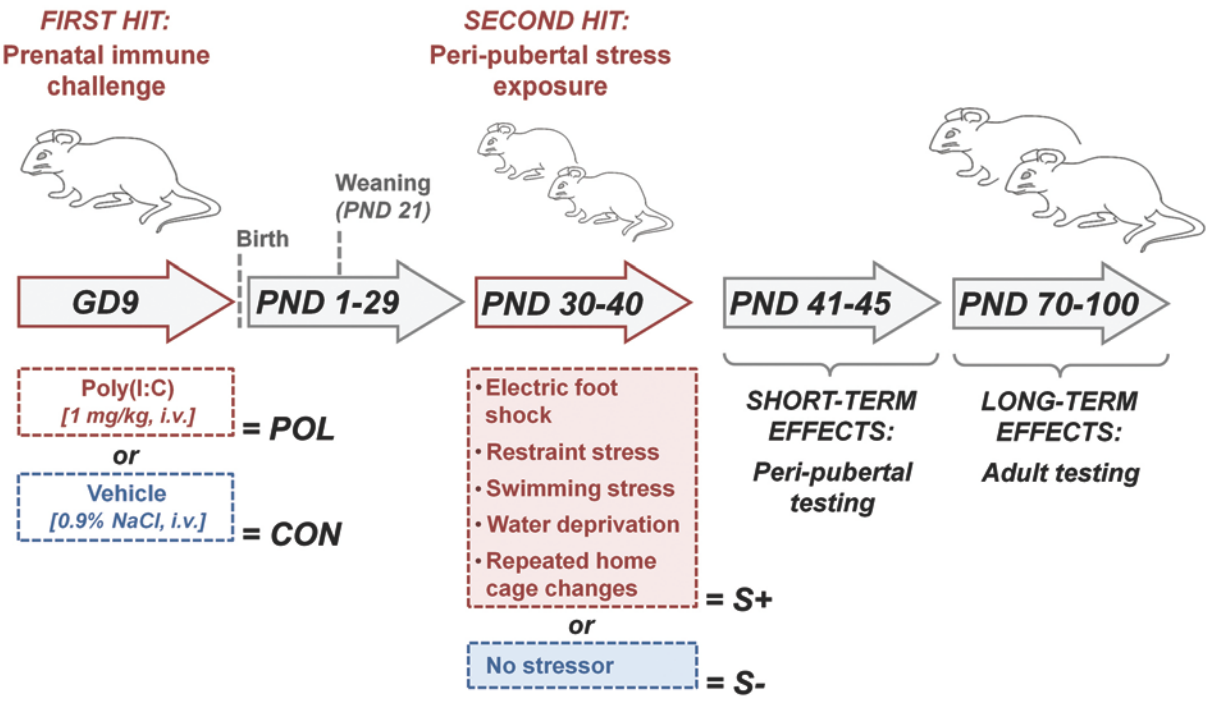
CD11b), the strongest staining intensities are yellow; the background is represented in dark purple (color scale bar). (E) Contents of IL-1β, TNF-α, and PGE2 in the HPC measured by using particle-based flow cytometry; **P < 0.01 and ***P < 0.001, post hoc comparisons. *N* = 10 to 12 per group. HPC levels of IL-6 and IL-10 protein were below detection limits. All data are means ± SEM.

We performed additional molecular analyses to explore whether the transient microglia changes in peri-puberty would be associated with dysfunctional neuron-microglia inhibitory signaling, which, under nonpathological conditions, aims to restrain microglia from activation (Ransohoff and Cardona, 2010). Besides other signaling pairs, contact-dependent neuron-microglia inhibitory signaling is governed by CD200-CD200 receptor (CD200R) and CD47-CD172a interactions, in which CD200 and CD47 are primarily expressed by neurons, and CD200R and CD172a by microglia (Ransohoff and Cardona, 2010). Previous investigations in rats have shown that exposure to severe stress impairs the CD200 expression in the HPC (Frank et al., 2007). Our real-time polymerase chain reaction analyses of these molecules demonstrated that exposure to an acute stressor was sufficient to severely impair hippocampal and prefrontal expression of CD200, CD200R, and CD47 specifically in prenatally immune-challenged animals (fig. S14). Again, these effects emerged without any group differences in plasma CORT secretion at basal conditions or after stress, which suggested that the observed changes in contact-dependent neuron-microglia inhibitory signaling are unlikely to be attributable to possible alterations in CORT-associated stress responses (fig. S15). However, it remains possible that altered expression of the selected neuron-microglia inhibitory signaling pairs reflects dynamic cellular adaptations, such as neuronal apoptosis and microglia differentiation or proliferation, and therefore, cell-specific expression of these signaling pairs awaits further validation in our double-hit model.

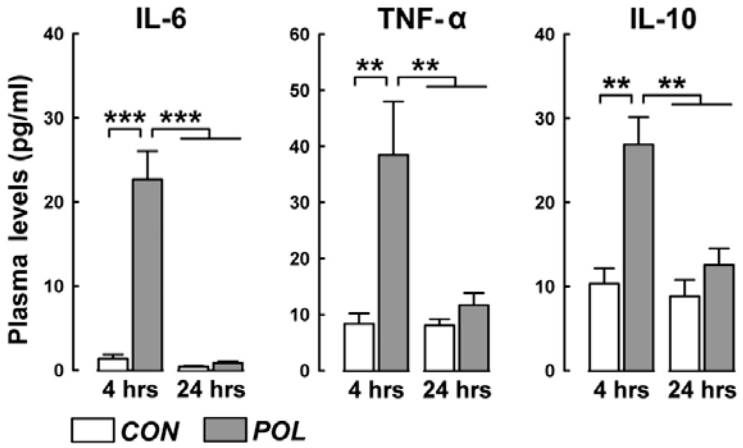
In conclusion, our results show synergistic interactions between two environmental risk factors that have individually been associated with developmental psychopathology. Prenatal adversities (here, in the form of in utero immune challenge) can thus function as a "disease primer" that increases the offspring's vulnerability to the detrimental neuropathological effects of subsequent stress exposure during peri-pubertal life. Prenatal infection and peri-pubertal stress may thus be important etiological risk factors for long-term mental illness especially upon combined exposure. The concept by which prenatal infection can "prime" the developing organism's

sensitivity to subsequent environmental challenges postnatally is consistent with other models demonstrating synergistic pathological effects between prenatal and postnatal insults, including prenatal exposure to air pollution and chronic high-fat diet consumption in adulthood (Bolton et al., 2004). The transient neuroimmunological changes emerging in peri-pubertal offspring that are exposed to combined immune activation and stress capture relevant aspects of neuroinflammatory processes. The precise inflammatory signature of these processes needs further elaboration and should be extended to other neuroimmunological aspects, including extension to other members of the cytokine network. Our data here may encourage attempts in this direction because rectifying altered immune responses during sensitive periods of peri-pubertal brain maturation could offer a valuable strategy to prevent possible neuronal maladaptations and subsequent psychopathology after exposure to multiple environmental adversities.

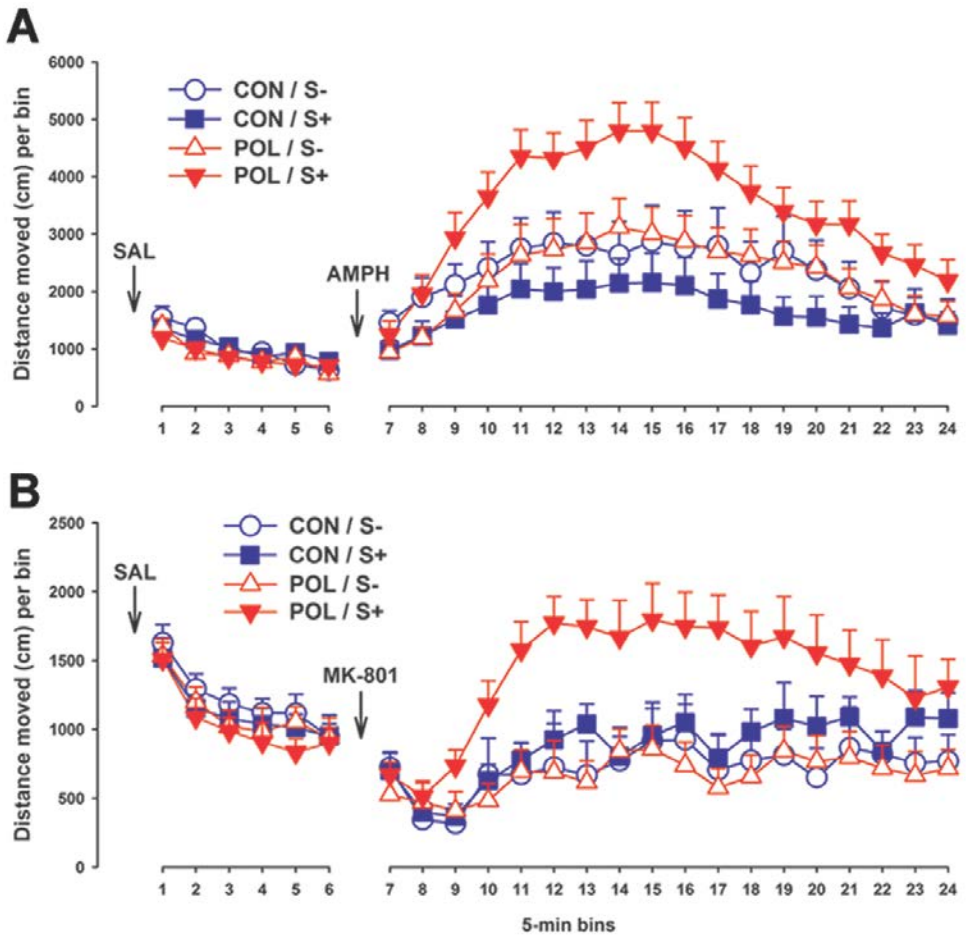
SUPPLEMENTARY RESULTS



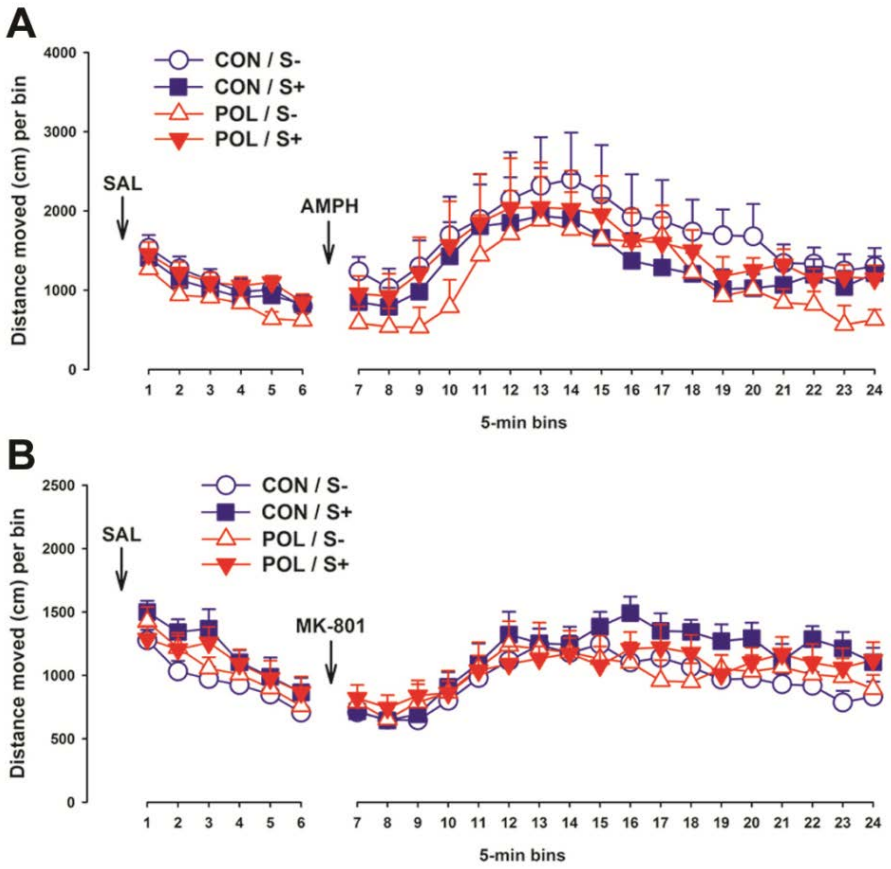
Supplementary Figure S1. Schematic representation of the two-hit environmental model. The first environmental hit was composed of prenatal viral-like immune activation induced by maternal administration of the synthetic double-stranded RNA poly(I:C) (= *polyriboinosinic-polyribocytidilic acid*; 1 mg/kg, i.v.) on gestation day (GD) 9. Control mothers received corresponding vehicle (physiological pyrogen-free saline [NaCl], i.v.) solution. Offspring born to poly(I:C)-treated (POL) or vehicle-treated (CON) mothers were weaned on postnatal day (PND) 21. They were then left undisturbed (= no stressor; S-) or exposed to variable and unpredictable stress between PND 30 and 40 (S+), the latter of which corresponded to the second environmental hit. An additional cohort of poly(I:C) or vehicle control offspring were subjected to an identical variable stress protocol between PND 50 and 60 (not shown). The stress protocol included five distinct stressors (1. electric foot shock; 2. restraint stress; 3. swimming stress; 4. water deprivation; 5. repeated home cage changes) applied on alternate days. The short-term effects of single or combined exposure to prenatal immune challenge and peri-pubertal stress were assessed when the offspring reached PND 41-45, and the long-term effects were assessed when the animals reached early adulthood (i.e., between PND 70 and 100).



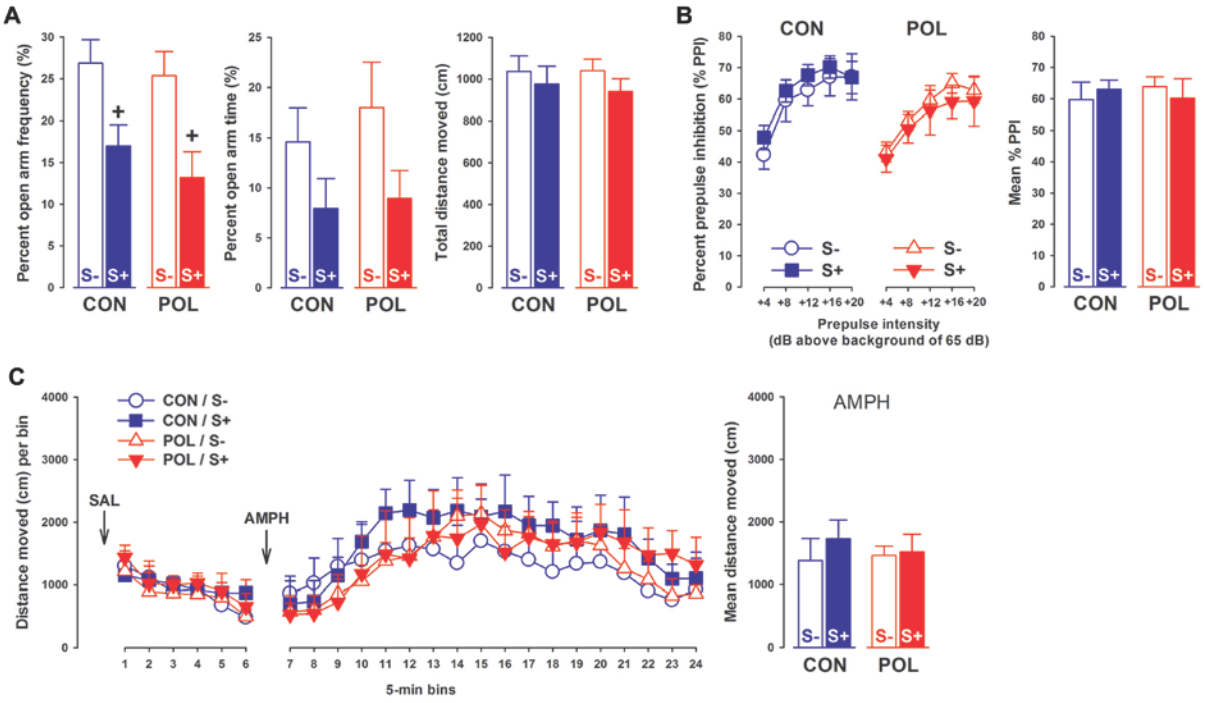
Supplementary Figure S2. Maternal cytokine levels at basal conditions and following viral-like immune activation in mid-pregnancy. Pregnant mice were exposed to poly(I:C) (1 mg/kg, i.v.; = POL) or control (= CON) vehicle (saline) solution on gestation day 9, and plasma levels of the pro-inflammatory (IL-1β, IL-6, TNF-α) and anti-inflammatory (IL-10) cytokines were measured 4 and 24 hrs post-treatment using particle-based flow cytometric cytokine assays. Note that the POL-induced elevation of cytokines was transient and normalized to basal levels within 24 hrs post-treatment. $^{**}P < 0.01$ and $^{***}P < 0.001$, based on post-hoc comparisons. N = 5-8 animals per treatment group/post-injection interval. Plasma levels of IL-1β were below detection limit (2.1 pg/ml) for each experimental condition. All data are means±SEM.



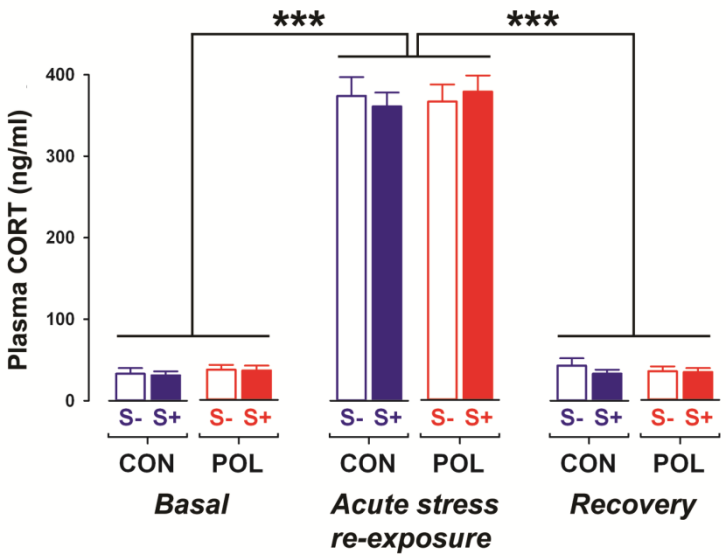
Supplementary Figure S3. Effects of single and combined prenatal immune activation and peri-pubertal stress on locomotor reactions to psychotomimetic drugs in adulthood. Pregnant C57BL/6 mice were exposed to poly(I:C) (POL) or control (CON) solution on gestation day 9, and the resulting offspring were subjected to sub-chronic stress (S+) or left undisturbed (S-) during peri-pubertal development (postnatal days [PNDs] 30~40). Behavioral assessment took place when the animals reached PND 70, i.e., 4-weeks after exposure to the last stressor. (A) Locomotor reaction to the indirect dopamine receptor agonist amphetamine (AMPH; 2.5 mg/kg, i.p.). The line plot depicts the distance moved in a standard open field arena to initial vehicle (saline, SAL) treatment and subsequent AMPH treatment as a function of successive 5-min bins. The corresponding mean distance moved is depicted in Figure 1D of the main document. *N*=8-10 per group. (B) Locomotor reaction to the non-competitive NMDA receptor antagonist dizocilpine (MK-801; 0.15 mg/kg, i.p.). The line plot shows the distance moved in a standard open field arena to initial vehicle (SAL) treatment and subsequent MK-801 treatment as a function of successive 5-min bins. The corresponding mean distance moved is depicted in Figure 1E of the main document. *N*=8 per group. All data are means±SEM.



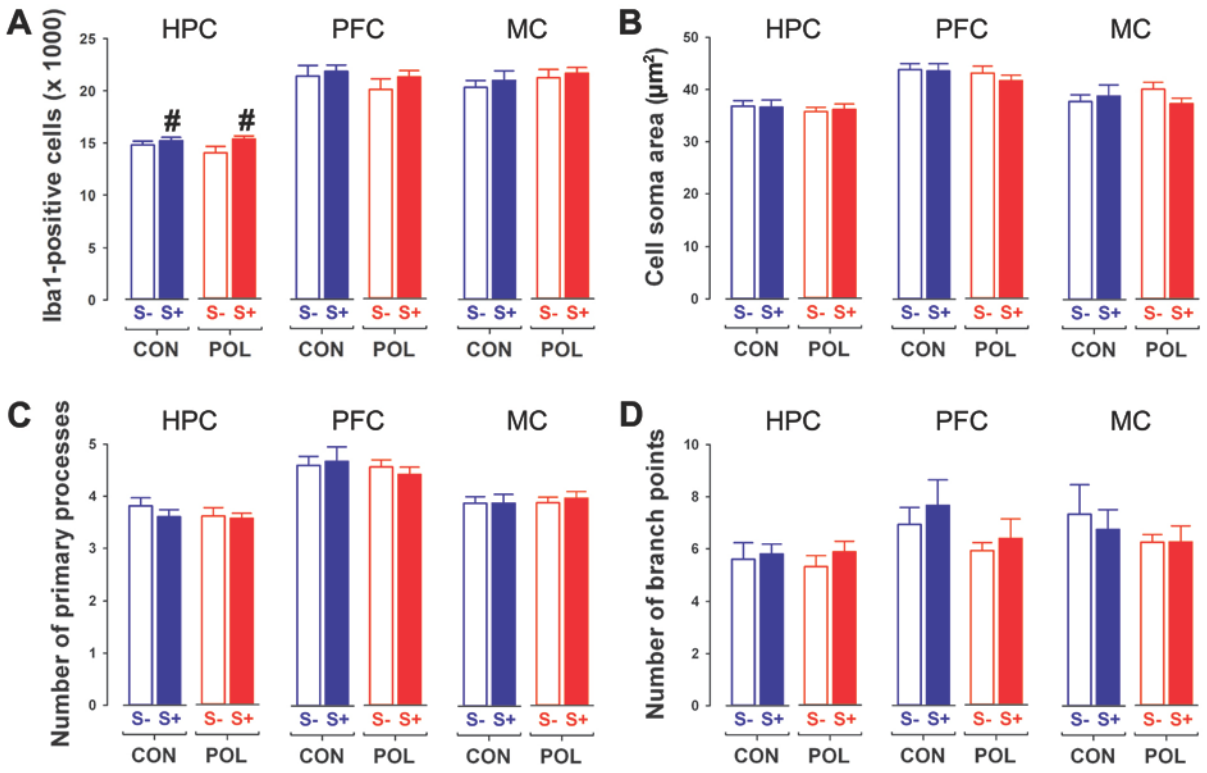
Supplementary Figure S4. Effects of single and combined prenatal immune activation and peri-pubertal stress on locomotor reactions to psychotomimetic drugs in pubescence. Pregnant C57BL/6 mice were exposed to poly(I:C) (POL) or control (CON) solution on gestation day 9, and the resulting offspring were subjected to sub-chronic stress (S+) or left undisturbed (S-) during peri-pubertal development (postnatal days [PNDs] 30~40). Behavioral assessment took place pubescence (PND 41~45). (A) Behavioral sensitivity to the indirect dopamine receptor agonist amphetamine (AMPH; 2.5 mg/kg, i.p.). Locomotor response (indexed by the distance moved in a standard open field arena) to initial vehicle (saline, SAL) treatment and subsequent AMPH treatment as a function of successive 5-min bins. The corresponding mean distance moved is depicted in Figure 2D of the main document. *N*=12-15 per group. (B) Behavioral sensitivity to the non-competitive NMDA receptor antagonist dizocilpine (MK-801; 0.15 mg/kg, i.p.). Locomotor response (indexed by the distance moved in a standard open field arena) to initial vehicle (SAL) treatment and subsequent MK-801 treatment as a function of successive 5-min bins. The corresponding mean distance moved is depicted in Figure 2E of the main document. *N*=10-11 per group. All data are means±SEM.



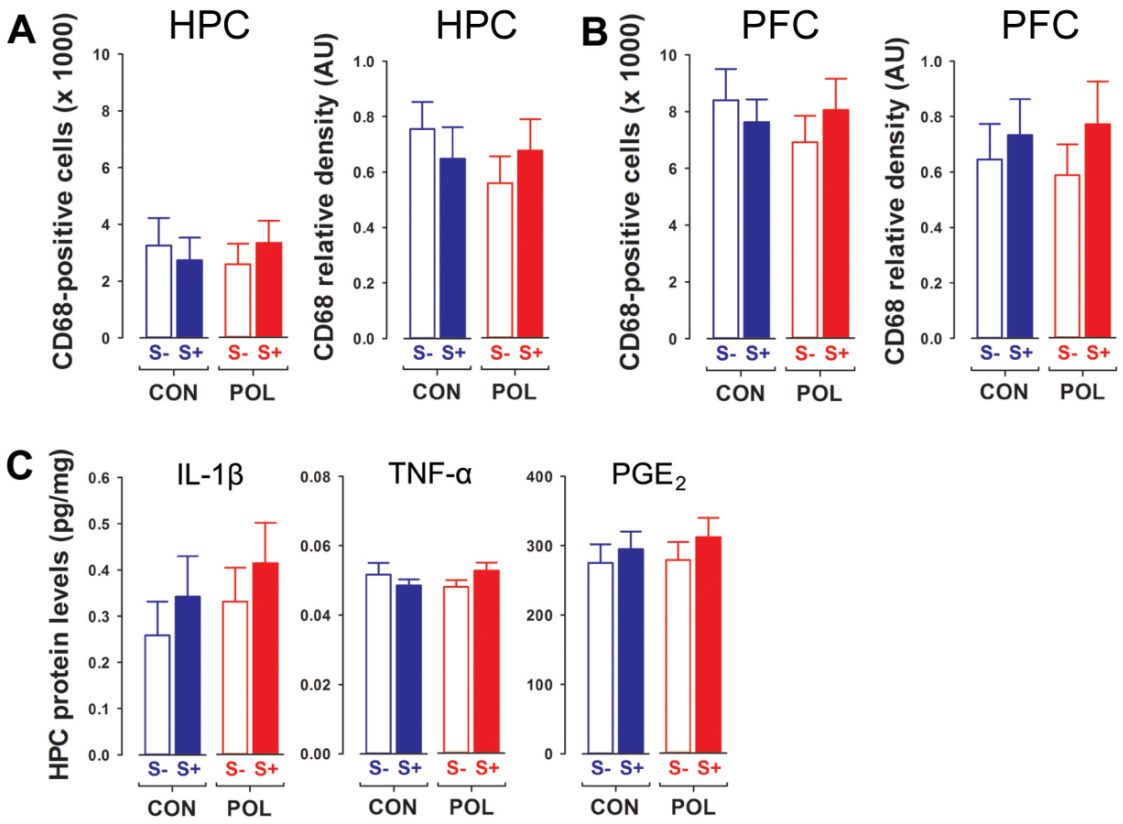
Supplementary Figure S5. Long-term effects of single or combined exposure to prenatal immune activation and adolescent stress on behavioral functions in adulthood. Pregnant C57BL/6 mice were exposed to poly(I:C) (POL) or control (CON) solution on gestation day 9, and the resulting offspring were subjected to sub-chronic stress (S+) or left undisturbed (S-) during adolescent development (postnatal days [PNDs] 50~60). Behavioral functions were assessed in adulthood (PND 90~110) starting 4 weeks after the last stress exposure in adolescence. (A) Anxiety-like behavior in the elevated plus maze test as indexed by the relative open arm frequency and relative time spent on the open arms; the total distance moved was taken to index general locomotor activity. $^{+}P < 0.01$, reflecting the significant main effect of adolescent stress based on ANOVA. *N*=7-12 per group. (B) Sensorimotor gating as assessed by the paradigm of prepulse inhibition (PPI) of the acoustic startle reflex. The line plot shows % PPI as a function of increasing prepulse intensities, and the bar plot depicts the mean % PPI across all prepulse levels. *N*=7-12 per group. (C) Behavioral sensitivity to the indirect dopamine receptor agonist amphetamine (AMPH; 2.5 mg/kg, i.p.). The line plot depicts the locomotor response (indexed by the distance moved in a standard open field arena) to initial vehicle (saline, SAL) treatment and subsequent AMPH treatment as a function of successive 5-min bins, and the bar plot shows the mean distance moved during the entire 90-min period following AMPH administration. *N*=5-8 per group. All data are means±SEM.



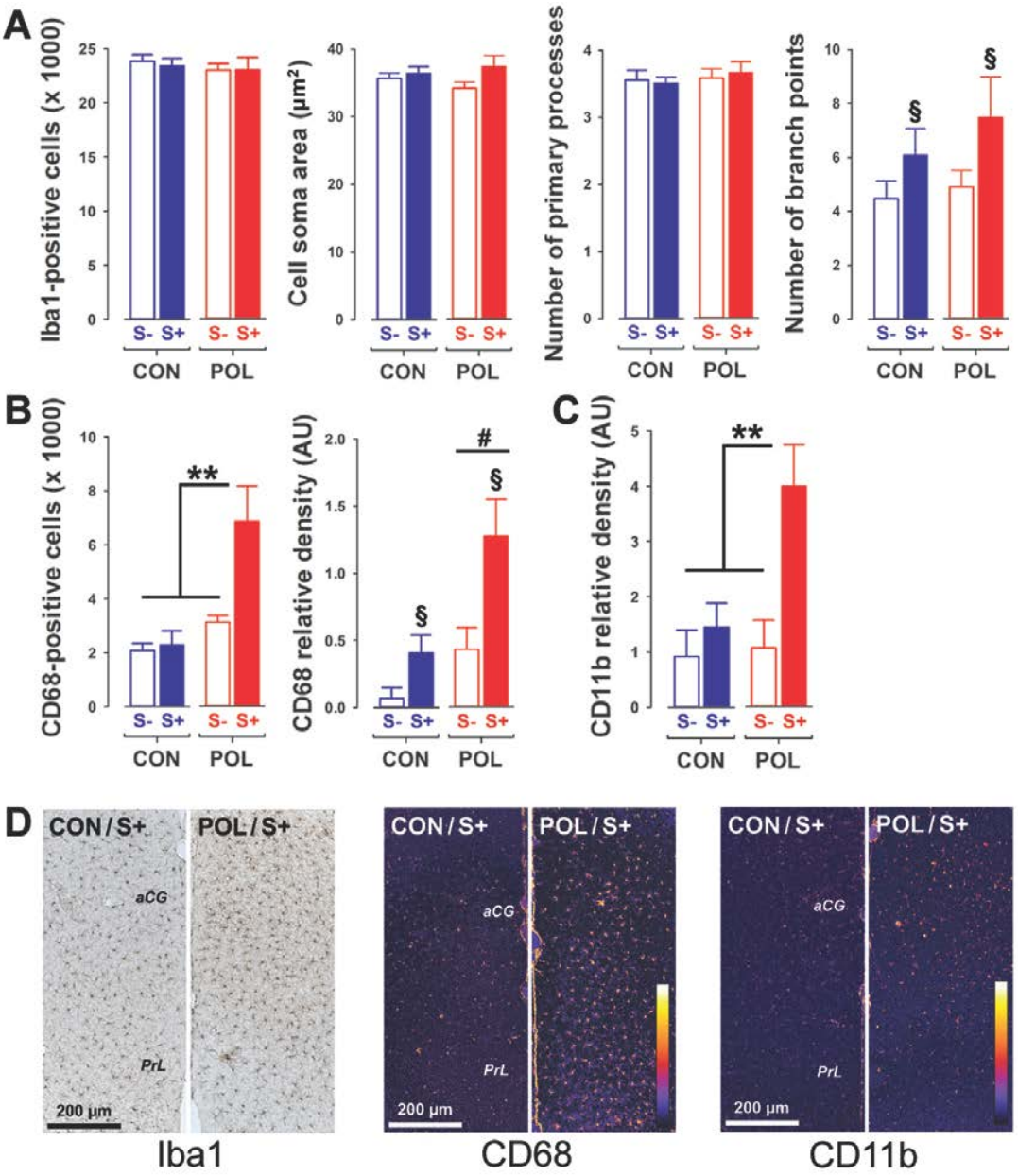
Supplementary Figure S6. Long-term effects of single or combined exposure to prenatal immune activation and peri-pubertal stress on plasma corticosterone responses to acute stress re-exposure in adulthood. Pregnant C57BL/6 mice were exposed to poly(I:C) (POL) or control (CON) solution on gestation day 9, and the resulting offspring were subjected to sub-chronic stress (S+) or left undisturbed (S-) during peri-pubertal development (postnatal days [PNDs] 30~40). Plasma corticosterone (CORT) levels were assessed 4 weeks after exposure to the last stressor (i.e., on PND 70) at basal conditions, immediately after acute (45 min restraint) stress, and following a recovery phase of 90 min post-stress in puberty (postnatal day 30). ***$P < 0.001$, reflecting the significant main effect of stress. N=12-13 per group and condition. All data are means±SEM.



Supplementary Figure S7. Long-term effects of single or combined exposure to prenatal immune activation and peri-pubertal stress on microglia numbers and morphology in adulthood. Pregnant C57BL/6 mice were exposed to poly(I:C) (POL) or control (CON) solution on gestation day 9, and the resulting offspring were subjected to sub-chronic stress (S+) or left undisturbed (S-) during peri-pubertal development (postnatal days [PNDs] 30~40). Brain specimens collected 4 weeks after exposure to the last stressor (i.e., on PND 70) were stained with anti-Iba1 to visualize the entire microglia cell population. All measurements were obtained in the hippocampal formation (HPC; including CA1-CA3 region and dentate gyrus), medial prefrontal cortex (PFC; including anterior cingulate, prelimbic and infralimbic cortices), and secondary motor cortex (MC; including M2 region of the neocortex). (A) Stereological estimates of Iba1-positive microglia cells (cells/mm^3). $^{\#}P < 0.05$, reflecting the significant main effect of peri-pubertal stress in the HPC. N=11-12 per group. (B) Cell soma area of Iba1-positive microglia cells (µm^2). N=11-12 per group. (C) Number of primary processes of Iba1-positive microglia cells. N=11-12 per group. (D) Number of branch points of Iba1-positive microglia cells. N=11-12 per group. All data are means±SEM.

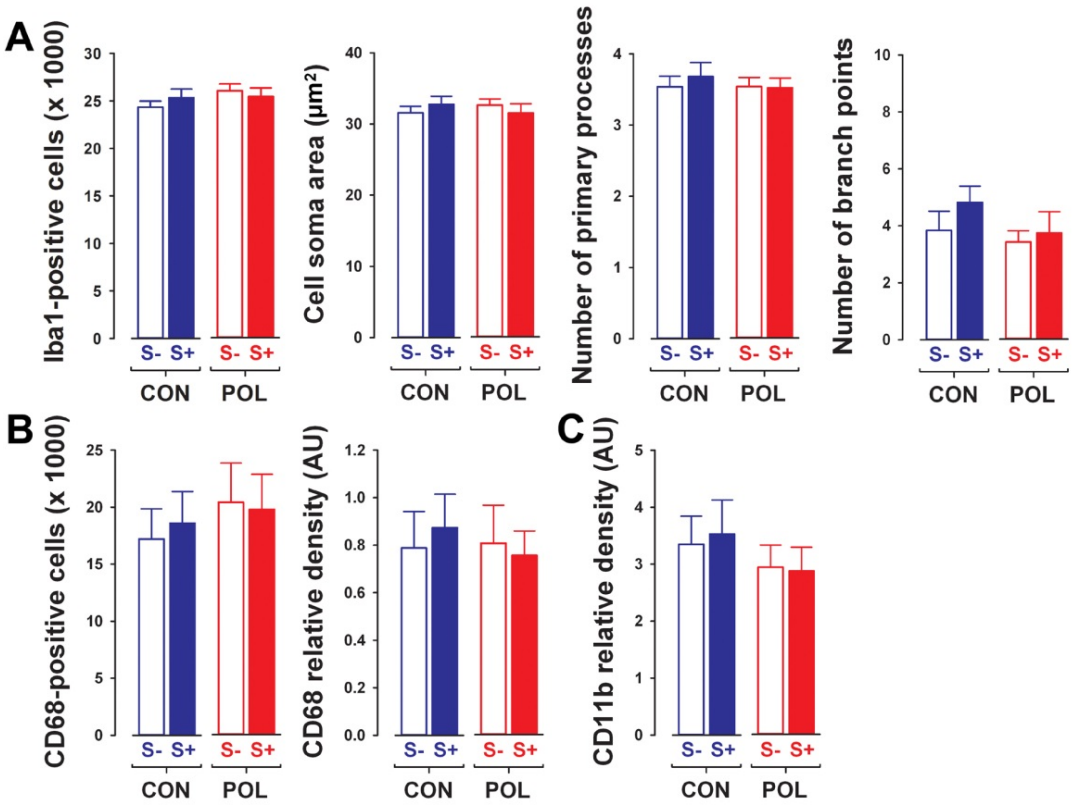


Supplementary Figure S8. Lack of neuroinflammatory signs in the adult CNS following single or combined exposure to prenatal immune activation and peri-pubertal stress. Pregnant C57BL/6 mice were exposed to poly(I:C) (POL) or control (CON) solution on gestation day 9, and the resulting offspring were subjected to sub-chronic stress (S+) or left undisturbed (S-) during peri-pubertal development (postnatal days [PNDs] 30~40). All immunohistochemical and cytokine protein measures were obtained on postnatal day 70, i.e. 4 weeks after exposure to the last peri-pubertal stressor. (A) The bar plots show the stereological estimates of CD68-positive microglia cells (cells/mm^3) and the relative optical density (in arbitrary units, AU) of CD68 immunoreactivity in the hippocampal formation (HPC; including CA1-CA3 region and dentate gyrus). *N*=11-12 per group. (B) The bar plots depict the stereological estimates of CD68-positive microglia cells (cells/mm^3) and the relative optical density (in arbitrary units, AU) of CD68 immunoreactivity in the medial prefrontal cortex (PFC; including anterior cingulate, prelimbic and infralimbic cortices). *N*=11-12 per group. (C) Tissue contents of IL-1β, TNF-α and PGE_2 in the HPC measured using particle-based flow cytometry. HPC levels of IL-6 and IL-10 protein were below detection limits. *N*=10-12 per group. All data are means±SEM.

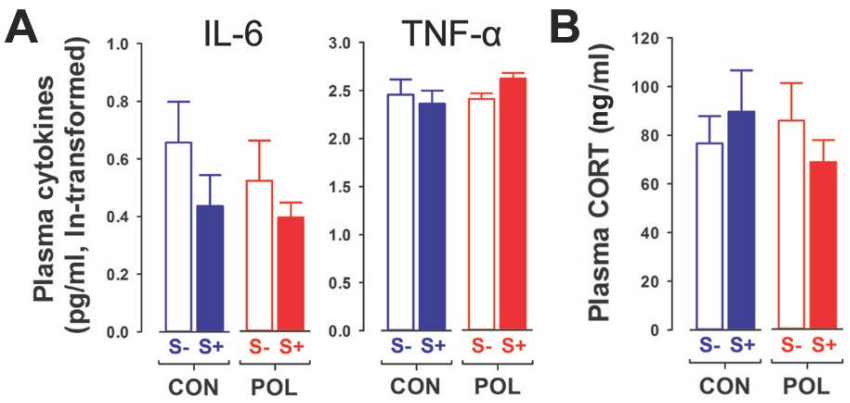


Supplementary Figure S9. Short-term effects (PND 41) of single or combined exposure to prenatal immune activation and peri-pubertal stress on cellular microglia parameters in the medial prefrontal cortex. Pregnant C57BL/6 mice were exposed to poly(I:C) (POL) or control (CON) solution on gestation day 9, and the resulting offspring were subjected to sub-chronic stress (S+) or left undisturbed (S-) during peri-pubertal development (postnatal days [PNDs] 30~40). Prefrontal sections were prepared from PND41 offspring. Hence, all measures were taken one day after exposure to the last peri-pubertal stressor. (A) The bar plots depict the stereological estimates of Iba1-positive microglia cells (cells/mm^3) and various morphological parameters (cell soma area, number of primary processes, and number of branch points). $^{\S}P < 0.05$, reflecting the significant main effect of peri-pubertal stress. *N*=11-12 per group. (B) The bar plots show the stereological estimates of CD68-positive microglia cells (cells/mm^3) and the relative optical density (in arbitrary units, AU) of CD68 immunoreactivity. $^{**}P < 0.01$, based on post-hoc

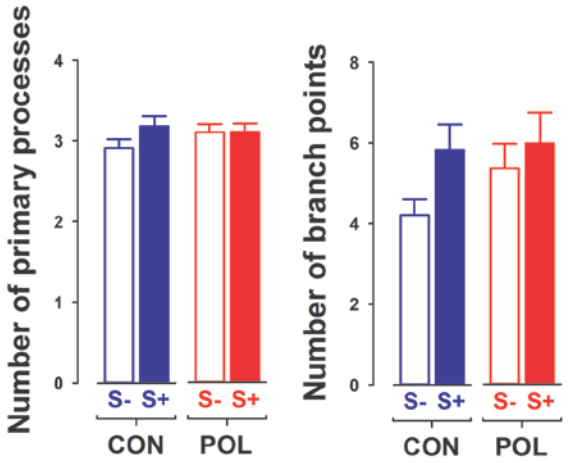
comparisons. $^{\S}P < 0.01$, reflecting the significant main effect of peri-pubertal stress; $^{\#}P < 0.01$, reflecting the significant main effect of prenatal immune activation. *N*=11-12 per group. (C) The bar plot depicts the relative optical density (in arbitrary units, AU) of CD11b immunoreactivity. $^{**}P < 0.01$, based on post-hoc comparisons. *N*=11-12 per group. (D) The photomicrographs show (color-coded) coronal brain sections of representative CON/S+ and POL/S+ offspring at the level of the PFC (highlighting anterior cingulate [aCG] and prelimbic [PrL] cortices) stained with anti-Iba1, -CD68, or -CD11b antibody. In color-coded sections (CD68 and CD11b), strongest staining intensities are shown in yellow, while the background is represented in dark purple (bar inset). All data are means±SEM.



Supplementary Figure S10. Neither single nor combined exposure to prenatal immune activation and peri-pubertal stress affect microglia parameters in the secondary motor cortex during puberty. Pregnant C57BL/6 mice were exposed to poly(I:C) (POL) or control (CON) solution on gestation day 9, and the resulting offspring were subjected to sub-chronic stress (S+) or left undisturbed (S-) during peri-pubertal development (postnatal days [PNDs] 30~40). Cortical sections containing the secondary motor cortex (M2) were prepared from PND 41 offspring. Hence, all measures were taken one day after exposure to the last peri-pubertal stressor. (A) The bar plots depict the stereological estimates of Iba1-positive microglia cells (cells/mm^3) and various morphological parameters (cell soma area, number of primary processes, and number of branch points.). *N*=11-12 per group. (B) The bar plots show the stereological estimates of CD68-positive microglia cells (cells/mm^3) and the relative optical density (in arbitrary units, AU) of CD68 immunoreactivity. *N*=11-12 per group. (C) The bar plot depicts the relative optical density (in arbitrary units, AU) of CD11b immunoreactivity. *N*=11-12 per group. All data are means±SEM.

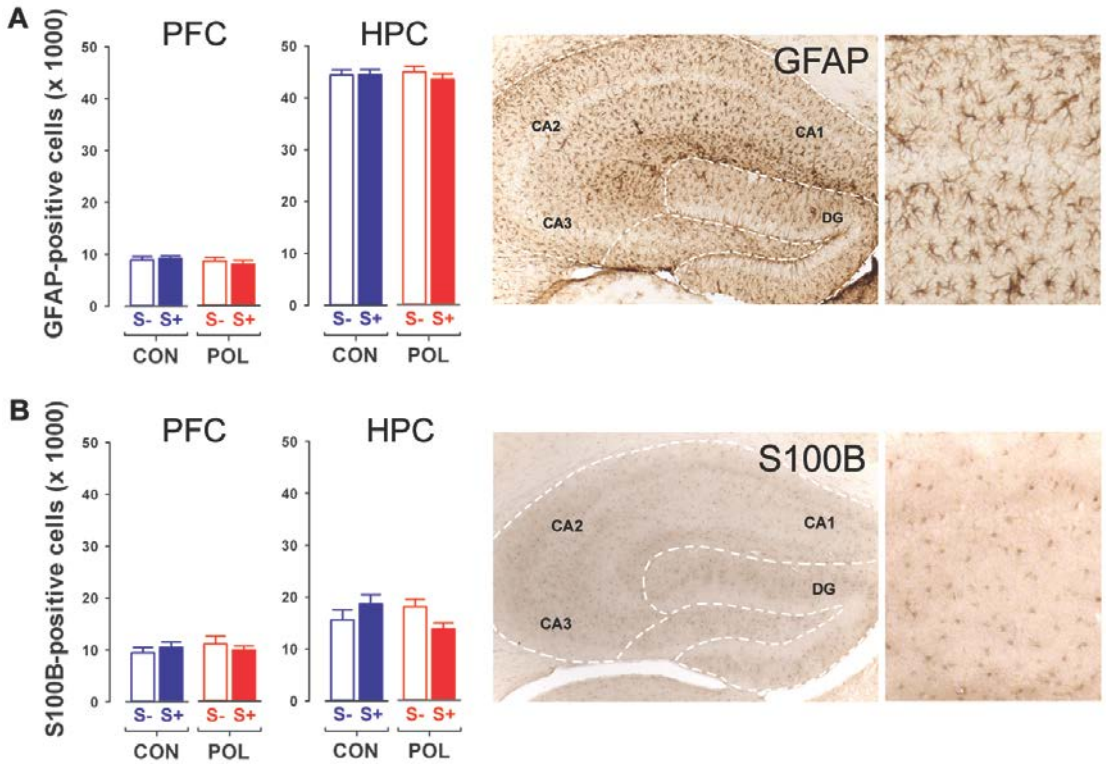


Supplementary Figure S11. Neither single nor combined exposure to prenatal immune activation and peri-pubertal stress affect plasma cytokine and corticosterone levels shortly after exposure to the last stressor in puberty. Pregnant C57BL/6 mice were exposed to poly(I:C) (POL) or control (CON) solution on gestation day 9, and the resulting offspring were subjected to sub-chronic stress (S+) or left undisturbed (S-) during peri-pubertal development (postnatal days [PNDs] 30~40). All measures were taken on postnatal day 41, i.e. one day after exposure to the last peri-pubertal stressor. (A) Plasma cytokine protein contents of IL-6 and TNF-α; IL-1β plasma levels were below detection limit (2.1 pg/ml). *N*=10-11 per group. (B) Plasma corticosterone (CORT) concentrations. *N*=12-14 per group. All data are means±SEM.

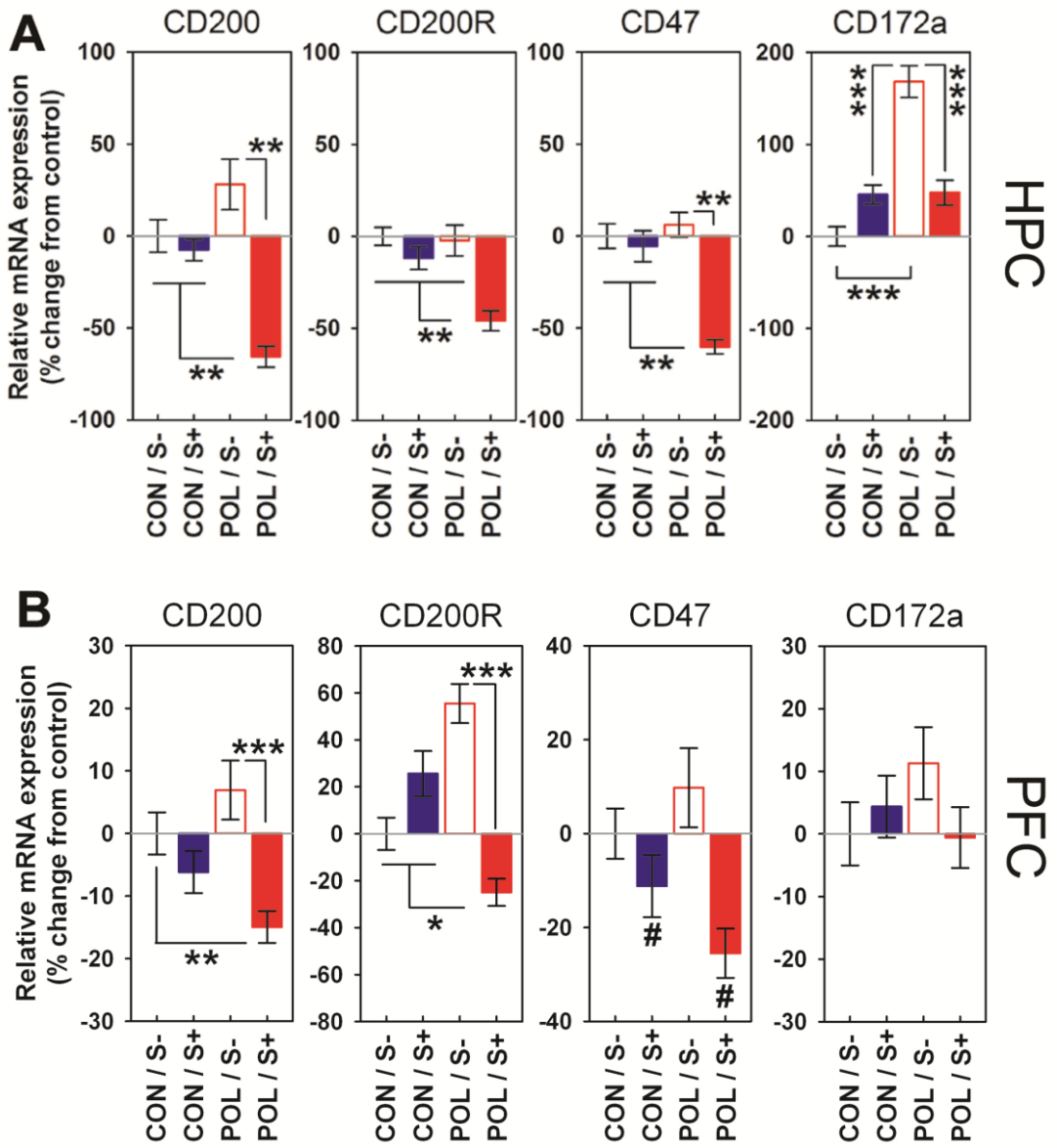


Supplementary Figure S12. Morphological parameters of microglia in the hippocampus of pubertal offspring exposed to single or combined prenatal immune activation and peri-pubertal stress. Pregnant C57BL/6 mice were exposed to poly(I:C) (POL) or control (CON) solution on gestation day 9, and the resulting offspring were subjected to sub-chronic stress (S+) or left undisturbed (S-) during peri-pubertal development (postnatal days [PNDs] 30~40). Hippocampal sections from PND 41 offspring were stained with anti-Iba1 to label the entire microglia population using standard immunohistochemical procedures. The bar plots depict the number of primary processes and number of branch points of randomly selected Iba1-positive cells in the CA3 region of the hippocampus. The cell soma areas of Iba1-postive cells in the

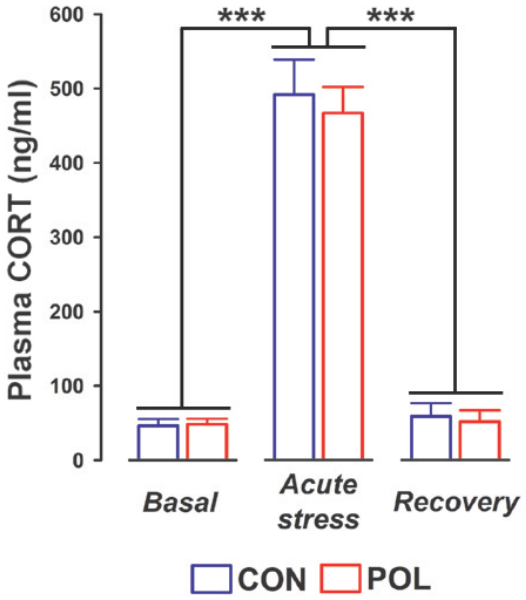
corresponding hippocampal sections are depicted in Figure 4A of the main document. *N*=11-12 per group. All data are means±SEM.



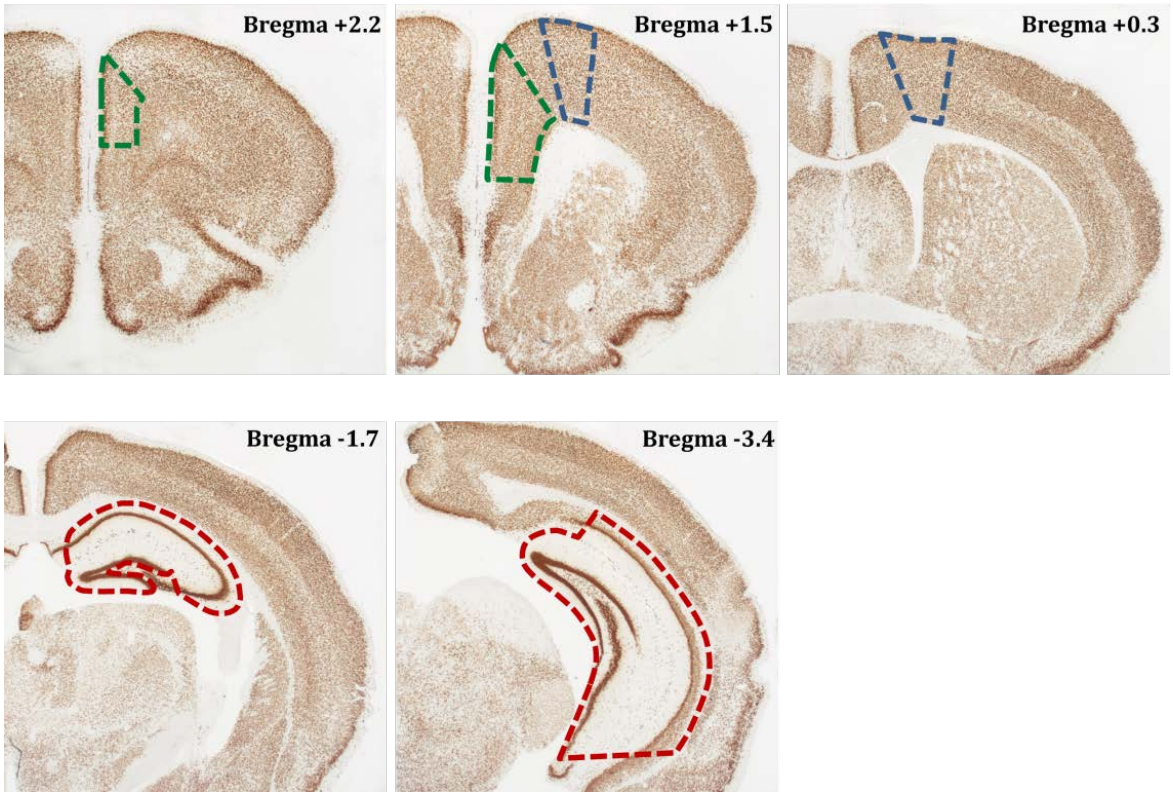
Supplementary Figure S13. Neither single nor combined exposure to prenatal immune activation and peri-pubertal stress affect astroglial markers in puberty. Pregnant C57BL/6 mice were exposed to poly(I:C) (POL) or control (CON) solution on gestation day 9, and the resulting offspring were subjected to sub-chronic stress (S+) or left undisturbed (S-) during peri-pubertal development (postnatal days [PNDs] 30~40). The astroglial markers glial fibrillary acidic protein (GFAP) and the S100 calcium binding protein B (S100B) were studied on postnatal day 41, i.e. one day after exposure to the last peri-pubertal stressor, using standard immunohistochemical procedures. (A) The bar plots show the stereological estimates of GFAP-positive cells in the medial prefrontal cortex (PFC; including anterior cingulate, prelimbic and infralimbic cortices) and hippocampal formation (HPC; including CA1-CA3 region and dentate gyrus); the photomicrographs show typical coronal brain sections stained with anti-GFAP antibody. *N*=11-12 per group. (B) The bar plots depicts the stereological estimates of S100B-positive cells in the PFC and HPC; the photomicrographs show typical coronal brain sections stained with anti-S100B antibody. *N*=11-12 per group. All data are means±SEM.



Supplementary Figure S14. Prenatal immune activation alters the sensitivity to stress-induced changes in neuron–microglia inhibitory signaling in the hippocampus and medial prefrontal cortex. (A) Real-time PCR analyses of contact-dependent neuron–microglia inhibitory signaling pairs (CD200-CD200R) and (CD47-CD172a) in the hippocampus (HPC) of prenatal poly(I:C) (POL) and control (CON) offspring that were subjected to acute (45 min restraint) stress (S+) or left non-stressed (S-) in puberty (postnatal day 30). *P < 0.05, **P < 0.01, and ***P < 0.001, based on post-hoc comparisons; $^{\#}P$ < 0.001, reflecting the significant main effect of stress. N=9-14 per group. (B) Real-time PCR analyses of contact-dependent neuron–microglia inhibitory signaling pairs (CD200-CD200R) and (CD47-CD172a) in the medial prefrontal cortex (PFC) of CON/S-, CON/S+, POL/S- and POL/S + offspring in puberty (postnatal day 30). *P < 0.05, **P < 0.01, and ***P < 0.001, based on post-hoc comparisons; $^{\#}P$ < 0.001, reflecting the significant main effect of stress. N=9-14 per group. All data are means±SEM.



Supplementary Figure S15. Plasma corticosterone responses to acute stress in puberty. Plasma corticosterone (CORT) levels in prenatal poly(I:C) (POL) and control (CON) offspring concentrations at basal conditions, immediately after acute (45 min restraint) stress, and following a recovery phase of 90 min post-stress in puberty (postnatal day 30). ***$P < 0.001$, reflecting the significant main effect of stress. N=11-23 per group. All data are means±SEM.



Supplementary Figure S16. Delineation of the brain regions of interest. The photomicrographs show NeuN peroxidase immunohistochemical stainings used to trace the regions of interest for the various immunohistochemical analyses. The green contours delineate the area of the medial prefrontal cortex, while the blue contours delineate the secondary motor cortex. The red contours delineate the hippocampal formation included in the analyses. Stereological and optical densitometric measurements in the medial prefrontal cortex were performed on sections ranging from Bregma +2.2 to +1.5 mm and included measures from the anterior cingulate, prelimbic, and infralimbic cortices. Analyses in the secondary motor cortex were performed on sections ranging from Bregma +1.5 to +0.3 mm. Stereological and optical densitometric measurements were performed on sections ranging from Bregma -1.30 to -3.45 mm and included measures from the CA1 to CA3 subfields and dentate gyrus (DG).

SUPPLEMENTARY TABLES

Cohort	PND	Test		CON/S-	CON/S+	POL/S-	POL/S+
1	70 - 73	EPM		16	18	19	16
	74 - 77	PPI		16	18	19	16
	80 - 90	AA-LI	NPE	7	9	9	9
			PE	9	9	10	7
	91 - 95	AMPH		8	9	10	8
	91 - 95	MK-801		8	8	8	8
2	41 - 42	EPM		15	14	14	13
	42 - 44	PPI		15	14	14	13
	45	AMPH		15	12	13	12
3	41 - 44	AA-LI	NPE	9	9	9	9
			PE	10	9	10	10
	45	MK-801		11	11	11	10
4	90 - 93	EPM		8	7	12	9
	96 - 100	PPI		8	7	12	9
	104 -110	AMPH		6	5	8	6

Supplementary Table 1. Summary of the number of offspring used and sequence of testing in the basic behavioral phenotyping. Pregnant C57BL/6 mice were exposed to poly(I:C) (POL) or control (CON) solution on gestation day 9, and the resulting offspring were subjected to sub-chronic stress (S+) or left undisturbed (S-) during either peri-pubertal (postnatal days [PNDs] 30~40) or adolescent (PNDs 50~60) maturation. In cohorts 1-3, the sub-chronic stress procedure was applied in peri-puberty (PNDs 30~40); cohort 4, the sub-chronic stress procedure was applied in peri-adolescence (PNDs 50~60). Offspring from cohort 1 were used to study the long-term behavioral effects in adulthood, i.e., when they reached postnatal days (PND) 70~100. Offspring were repeatedly tested in the following paradigms: Elevated plus maze (EPM) test, prepulse inhibition (PPI) test, latent inhibition test in conditioned active avoidance (AA-LI), locomotor response to systemic amphetamine (AMPH) treatment, and locomotor response to systemic dizocilpine (MK-801) treatment. The AMPH and MK-801 tests were performed using two distinct sub-cohorts of animals to avoid repeated exposure to psychotomimetic drugs. Offspring from cohort 2 were used to ascertain the short-term behavioral effects of the experimental manipulations on performance in the EPM, PPI and AMPH tests between PNDs 41 and 45, and offspring from cohort 3 were used to assess the short-term behavioral effects of the experimental manipulations on performance in the AA-LI and MK-801 tests in pubescence (i.e., between PNDs 41 and 45). Offspring from cohort 4 were used to study the long-term behavioral effects in adulthood, i.e., when they reached postnatal days (PND) 90~110, and included the EPM, AMPH and MK-801 tests. Again, the AMPH and MK-801 tests were performed using two distinct sub-cohorts of animals to avoid repeated exposure to psychotomimetic drugs. Offspring in each cohort derived from multiple independent litters (> 10) to avoid confounds from litter effects. Both male and female subjects were included in all behavioral tests; however, initial statistical analyses showed that there were no significant sex-specific effects associated with single or combined exposure to prenatal immune activation and postnatal stress. Therefore, the data of the two

sexes were combined for the final analyses and presentation of the data (Supplementary Table 7 to 19). NPE, non-pre-exposed; PE, pre-exposed.

Cohort	PND	Markers/Proteins	CON/S-	CON/S+	POL/S-	POL/S+
5	41	Iba1 CD68 CD11b GFAP S100b	12	12	12	11
6	70	Iba1 CD68	12	11	11	12
7	41	IL-1β *(HPC)* IL-6 *(HPC)* IL-10 *(HPC)* TNF-α *(HPC)* PGE2 *(HPC)*	13	14	12	12
8	41	IL-1β *(Plasma)* IL-6 *(Plasma)* TNF-α *(Plasma)*	10	11	11	11
9	70	IL-1β *(HPC)* IL-6 *(HPC)* IL-10 *(HPC)* TNF-α *(HPC)* PGE2 *(HPC)*	12	12	10	12

Supplementary Table 2. Summary of the number of offspring used for the immunohistochemical and protein analyses in the hippocampus and plasma. Pregnant C57BL/6 mice were exposed to poly(I:C) (POL) or control (CON) solution on gestation day 9, and the resulting offspring were subjected to sub-chronic stress (S+) or left non-stressed (S-) during the peri-pubertal (postnatal days [PNDs] 30~40) period. Animals from cohort 5 and 6 were sacrificed for immunohistochemical studies of the indicated markers on PND 41 and 70, respectively. Animals from cohort 7 and 8 were sacrificed for analyses of the indicated proteins in the hippocampus (HPC) and plasma on PND 41, and animals from cohort 9 were sacrificed for HPC protein analyses on PND 70. Offspring in each cohort derived from multiple independent litters (> 10) to avoid confounds from litter effects. Both male and female subjects were included in all immunohistochemical and protein analyses; however, initial statistical analyses showed that there were no significant sex-specific effects associated with single or combined exposure to prenatal immune activation and peri-pubertal stress. Therefore, the data of the two sexes were combined for the final analyses and presentation of the data (Supplementary Table 7 to 19).

Cohort	PND	Genes	CON/basal	CON/ acute stress	POL/basal	POL/ acute stress
10	30	CD200 CD200R CD47 CD172a	12	14	9	11

Supplementary Table 3. Summary of the number of offspring used for the real-time PCR gene expression analyses. Pregnant C57BL/6 mice were exposed to poly(I:C) (POL) or control (CON) solution on gestation day 9. Expression analyses of the indicated genes were conducted in offspring that were subjected to acute (45 min restraint) stress or left non-stressed (= basal) on postnatal day 30. Offspring in each cohort derived from multiple independent litters (> 8) to avoid confounds from litter effects. Both male and female subjects were included in all PCR analyses; however, initial statistical analyses showed that there

were no significant sex-specific effects associated with single or combined exposure to prenatal immune activation and acute stress exposure. Therefore, the data of the two sexes were combined for the final analyses and presentation of the data (Supplementary Table 7 to 19).

Cohort	PND	Marker	CON/S-	CON/S+	POL/S-	POL/S+		
11	41	CORT	13	14	12	12		
			CON basal	**CON acute stress**	**CON recovery**	**POL basal**	**POL acute stress**	**POL recovery**
12	30	CORT	23	14	11	12	11	12

Supplementary Table 4. Summary of the number of peri-pubertal offspring used for the plasma corticosterone (CORT) analyses. Pregnant C57BL/6 mice were exposed to poly(I:C) (POL) or control (CON) solution on gestation day 9. Offspring from cohort 11 were subjected to sub-chronic stress (S+) or left undisturbed (S-) during the peri-pubertal (postnatal days [PNDs] 30~40) period and then sacrificed on PND 41 for blood collection and subsequent CORT analyses in plasma. CON and POL offspring from cohort 12 were subjected to blood collection under basal condition, immediately after acute (45 min restraint) stress, or 90 min after acute stress exposure (= recovery) on PND 30. Offspring in each cohort derived from multiple independent litters (> 6) to avoid confounds from litter effects. Both male and female subjects were included in all CORT analyses; however, initial statistical analyses showed that there were no significant sex-specific effects associated with single or combined exposure to prenatal immune activation and stress. Therefore, the data of the two sexes were combined for the final analyses and presentation of the data (Supplementary Table 7 to 19).

Cohort	PND	Marker	CON/S-	CON/S+	POL/S-	POL/S+
13	70	CORT basal	12	12	12	12
		CORT acute Stress	13	13	12	12
		CORT recovery	12	12	12	12

Supplementary Table 5. Summary of the number of offspring used for the plasma corticosterone (CORT) analyses in adulthood. Pregnant C57BL/6 mice were exposed to poly(I:C) (POL) or control (CON) solution on gestation day 9, and the resulting offspring were subjected to sub-chronic stress (S+) or left non-stressed (S-) during the peri-pubertal (postnatal days [PNDs] 30~40) period. CON/S-, CON/S+, POL/S- and POL/S+ animals were subjected to blood collection and subsequent plasma CORT analysis under basal condition, immediately after acute (45 min restraint) stress, or 90 min after acute stress exposure (= recovery) on PND 70. Offspring derived from multiple independent litters (> 8) to avoid confounds from litter effects. Both male and female subjects were included in all CORT analyses; however, initial statistical analyses showed that there were no significant sex-specific effects associated with single or combined exposure to prenatal immune activation and stress. Therefore, the data of the two sexes were combined for the final analyses and presentation of the data (Supplementary Table 7 to 19).

Cohort	PND	Transmitter *(Region)*	CON/S-	CON/S+	POL/S-	POL/S+
13	80	DA (*HPC)*	10	11	8	9
		DA *(PFC)*	11	10	11	9
		DA (*NAc)*	10	10	9	8
		5-HT (*HPC)*	11	10	11	9
		5-HT (*PFC)*	11	10	11	9
		5-HT (*NAc)*	10	10	9	8

Supplementary Table 6. Summary of the number of offspring used for neurochemical analyses. Pregnant C57BL/6 mice were exposed to poly(I:C) (POL) or control (CON) solution on gestation day 9, and the resulting offspring were subjected to sub-chronic stress (S+) or were left undisturbed (S-) during peri-pubertal development (postnatal days [PNDs] 30~40). Animals were sacrificed in adulthood (PND 80) for brain tissue collection and subsequent neurochemical analyses. Offspring in each cohort derived from multiple independent litters (> 8) to avoid confounds from litter effects. Both male and female subjects were included in all neurochemical analyses; however, initial statistical analyses showed that there were there were no significant sex-specific effects associated with single or combined exposure to prenatal immune activation and stress. Therefore, the data of the two sexes were combined for the final analyses and presentation of the data (Supplementary Table 7 to 19). 5-HT, serotonin; DA, dopamine; HPC, hippocampus; NAc, nucleus accumbens; PFC, medial prefrontal cortex.

Cytokine	Dependent Measure	ANOVA	Effects	DF	F-Value	P-Value	Figure
IL-1β	Plasma concentration (pg/ml)		*below detection limit (2.1 pg/ml)*				
IL-6	Plasma concentration (pg/ml)	2 × 2 (MIA × Delay)	MIA	(1,22)	33.364	**<0.0001**	S2
			Delay	(1,22)	36.562	**<0.0001**	
			MIA × Delay	(1,22)	30.731	**<0.0001**	
IL-10	Plasma concentration (pg/ml)	2 × 2 (MIA × Delay)	MIA	(1,22)	17.274	**0.0004**	S2
			Delay	(1,22)	10.469	**0.0038**	
			MIA × Delay	(1,22)	6.925	**0.0152**	
TNF-α	Plasma concentration (pg/ml)	2 × 2 (MIA × Delay)	MIA	(1,22)	9.615	**0.0052**	S2
			Delay	(1,22)	6.187	**0.0209**	
			MIA × Delay	(1,22)	5.918	**0.0236**	

Supplementary Table 7. Summary of the statistical tests and outcomes for the cytokine analyses conducted in immune-challenged and non-challenged pregnant mice. The table specifies the dependent measures (plasma cytokine concentrations) for each cytokine of interest and summarizes the main effects of and interactions between the two between-subjects factors (maternal immune activation *N* = MIA; post-treatment interval = Delay). The table also specifies the corresponding degrees of freedom (DF) and F-values. Significant effects ($P < 0.05$) are given in bold font. IL, interleukin; TNF, tumor necrosis factor.

Behavioral Test	Dependent Measures	ANOVA	Effects	DF	F-Value	P-Value	Figure
Elevated plus maze	Relative open arm entries (%)	2 × 2 (MIA × Stress)	MIA	(1,65)	0.572	0.4521	
			Stress	(1,65)	6.772	**0.0115**	
			MIA × Stress	(1,65)	0.140	0.7091	
	Relative time in open arm (%)	2 × 2 (MIA × Stress)	MIA	(1,65)	2.429	0.1240	Fig. 1A
			Stress	(1,65)	5.534	**0.0217**	
			MIA × Stress	(1,65)	0.866	0.3555	
	Total distance moved (cm)	2 × 2 (MIA × Stress)	MIA	(1,65)	1.747	0.1909	Fig. 1A
			Stress	(1,65)	0.089	0.7664	
			MIA × Stress	(1,65)	1.133	0.2911	
Prepulse inhibition	PPI (%)	2 × 2 × 5 (MIA × Stress × Prepulse)	MIA	(1,65)	1.393	0.2424	Fig. 1C
			Stress	(1,65)	2.203	0.1429	
			MIA × Stress	(1,65)	4.233	**0.0439**	
			Prepulse	(4,260)	90.913	**<0.0001**	
	Startle reactivity (AU)	2 × 2 (MIA × Stress)	MIA	(1,65)	3.535	0.0646	
			Stress	(1,65)	2.931	0.0917	
			MIA × Stress	(1,65)	0.028	0.8670	
	Prepulse-induced reactivity (AU)	2 × 2 × 5 (MIA × Stress × Prepulse)	MIA	(1,65)	0.150	0.6999	
			Stress	(1,65)	0.637	0.4278	
			MIA × Stress	(1,65)	0.498	0.4829	
			Prepulse	(4,260)	41.760	**<0.0001**	
Latent inhibition in conditioned active avoidance	Response latency (sec)	2 × 2 × 2 × 5 (MIA × Stress × Pre-exposure × Blocks)	MIA	(1,61)	2.997	0.0885	Fig. 1B
			Stress	(1,61)	9.524	**0.0030**	
			Pre-exposure	(1,61)	12.569	**0.0008**	
			Pre-exposure × MIA	(1,61)	2.329	0.1322	
			Pre-exposure × Stress	(1,61)	0.712	0.4020	
			Pre-exposure × MIA × Stress	(1,61)	3.300	0.0742	
			Blocks	(4,244)	68.209	**<0.0001**	
Locomotor response to AMPH	Total distance moved following vehicle (cm)	2 × 2 × 6 (MIA × Stress × Bins)	MIA	(1,31)	2.258	0.4131	S3A
			Stress	(1,31)	0.505	0.4827	
			MIA × Stress	(1,31)	0.632	0.4326	
			Bins	(5,155)	48.323	**<0.0001**	
			Bins × MIA × Stress	(5,155)	1.567	0.1726	
	Total distance moved following AMPH (cm)	2 × 2 × 18 (MIA × Stress × Bins)	MIA	(1,31)	4.557	**0.0408**	Fig. 1D, S3A
			Stress	(1,31)	0.453	0.5058	
			MIA × Stress	(1,31)	5.217	**0.0294**	
			Bins	(17,527)	33.354	**<0.0001**	
			Bins × MIA × Stress	(17,527)	1.797	**0.0255**	
Locomotor response to MK-801	Total distance moved following vehicle (cm)	2 × 2 × 6 (MIA × Stress × Bins)	MIA	(1,28)	1.409	0.2452	S3B
			Stress	(1,28)	0.402	0.5313	
			MIA × Stress	(1,28)	0.098	0.7560	
			Bins	(5,140)	38.983	**<0.0001**	
			Bins × MIA × Stress	(5,140)	0.653	0.6597	
	Total distance moved following MK-801 (cm)	2 × 2 × 18 (MIA × Stress × Bins)	MIA	(1,28)	3.301	0.0800	Fig. 1E, S3B
			Stress	(1,28)	6.459	**0.0169**	
			MIA × Stress	(1,28)	1.435	**0.0443**	
			Bins	(17,476)	13.720	**<0.0001**	
			Bins × MIA × Stress	(17,476)	2.072	**0.0072**	

Supplementary Table 8. Summary of the statistical tests and outcomes for the behavioral analyses conducted in adult (postnatal day 70~100) offspring following prenatal immune activation and/or peri-pubertal stress. The table specifies the dependent measures for each behavioral test and summarizes the main effects of and interactions between the main between-subjects factors (maternal immune activation

N = MIA; peri-pubertal stress = Stress), as well as the main effects of and interactions between additional independent factors specific to each behavioral test. The table also specifies the corresponding degrees of freedom (DF) and F-values. Significant effects (P < 0.05) are given in bold font. AU, arbitrary units; AMPH, amphetamine; MK-801, dizocilpine; PPI, prepulse inhibition.

TEST	Dependent Measures	ANOVA	Effects	DF	F-Value	P-Value	Figure
Elevated plus maze	Relative open arm entries (%)	2 × 2 (MIA × Stress)	MIA	(1,52)	0.035	0.8523	
			Stress	(1,52)	5.393	**0.0242**	
			MIA × Stress	(1,52)	0.797	0.3761	
	Relative time in open arm (%)	2 × 2 (MIA × Stress)	MIA	(1,52)	0.171	0.6812	Fig. 2A
			Stress	(1,52)	4.249	**0.0443**	
			MIA × Stress	(1,52)	0.018	0.8933	
	Total distance moved (cm)	2 × 2 (MIA × Stress)	MIA	(1,52)	0.502	0.4816	Fig. 2A
			Stress	(1,52)	1.181	0.2822	
			MIA × Stress	(1,52)	2.481	0.1213	
Prepulse inhibition	PPI %	2 × 2 × 5 (MIA × Stress × Prepulse)	MIA	(1,52)	0.232	0.9270	Fig. 2C
			Stress	(1,52)	0.045	0.6318	
			MIA × Stress	(1,52)	0.008	0.8337	
			Prepulse	(4,208)	78.986	**<0.0001**	
	Startle reactivity (AU)	2 × 2 (MIA × Stress)	MIA	(1,52)	0.013	0.9080	
			Stress	(1,52)	1.373	0.2466	
			MIA × Stress	(1,52)	0.384	0.5380	
	Prepulse induced reactivity (AU)	2 × 2 × 5 (MIA × Stress × Prepulse)	MIA	(1,52)	0.554	0.4601	
			Stress	(1,52)	0.095	0.7591	
			MIA × Stress	(1,52)	0.972	0.4239	
			Prepulse	(4,208)	36.042	**<0.0001**	
Latent inhibition	Response latency (sec)	2 × 2 × 2 × 5 (MIA × Stress × Pre-exposure × Blocks)	MIA	(1,67)	0.374	0.5428	Fig. 2B
			Stress	(1,67)	1.021	0.3160	
			Pre-exposure	(1,67)	20.386	**<0.0001**	
			Pre-exposure × MIA	(1,67)	0.385	0.5372	
			Pre-exposure × Stress	(1,67)	0.424	0.5169	
			Pre-exposure × MIA × Stress	(1,67)	0.765	0.3848	
			Blocks	(4,268)	37.852	**<0.0001**	
Locomotor response to AMPH	Total distance moved following vehicle (cm)	2 × 2 × 6 (MIA × Stress × Bins)	MIA	(1,48)	0.539	0.4665	S4A
			Stress	(1,48)	0.708	0.4042	
			MIA × Stress	(1,48)	2.632	0.1113	
			Bins	(5,240)	40.398	**<0.0001**	
			Bins × MIA × Stress	(5,240)	0.709	0.6169	
	Total distance moved following AMPH (cm)	2 × 2 × 18 (MIA × Stress × Bins)	MIA	(1,48)	1.103	0.2989	Fig. 2D, S4A
			Stress	(1,48)	0.034	0.8547	
			MIA × Stress	(1,48)	2.339	0.1327	
			Bins	(17,816)	11.736	**<0.0001**	
			Bins × MIA × Stress	(17,816)	0.466	0.9673	
Locomotor response to MK-801	Total distance moved following vehicle (cm)	2 × 2 × 6 (MIA × Stress × Bins)	MIA	(1,40)	0.105	0.7476	S4B
			Stress	(1,40)	2.969	0.0926	
			MIA × stress	(1,40)	1.010	0.3209	
			Bins	(5,200)	20.676	**<0.0001**	
			Bins × MIA × Stress	(5,200)	1.189	0.3157	
	Total distance moved following MK-801 (cm)	2 × 2 × 18 (MIA × Stress × Bins)	MIA	(1,40)	0.001	0.9801	Fig. 2D, S4B
			Stress	(1,40)	2.311	0.1363	
			MIA × Stress	(1,40)	0.968	0.3311	
			Bins	(17,680)	15.904	**<0.0001**	
			Bins × MIA × Stress	(17,680)	1.030	0.4228	

Supplementary Table 9 (previous page). Summary of the statistical tests and outcomes for the behavioral analyses conducted in peri-pubertal (postnatal day 41~45) offspring. The table specifies the dependent measures for each behavioral test and summarizes the main effects of and interactions between the main between-subjects factors (maternal immune activation *N* = MIA; peri-pubertal stress = Stress), as well as the main effects of and interactions between additional independent factors specific to each behavioral test. The table also specifies the corresponding degrees of freedom (DF) and F-values. Significant effects ($P < 0.05$) are given in bold font. AU, arbitrary units; AMPH, amphetamine; MK-801, dizocilpine.

TEST	Dependent Measures	ANOVA	Effects	DF	F-Value	P-Value	Figure
Elevated plus maze	Relative open arm entries (%)	2 × 2 (MIA × Stress)	MIA	(1,32)	1.217	0.2781	S5A
			Stress	(1,32)	8.228	**0.0072**	
			MIA × Stress	(1,32)	1.553E^{-5}	0.9969	
	Relative time in open arm (%)	2 × 2 (MIA × Stress)	MIA	(1,32)	0.329	0.5701	S5A
			Stress	(1,32)	2.137	0.1535	
			MIA × Stress	(1,32)	0.108	0.7442	
	Total distance moved (cm)	2 × 2 (MIA × Stress)	MIA	(1,32)	0.159	0.6931	S5A
			Stress	(1,32)	0.195	0.6620	
			MIA × Stress	(1,32)	1.805	0.1885	
Prepulse inhibition	PPI (%)	2 × 2 × 5 (MIA × Stress × Prepulse)	MIA	(1,32)	0.007	0.9777	S5B
			Stress	(1,32)	0.022	0.8841	
			MIA × Stress	(1,32)	0.713	0.4048	
			Prepulse	(4,128)	42.666	**<0.0001**	
	Startle reactivity (AU)	2 × 2 (MIA × Stress)	MIA	(1,32)	0.052	0.8205	
			Stress	(1,32)	1.549	0.2223	
			MIA × Stress	(1,32)	0.753	0.3919	
	Prepulse induced reactivity (AU)	2 × 2 × 5 (MIA × Stress × Prepulse)	MIA	(1,32)	0.183	0.6720	
			Stress	(1,32)	1.044	0.3147	
			MIA × Stress	(1,32)	0.778	0.3844	
			Prepulse	(4,128)	18.116	**<0.0001**	
Locomotor response to AMPH	Total distance moved following vehicle (cm)	2 × 2 × 6 (MIA × Stress × Bins)	MIA	(1,21)	0.256	0.6183	S5C
			Stress	(1,21)	2.013	0.1706	
			MIA × Stress	(1,21)	0.016	0.9002	
			Bins	(5,105)	29.563	**<0.0001**	
			Bins × MIA × Stress	(5,105)	1.856	0.1083	
	Total distance moved following AMPH (cm)	2 × 2 × 18 (MIA × Stress × Bins)	MIA	(1,21)	0.915	0.3497	S5C
			Stress	(1,21)	0.731	0.4021	
			MIA × Stress	(1,21)	0.065	0.8010	
			Bins	(17,357)	19.267	**<0.0001**	
			Bins × MIA × Stress	(17,357)	0.485	0.9590	

Supplementary Table 10. Summary of the statistical tests and outcomes for the behavioral analyses conducted in adult (postnatal day 90~110) offspring following prenatal immune activation and/or peri-adolescent stress. The table specifies the dependent measures for each behavioral test and summarizes the main effects of and interactions between the main between-subjects factors (maternal immune activation *N* = MIA; peri-adolescent stress = Stress), as well as the main effects of and interactions between additional independent factors specific to each behavioral test. The table also specifies the

corresponding degrees of freedom (DF) and F-values. Significant effects ($P < 0.05$) are given in bold font. AU, arbitrary units; AMPH, amphetamine; PPI, prepulse inhibition.

Cellular Marker	Region	Dependent Measures	Effects	DF	F-Value	P-Value	Figure
Iba1	HPC	Number of cells (cells/mm³)	MIA	(1,44)	1.162	0.2869	Fig. 4A
			Stress	(1,44)	4.107	**0.0488**	
			MIA × Stress	(1,44)	0.286	0.5953	
	PFC	Number of cells (cells/mm³)	MIA	(1,43)	0.707	0.4051	S9A
			Stress	(1,43)	0.075	0.7852	
			MIA × Stress	(1,43)	0.087	0.7695	
	MC	Number of cells (cells/mm³)	MIA	(1,43)	2.048	0.1596	S10A
			Stress	(1,43)	0.400	0.5305	
			MIA × Stress	(1,43)	1.863	0.1794	
CD68	HPC	Number of cells (cells/mm³)	MIA	(1,44)	11.601	**0.0014**	Fig. 4B
			Stress	(1,44)	17.654	**0.0001**	
			MIA × Stress	(1,44)	10.091	**0.0027**	
	PFC	Number of cells (cells/mm³)	MIA	(1,44)	8.103	**0.0067**	S9B
			Stress	(1,44)	14.984	**0.0004**	
			MIA × Stress	(1,44)	9.987	**0.0029**	
	MC	Number of cells (cells/mm³)	MIA	(1,44)	0.003	0.9582	S10B
			Stress	(1,44)	0.015	0.9021	
			MIA × Stress	(1,44)	1.443E-6	0.9990	
	HPC	Relative optical density (AU)	MIA	(1,44)	4.225	**0.0458**	
			Stress	(1,44)	6.535	**0.0141**	
			MIA × Stress	(1,44)	7.547	**0.0087**	
	PFC	Relative optical density (AU)	MIA	(1,44)	12.096	**0.0012**	S9B
			Stress	(1,44)	11.211	**0.0017**	
			MIA × Stress	(1,44)	2.049	0.1594	
	MC	Relative optical density (AU)	MIA	(1,44)	0.541	0.4660	S10B
			Stress	(1,44)	0.058	0.8115	
			MIA × Stress	(1,44)	0.731	0.3971	
CD11b	HPC	Relative optical density (AU)	MIA	(1,44)	14.766	**0.0004**	Fig. 4C
			Stress	(1,44)	20.112	**<.0001**	
			MIA × Stress	(1,44)	9.811	**0.0031**	
	PFC	Relative optical density (AU)	MIA	(1,44)	8.220	**0.0063**	S9C
			Stress	(1,44)	13.881	**0.0006**	
			MIA × Stress	(1,44)	4.107	**0.0488**	
	MC	Relative optical density (AU)	MIA	(1,44)	1.699	0.1992	S10C
			Stress	(1,44)	0.018	0.8951	
			MIA × Stress	(1,44)	0.089	0.7666	
GFAP	HPC	Number of cells (cells/mm³)	MIA	(1,44)	0.022	0.8816	S13A
			Stress	(1,44)	0.026	0.8726	
			MIA × Stress	(1,44)	0.053	0.8186	
	PFC	Number of cells (cells/mm³)	MIA	(1,44)	1.307	0.2591	S13A
			Stress	(1,44)	0.048	0.8277	
			MIA × Stress	(1,44)	0.476	0.4940	
S100B	HPC	Number of cells (cells/mm³)	MIA	(1,44)	0.793	0.3780	S13B
			Stress	(1,44)	0.013	0.9108	
			MIA × Stress	(1,44)	3.303	0.0760	
	PFC	Number of cells (cells/mm³)	MIA	(1,44)	0.183	0.6708	S13B
			Stress	(1,44)	0.011	0.9172	
			MIA × Stress	(1,44)	1.029	0.3160	

Supplementary Table 11. Summary of the statistical tests and outcomes for the immunohistochemcial analyses conducted in peri-pubertal (postnatal day 41) offspring following prenatal immune activation and/or peri-pubertal stress. The table specifies the region of interest in which each immunohistochemical marker was evaluated, and summarizes the main effects of and interactions between the main between-subjects factors (maternal immune activation *N* = MIA; peri-pubertal stress = Stress). The table also specifies the corresponding degrees of freedom (DF) and F-values. Significant effects ($P < 0.05$) are given in bold font. AU, arbitrary units; HPC, hippocampus; MC, secondary motor cortex; PFC, medial prefrontal cortex.

Cellular Marker	Region	Dependent Measures	Effects	DF	F-Value	P-Value	Figure
Iba1 Cell Morphology PND41	HPC	Cell soma area (µm²)	MIA	(1,43)	0.399	0.5310	Fig. 4A
			Stress	(1,43)	7.842	**0.0076**	
			MIA × Stress	(1,43)	0.523	0.4736	
	PFC	Cell soma area (µm²)	MIA	(1,43)	1.438	0.2371	S9A
			Stress	(1,43)	0.003	0.9576	
			MIA × Stress	(1,43)	1.171	0.2853	
	MC	Cell soma area (µm²)	MIA	(1,43)	0.024	0.8787	S10A
			Stress	(1,43)	0.001	0.9821	
			MIA × Stress	(1,43)	1.154	0.2890	
	HPC	Primary processes (number/cell)	MIA	(1,43)	0.224	0.6381	S12
			Stress	(1,43)	1.589	0.2142	
			MIA × Stress	(1,43)	1.518	0.2246	
	PFC	Primary processes (number/cell)	MIA	(1,43)	0.420	0.5202	S9A
			Stress	(1,43)	0.008	0.9309	
			MIA × Stress	(1,43)	0.249	0.6202	
	MC	Primary processes (number/cell)	MIA	(1,43)	1.409	0.2421	S10A
			Stress	(1,43)	0.059	0.8098	
			MIA × Stress	(1,43)	0.017	0.8977	
	HPC	Branch points (number/ cell)	MIA	(1,43)	1.107	0.2986	S12
			Stress	(1,43)	3.391	0.0725	
			MIA × Stress	(1,43)	0.651	0.4241	
	PFC	Branch points (number/ cell)	MIA	(1,43)	0.027	0.8708	S9A
			Stress	(1,43)	4.689	**0.0359**	
			MIA × Stress	(1,43)	0.890	0.3508	
	MC	Branch points (number/ cell)	MIA	(1,43)	1.578	0.2162	S10A
			Stress	(1,43)	1.138	0.2923	
			MIA × Stress	(1,43)	0.300	0.5870	

Supplementary Table 12. Summary of the statistical tests and outcomes for the microglia morphological analyses conducted in peri-pubertal (postnatal day 41) offspring following prenatal immune activation and/or peri-pubertal stress. The table specifies the region of interest in which microglia morphology was evaluated, and summarizes the main effects of and interactions between the main between-subjects factors (maternal immune activation *N* = MIA; peri-pubertal stress = Stress). The table also specifies the corresponding degrees of freedom (DF) and F-values. Significant effects ($P < 0.05$) are given in bold font. AU, arbitrary units; HPC, hippocampus; MC, secondary motor cortex; PFC, medial prefrontal cortex.

Protein/Marker	Dependent Measure	ANOVA	Effects	DF	F-Value	P-Value	Figure
IL-1β	HPC concentration (pg/mg)	2 × 2 (MIA × Stress)	MIA	(1,47)	12.590	**0.0009**	Fig. 4E
			Stress	(1,47)	17.991	**0.0001**	
			MIA × Stress	(1,47)	11.888	**0.0012**	
TNF-α	HPC concentration (pg/mg)	2 × 2 (MIA × Stress)	MIA	(1,47)	7.840	**0.0047**	Fig. 4E
			Stress	(1,47)	7.097	**0.0105**	
			MIA × Stress	(1,47)	3.398	0.0716	
PGE_2	HPC concentration (pg/mg)	2 × 2 (MIA × Stress)	MIA	(1,47)	0.665	0.4190	Fig. 4E
			Stress	(1,47)	1.563	0.2175	
			MIA × Stress	(1,47)	0.515	0.4766	
IL-6	Plasma concentration (pg/ml, ln-transformed)	2 × 2 (MIA × Stress)	MIA	(1,39)	1.063	0.3089	S11A
			Stress	(1,39)	2.282	0.1390	
			MIA × Stress	(1,39)	0.652	0.4242	
TNF-α	Plasma concentration (pg/ml, ln-transformed)	2 × 2 (MIA × Stress)	MIA	(1,39)	0.152	0.6987	S11A
			Stress	(1,39)	0.004	0.9493	
			MIA × Stress	(1,39)	0.644	0.4273	

Supplementary Table 13. Summary of the statistical tests and outcomes for the analyses of hippocampal and plasma inflammatory proteins in peri-pubertal (postnatal day 41) offspring following prenatal immune activation and/or peri-pubertal stress. The table specifies the dependent measures for each inflammatory protein and summarizes the main effects of and interactions between the main between-subjects factors (maternal immune activation *N* = MIA; peri-pubertal stress = Stress). The table also specifies the corresponding degrees of freedom (DF) and F-values. Significant effects ($P < 0.05$) are given in bold font. HPC, hippocampus.

Corticosterone	Dependent Measure	ANOVA	Effects	DF	F-Value	P-Value	Figure
Peri-puberty following sub-chronic stress (PND 41)	Plasma concentration (ng/ml)	2 × 2 (MIA × SC-Stress)	MIA	(1,46)	0.006	0.9411	S11B
			SC-Stress	(1,46)	0.013	0.9093	
			MIA × SC-Stress	(1,46)	0.470	0.4963	
Peri-puberty following acute stress (PND 30)	Plasma concentration (ng/ml)	2 × 3 (MIA × A-Stress)	MIA	(2,82)	0.287	0.5938	S15
			A-Stress	(2,82)	228.195	**<0.0001**	
			MIA × A-Stress	(2,82)	0.195	0.8235	
Adulthood following acute stress (PND 70)	Plasma concentration (ng/ml)	2 × 2 × 3 (MIA × SC-Stress × A-Stress)	MIA	(1,132)	0.188	0.6656	S6
			SC-Stress	(1,132)	0.460	0.4986	
			A-Stress	(2,132)	302.665	**<0.0001**	
			MIA × SC-Stress × A-Stress	(2,132)	0.674	0.5115	

Supplementary Table 14. Summary of the statistical tests and outcomes for the plasma corticosterone (CORT) analyses in peri-pubertal and adult offspring following prenatal immune activation and/or peri-pubertal stress. The table specifies the dependent measures and summarizes the main effects of and interactions between the main between-subjects factors (maternal immune activation *N* = MIA; peri-pubertal stress, either in the form of sub-chronic [SC] stress between PND30 and 40 and/or in the form of acute [A] stress on PND 30 or 70). The table also specifies the corresponding degrees of freedom (DF) and F-values. Significant effects ($P < 0.05$) are given in bold font. PND, postnatal day.

Gene	Region	Dependent Measure	ANOVA	Effects	DF	F-Value	P-Value	Figure
CD200	HPC	Relative mRNA expression (%)	2 × 2 (MIA × Stress)	MIA	(1,43)	0.128	0.7223	S14A
				Stress	(1,43)	20.416	**<0.0001**	
				MIA × Stress	(1,43)	13.229	**0.0007**	
	PFC	Relative mRNA expression (%)	2 × 2 (MIA × Stress)	MIA	(1,43)	0.071	0.7912	S14B
				Stress	(1,43)	16.265	**0.0002**	
				MIA × Stress	(1,43)	5.092	**0.0292**	
CD200R	HPC	Relative mRNA expression (%)	2 × 2 (MIA × Stress)	MIA	(1,43)	5.962	**0.0188**	S14A
				Stress	(1,43)	24.389	**<0.0001**	
				MIA × Stress	(1,43)	4.271	**0.0448**	
	PFC	Relative mRNA expression (%)	2 × 2 (MIA × Stress)	MIA	(1,43)	0.098	0.7557	S14B
				Stress	(1,43)	11.909	**0.0013**	
				MIA × Stress	(1,43)	44.447	**<0.0001**	
CD47	HPC	Relative mRNA expression (%)	2 × 2 (MIA × Stress)	MIA	(1,43)	2.989	0.0910	S14A
				Stress	(1,43)	9.467	**0.0036**	
				MIA × Stress	(1,43)	6.805	**0.0125**	
	PFC	Relative mRNA expression (%)	2 × 2 (MIA × Stress)	MIA	(1,43)	0.557	0.4595	S14B
				Stress	(1,43)	16.279	**0.0002**	
				MIA × Stress	(1,43)	2.236	0.1422	
CD172a	HPC	Relative mRNA expression (%)	2 × 2 (MIA × Stress)	MIA	(1,43)	34.869	**<0.0001**	S14A
				Stress	(1,43)	7.790	**0.0078**	
				MIA × Stress	(1,43)	33.381	**<0.0001**	
	PFC	Relative mRNA expression (%)	2 × 2 (MIA × Stress)	MIA	(1,43)	0.996	0.3239	S14B
				Stress	(1,43)	1.238	0.2720	
				MIA × Stress	(1,43)	3.819	0.0672	

Supplementary Table 15. Summary of the statistical tests and outcomes for the analyses of gene expression following prenatal immune activation and additional acute stress exposure in peri-puberty (postnatal day 30). The table specifies the selected brain region and the dependent measures for each gene of interest. The table also summarizes the main effects of and interactions between the main between-subjects factors (maternal immune activation *N* = MIA; acute stress exposure = Stress). The table also specifies the corresponding degrees of freedom (DF) and F-values. Significant effects ($P < 0.05$) are given in bold font. HPC, hippocampus; PFC, medial prefrontal cortex.

Cellular Marker	Region	Dependent Measures	Effects	DF	F-Value	P-Value	Figure
Iba1	HPC	Number of cells (cells/mm^3)	MIA	(1,42)	0.656	0.4227	S7A
			Stress	(1,42)	5.191	**0.0279**	
			MIA × Stress	(1,42)	1.187	0.2821	
	PFC	Number of cells (cells/mm^3)	MIA	(1,42)	0.020	0.8892	S7A
			Stress	(1,42)	0.008	0.9292	
			MIA × Stress	(1,42)	0.737	0.3955	
	MC	Number of cells (cells/mm^3)	MIA	(1,42)	0.709	0.4045	S7A
			Stress	(1,42)	1.172	0.2852	
			MIA × Stress	(1,42)	0.017	0.8968	
CD68	HPC	Relative optical density (AU)	MIA	(1,42)	0.189	0.6590	S8A
			Stress	(1,42)	0.006	0.9399	
			MIA × Stress	(1,42)	1.576	0.2163	
	PFC	Relative optical density (AU)	MIA	(1,42)	0.039	0.8447	S8B
			Stress	(1,42)	1.125	0.2949	
			MIA × Stress	(1,42)	0.208	0.6504	
	HPC	Number of cells (cells/mm^3)	MIA	(1,42)	0.020	0.8875	S8A
			Stress	(1,42)	0.164	0.6877	
			MIA × Stress	(1,42)	2.472	0.1234	
	PFC	Number of cells (cells/mm^3)	MIA	(1,42)	0.562	0.4575	S8B
			Stress	(1,42)	0.005	0.9426	
			MIA × Stress	(1,42)	1.046	0.3124	

Supplementary Table 16. Summary of the statistical tests and outcomes for the immunohistochemcial analyses conducted in adult (postnatal day 70) offspring following prenatal immune activation and/or peri-pubertal stress. The table specifies the region of interest in which each immunohistochemical marker was evaluated, the dependent measures for each gene of interest, and summarizes the main effects of and interactions between the main between-subjects factors (maternal immune activation *N* = MIA; peri-pubertal stress = Stress). The table also specifies the corresponding degrees of freedom (DF) and F-values. Significant effects ($P < 0.05$) are given in bold font. AU, arbitrary units; HPC, hippocampus; MC, secondary motor cortex; PFC, medial prefrontal cortex.

Cellular Marker	Region	Dependent Measures	Effects	DF	F-Value	P-Value	Figure
Iba1 Cell Morphology PND70	HPC	Cell soma area (µm²)	MIA	(1,42)	0.549	0.4627	S7B
			Stress	(1,42)	0.005	0.9462	
			MIA × Stress	(1,42)	0.069	0.7948	
	PFC	Cell soma area (µm²)	MIA	(1,42)	1.123	0.2953	S7B
			Stress	(1,42)	0.554	0.4610	
			MIA × Stress	(1,42)	0.278	0.6007	
	MC	Cell soma area (µm²)	MIA	(1,42)	0.041	0.8409	S7B
			Stress	(1,42)	0.282	0.5981	
			MIA × Stress	(1,42)	1.527	0.2234	
	HPC	Primary processes (number/cell)	MIA	(1,42)	0.778	0.3829	S7C
			Stress	(1,42)	0.834	0.3664	
			MIA × Stress	(1,42)	0.303	0.5850	
	PFC	Primary processes (number/cell)	MIA	(1,42)	0.632	0.4312	S7C
			Stress	(1,42)	0.033	0.8570	
			MIA × Stress	(1,42)	0.334	0.5666	
	MC	Primary processes (number/cell)	MIA	(1,42)	0.060	0.8081	S7C
			Stress	(1,42)	0.144	0.7066	
			MIA × Stress	(1,42)	0.132	0.7184	
	HPC	Branch points (number/ cell)	MIA	(1,42)	0.673	0.4165	S7D
			Stress	(1,42)	0.086	0.7702	
			MIA × Stress	(1,42)	0.119	0.7319	
	PFC	Branch points (number/ cell)	MIA	(1,42)	2.538	0.1187	S7D
			Stress	(1,42)	0.646	0.4261	
			MIA × Stress	(1,42)	0.047	0.8291	
	MC	Branch points (number/ cell)	MIA	(1,42)	1.767	0.1909	S7D
			Stress	(1,42)	0.490	0.4876	
			MIA × Stress	(1,42)	0.474	0.4950	

Supplementary Table 17. Summary of the statistical tests and outcomes for the microglia morphological analyses conducted in adult (postnatal day 70) offspring following prenatal immune activation and/or peri-pubertal stress. The table specifies the region of interest in which microglia morphology was evaluated, and summarizes the main effects of and interactions between the main between-subjects factors (maternal immune activation *N* = MIA; peri-pubertal stress = Stress). The table also specifies the corresponding degrees of freedom (DF) and F-values. AU, arbitrary units; HPC, hippocampus; MC, secondary motor cortex; PFC, medial prefrontal cortex.

Protein/Marker	Dependent Measure	ANOVA	Effects	DF	F-Value	P-Value	Figure
IL-1β	HPC concentration (pg/mg)	2 × 2 (MIA × Stress)	MIA	(1,42)	2.587	0.1153	S8C
			Stress	(1,42)	3.169	0.0823	
			MIA × Stress	(1,42)	0.329	0.5690	
TNF-α	HPC concentration (pg/mg)	2 × 2 (MIA × Stress)	MIA	(1,42)	0.006	0.9399	S8C
			Stress	(1,42)	0.102	0.7516	
			MIA × Stress	(1,42)	2.502	0.1212	
PGE_2	HPC concentration (pg/mg)	2 × 2 (MIA × Stress)	MIA	(1,42)	0.139	0.7113	S8C
			Stress	(1,42)	0.975	0.3291	
			MIA × Stress	(1,42)	0.061	0.8058	

Supplementary Table 18. Summary of the statistical tests and outcomes for the analyses of hippocampal inflammatory proteins in adult (postnatal day 70) offspring following prenatal immune activation and/or peri-pubertal stress. The table specifies the dependent measures for each inflammatory protein and summarizes the main effects of and interactions between the main between-subjects factors (maternal immune activation *N* = MIA; peri-pubertal stress = Stress). The table also specifies the corresponding degrees of freedom (DF) and F-values. HPC, hippocampus.

Transmitter	Region	Dependent Measure	ANOVA	Effects	DF	F-Value	P-Value	Figure
Dopamine	HPC	Content (nM/mg, sqrt-transformed)	2 × 2 (MIA × Stress)	MIA	(1,34)	2.765	0.1056	Fig. 3A
				Stress	(1,34)	4.298	**0.0458**	
				MIA × Stress	(1,34)	3.268	0.0795	
	PFC	Content (nM/mg, sqrt-transformed)	2 × 2 (MIA × Stress)	MIA	(1,37)	0.040	0.8431	Fig. 3A
				Stress	(1,37)	0.057	0.8121	
				MIA × Stress	(1,37)	0.133	0.7172	
	NAc	Content (nM/mg, sqrt-transformed)	2 × 2 (MIA × Stress)	MIA	(1,33)	5.954	**0.0202**	Fig. 3A
				Stress	(1,33)	0.326	0.5718	
				MIA × Stress	(1,33)	0.722	0.4016	
Serotonin	HPC	Content (nM/mg, sqrt-transformed)	2 × 2 (MIA × Stress)	MIA	(1,37)	0.158	0.6926	Fig. 3B
				Stress	(1,37)	0.105	0.7483	
				MIA × Stress	(1,37)	0.405	0.5285	
	PFC	Content (nM/mg, sqrt-transformed)	2 × 2 (MIA × Stress)	MIA	(1,37)	1.553	0.2206	Fig. 3B
				Stress	(1,37)	4.736	**0.0360**	
				MIA × Stress	(1,37)	0.034	0.8556	
	NAc	Content (nM/mg, sqrt-transformed)	2 × 2 (MIA × Stress)	MIA	(1,33)	$9.643E^{-5}$	0.9922	Fig. 3B
				Stress	(1,33)	0.275	0.6033	
				MIA × Stress	(1,33)	2.704	0.1096	

Supplementary Table 19. Summary of the statistical tests and outcomes for the neurochemical analyses conducted in adult (postnatal day 70) offspring following single or combined prenatal immune activation and/or stress. The table specifies the region of interest in which each neurochemical marker was evaluated, the dependent measures for each transmitter/region, and summarizes the main effects of and interactions between the main between-subjects factors (maternal immune activation and stress = MIA; peri-pubertal stress = Stress). The table also specifies the corresponding degrees of freedom (DF) and F-values. Significant effects ($P < 0.05$) are given in bold font. HPC, hippocampus; NAc, nucleus accumbens; PFC, medial prefrontal cortex.

SUPPLEMENTARY MATERIALS & METHODS

Animals

C57BL/6 mice were used throughout the study. Female and male breeders were obtained from our in-house specific pathogen free (SPF) colony at the age of 10-14 weeks. Breeding began after 1-2 weeks of acclimatization to the new animal holding room, which was a temperature- and humidity-controlled (21±1 °C, 55±5 %) holding facility under a reversed light-dark cycle (lights off: 8:00 A.M. to 8:00 P.M.). All animals had *ad libitum* access to food (Kliba 3430, Klibamuehlen, Kaiseraugst, Switzerland) and water unless specified otherwise. All procedures described in the present study had been previously approved by the Cantonal Veterinarian's Office of Zurich and are in agreement with the principles of laboratory animal care in the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication, 2011). All efforts were made to minimize the number of animals used and their suffering.

Maternal immune activation during pregnancy

For the purpose of the maternal immunological manipulation during pregnancy, female mice were subjected to a timed-mating procedure as described previously *(*Meyer et al., 2005*)*. Pregnant dams on gestation day 9 (GD 9) received either a single injection of poly(I:C) (potassium salt; Sigma-Aldrich) at a dose of 1 mg/kg or vehicle (sterile pyrogen-free 0.9% NaCl) according to protocols established before (Meyer et al., 2005*)*. The gestational stage (i.e., GD 9) was selected because of our previous findings showing that GD 9 poly(I:C) treatment is capable of inducing long-term abnormalities in multiple behavioral and cognitive domains *(*Meyer et al., 2005*)*. Furthermore, the GD 9 window in the mouse roughly corresponds to the middle of the first trimester of human pregnancy, which in turn is the gestational stage known to be highly sensitive with respect to increasing the risk of schizophrenia and autism following maternal infection *(*Brown et al., 2004; Atladóttir *et al., 2010)*. A low dose of poly(I:C) (i.e., 1 mg/kg, i.v.) was used based on our previous dose-response studies in C57BL/6 mice showing that prenatal exposure to low doses of poly(I:C) lead only to restricted behavioral abnormalities in adulthood, thereby avoiding possible ceiling effects of the prenatal immunological manipulation on long-term brain and behavioral functions *(*Meyer *et al., 2009;* Meyer and Feldon, 2012*)*.

Poly(I:C) was dissolved in sterile pyrogen-free 0.9% NaCl (= vehicle) solution to yield a final concentration of 0.2 mg/ml and was administered via the intravenous (i.v) route at the tail vein under mild physical constraint as described in detail elsewhere *(*Meyer et al., 2005*)*. All solutions were freshly prepared at the day of administration and injected with a volume of 5 ml/kg.

Peri-pubertal and adolescent stress exposure

Offspring born to poly(I:C)- or saline-treated mothers were weaned and sexed on postnatal day (PND) 21. They were caged as littermates of 2-3 animals in Makrolon Type III cages (Techniplast, Milan, Italy). Litters with four or more animals per sex were split into two cages. All animals of a particular housing cage underwent the same peri-pubertal stress procedure (see below). To minimize potential confounds associated with litter effects, equal numbers of offspring derived from a given litter were subjected to either peri-pubertal stress or peri-pubertal control treatment.

Peri-pubertal stress was induced using a variable sub-chronic stress protocol, which included five distinct stressors applied on alternate days starting from PND 30. Hence, the peri-pubertal stress protocol was implemented between PNDs 30 and 40. A separate cohort of animals was subjected to identical stress exposure in adolescence starting from PND 50 and ending on PND 60. Offspring assigned to peri-pubertal or adolescent control treatment were not exposed to any specific stressor but were briefly handled on alternate days by the same experimenter. Handling was in the form of removing the animal from its home cage and fixing its tail with two fingers while holding it on one arm (10-15 sec).

The peri-pubertal period (i.e., PND 30-40) was selected because it is known to be highly sensitive with respect to precipitating long-term behavioral, neuroanatomical, and endocrine consequences of (severe) stress exposure in rodents (Spear, 2009). This developmental period in the mouse roughly corresponds to the age of 8 to 14 in humans (Spear, 2000). This period seems particularly critical with respect to the association between exposure to traumatizing experiences and subsequent psychosis-related disease in adulthood (Fisher et al., 2010). The adolescent period (i.e., PND 50-60) was chosen because it roughly corresponds to late adolescence in humans (Spear, 2000).

This period is critical for high-risk subjects to trigger acute psychosis when challenged with stressful life events (Pruessner et al., 2011).

The five distinct stressors are described in detail below and were applied in the following order: 1. Electric foot shock (day 1); 2. Restraint stress (day 3); 3. Water deprivation (day 5); 4. Forced swimming stress (day 7); 5. Multiple changes of home cage embedding (day 9). A one-day resting period followed each stressor day.

• *Exposure to electric foot shock (day 1).* Four identical freezing chambers (Coulbourn Instruments, Allentown, PA) were used, each installed in a sound insulated, wooden cabinet. Each cabinet was equipped with 16 stainless steel bars (diameter: 4 mm) spaced 10 mm apart from center to center, through which scrambled electric foot shocks could be delivered by a Coulbourn Precision Regulated Animal Shocker (Model E13-12). Animals were restricted to a semi-circular (19 cm in diameter; 30 cm height) Plexiglas enclosure, which was positioned over the grid. The lights were turned off throughout the entire period, and additional odor stimuli were presented by placing a compressed tissue saturated with Sterilium™ solution (Bode Chemie Hamburg, Germany) into the wooden cabinets. The chambers were cleaned after each run, and Sterilium™ tissues were saturated again. Each animal was first placed into the chamber for an initial period of 3 min, after which it received 3 mild electric foot shocks (0.3 mA), each lasting 1 sec. The electric foot shocks were separated by intervals of 3 min. The session ended with an additional 3 min period, in which no shocks were delivered.

• *Exposure to restraint stress (day 3). Each* animal assigned to the peri-pubertal stress exposure was kept in transparent plastic tube (diameter: 3 cm; length: 11.5 cm) for 45 min. The restrainer tubes contained drilled holes (2 mm in diameter) so as to facilitate oxygen supply. The tubes were tapped on a wooden table which was placed in a brightly lit testing room. The animals were immediately returned to their home cages at the end of the restraint stress procedure.

• *Exposure to water deprivation (day 5).* Littermates assigned to peri-pubertal stress were subjected to 16-hrs water deprivation. For this purpose, animals were kept in their homes cages, and the water bottles were removed at 16h00 on day 5 and added

again the next day at 08h00. Animals had free access to food during the entire water deprivation period.

• *Exposure to forced swim stress (day 7).* The apparatus used for the forced swim stress was made of a circular white fiberglass tank (diameter: 100 cm, height: 36 cm) filled with water (temperature: 18 °C, depth: 20 cm). The water tank was placed in a brightly lit testing room. Each animal underwent two session of forced swimming, each lasting 1 min. The two swimming session were separated by a 3-min interval, during which the animals were kept in a waiting box containing sawdust embedding. The animals were dried with a towel and immediately brought back to their homes cages after the second swimming session.

• *Exposure to repeated changing of home cages (day 9).* The last stressor was in the form of repeated changing of home cages. For this purpose, the animals were transferred from their original home cages to new cages containing fresh sawdust embedding. Food and water was supplied to the animals immediately after transferring them from one cage to another in order to avoid additional food or water deprivation. This procedure was repeated five times at irregular intervals during the dark phase of the light-dark cycle.

Behavioral Testing

The short- and long-term behavioral effects of the prenatal and postnatal manipulations were assessed when the offspring reached the peri-pubertal (PND 41-45) or adult (PND 70-110) stage of development, respectively. Peri-pubertal and adult stages were defined based on the gradual attainment of sexual maturity and age-specific behavioral discontinuities from younger to older animals *(*Spear, 2000*)*. These two developmental stages approximately correspond to periods between 11 and 16 years and from 20 years onward, respectively, in humans (Spear, 2000*).* Independent cohorts of animals were used for the behavioral investigations conducted in peri-puberty and adulthood to avoid potential confounding carryover effects from behavioral testing during peri-pubertal brain maturation. In an additional series of experiments, prenatal poly(I:C) or control offspring subjected to adolescent (PND 50-60) stress or not were tested behaviorally between PND 90-110 to assess the long-term behavioral effects of single or combined exposure to prenatal immune activation and adolescent stress. Both

male and female subjects were included in each phenotypic characterization in order to identify possible sex-dependent effects of the experimental manipulations. However, initial analyses showed that there were no significant sex-specific effects associated with single or combined exposure to prenatal immune activation and peri-pubertal stress. Therefore, the data of the two sexes were combined for final analyses. The numbers of animals included in each of the four experimental groups and individual tests are summarized in Supplementary Tables 1-6, and so are the sequences of behavioral testing. Unless specified otherwise, all animals were housed in groups of 2-3 throughout the entire testing period. All testing was conducted during the dark phase of the light-dark cycle.

The selected behavioral tests (*see below*) were chosen based on a plethora of previous investigations demonstrating reduced prepulse inhibition, loss of latent inhibition, and enhanced behavioral sensitivity to psychotomimetic drugs following prenatal poly(I:C)-induced immune activation considerably higher intensities *(Meyer and Feldon, 2012)*. We hypothesized that stress in puberty would have the potential to unmask latent behavioral pathology in neurodevelopmentally vulnerable subjects that had previously been subjected to mild prenatal immune challenge. In addition, we included behavioral functions known to be modulated by traumatizing experiences, namely anxiety-related behavior.

■ *Elevated plus maze test. The* elevated plus maze tests served as tests for innate anxiety-like behavior. The apparatus was made of Plexiglas painted in gray and consisted of 4 equally spaced arms (5 x 30 cm^2) radiating from a square center (5 × 5 cm^2). One pair of opposing arms was enclosed with opaque walls (height: 15 cm) except for the side adjoining the central square (CZ). The remaining two arms were exposed with a parameter border (height: 3 mm) along the outer edges. The maze was elevated 70 cm above floor level and positioned in a testing room with diffused lighting (approximately 20 Lux in open arm and 10 Lux in closed arm). A digital camera was mounted above the plus maze, captured images at a rate of 5 Hz and transmitted them to a PC running the Ethovision (Noldus Technology, Wageningen, The Netherlands) tracking system.

A test session began by placing the animal into the CZ with it facing one of the closed arms. It was then left to explore freely for 5 min before being returned to the home cage. After each trial, the apparatus was cleansed with water and dried before a new trial began.

The relative (percent) time spent in the open arms and relative open arm entries during the entire 5-min test period were analyzed in order to index anxiety-related behavior. The percent time spent in the open arms and percent open arm frequency were calculated using the formula [(time spent in the open arms) / (time spent in all arms) × 100] and [(open arm entries) / (total arm entries) × 100]. In addition, total distance moved in the entire maze was analyzed in order to assess general locomotor activity.

■ *Prepulse inhibition of the acoustic startle reflex.* Sensorimotor gating was assessed using the paradigm of prepulse inhibition (PPI) of the acoustic startle reflex. PPI of the acoustic startle reflex refers to the reduction in startle reaction in response to a startle-eliciting pulse stimulus when it is shortly preceded by a weak prepulse stimulus. The apparatus consisted of four startle chambers for mice (San Diego Instruments, San Diego, CA, USA). The test apparatus has been fully described elsewhere *(25)*.

During a 45-min test session, subjects were presented with a series of discrete test trials comprising a mixture of four trial-types as fully described previously *(25,31)*. In brief, these trial stimuli included pulse-alone, prepulse-plus-pulse and prepulse-alone trials, as well as no-stimulus trials in which no discrete stimulus other than the constant background noise (65 dB_A) was presented. The startle program consisted of one 40-ms pulse of white noise (120 dB_A) combined with five different pre-pulses. Pre-pulses were a 20-ms burst of white noise at five different intensities (69, 73, 77, 81, 85 dB_A, which corresponded to 4, 8, 12, 16, and 20 dB_A above background). The stimulus onset asynchrony (SOA) of the pre-pulse and pulse stimuli in prepulse-plus-pulse trials was 100 ms. Following a 2-min acclimatization period, 6 consecutive pulse-alone trials were presented in order to habituate and stabilize the animals' startle response. Subsequent to this startle habituation phase, each trial stimulus was presented 12 times in a pseudorandom order with an average interval between successive trials (ITI) of 15 ± 5

sec. The session was concluded with 6 consecutive pulse-alone trials. Boxes were cleansed with warm tap water and dried between each animal. Each box was used by one sex only.

For each subject and at each of the five possible prepulse intensities, PPI was indexed by percent inhibition of startle response obtained in the prepulse-plus-trials compared to pulse-alone trials by following expression: [1-(mean reactivity on prepulse-plus-pulse trials / mean reactivity on pulse-alone trials) × 1/100]. The first and last six trials were not included in the calculation and analysis of percent PPI. In addition to PPI, reactivates to pulse-alone trials and prepulse-alone trials were also analyzed.

■ *Latent inhibition.* Selective attention was studied using the paradigm of latent inhibition (LI). LI refers to the phenomenon, in which prior repeated pre-exposures to the to-be-conditioned stimulus (CS) impairs the subsequent development of the conditioned response (CR) following explicit pairings between the same CS and an unconditioned stimulus (US). LI was assessed in a two-way active avoidance procedure using four identical two-way shuttle boxes (model H10–11M-SC; Coulbourn Instruments, Allentown, PA, USA) as fully described before (Meyer et al., 2008).

The test procedures consisted of two phases: Pre-exposure and conditioning, conducted 24 hrs apart. Animals from each of the four treatment groups were allocated to one of the two conditions: CS-pre-exposure (PE) and non-pre-exposure (NPE).

In the pre-exposure phase, the PE subjects were placed in the appropriate test chambers and presented with 100 pre-exposures to a 5-sec white noise (85 dB_A) CS according to a random inter-stimulus interval schedule of 40±15 sec. The NPE subjects were confined to the chambers for an equivalent period of time without any stimulus presentation. On conditioning day, the subjects were returned to the same shuttle boxes and received a total of 50 avoidance trials presented with an inter-trial interval of 40±15 sec. A trial began with the onset of the white noise CS. If the animal shuttled within 5 sec of CS onset, the CS was terminated and the animal avoided the electric shock on that trial. Avoidance failure led immediately to an electric foot shock presented in coincidence to the CS. This could last for a maximum of 2 sec but could be terminated by a shuttle response during this period (that is, an escape response).

■ Behavioral sensitivity to psychotomimetic drugs. The sensitivity to psychotomimetic drugs was assessed by measuring drug-induced locomotor activity in an open field apparatus. The apparatus consisted of 4 identical square arenas (40 × 40 × 35 cm high) made of wood and painted gray as fully described elsewhere *(31)*. Two distinct psychotomimetic drugs were included, namely the indirect dopamine receptor agonist amphetamine (AMPH) and the non-competitive NMDA receptor antagonist dizocilpine (MK-801). First, the animals were injected with vehicle (isotonic 0.9% NaCl) solution and immediately placed in the apparatus to measure basal locomotor activity for 30 min. Subsequently, the animals were removed from the apparatus and administered with either AMPH (D-amphetamine sulfate, Sigma-Aldrich; 2.5 mg/kg, intraperitoneally [i.p.]) or MK-801 (Merck, Sharp & Dohme, Hertfordshire, UK; 0.15 mg/kg, i.p.). They were then immediately returned to the same arena again, and the locomotor response to the acute drug challenge was monitored for a period of 90 min.

Both drugs were dissolved in isotonic 0.9% NaCl solution to achieve the desired concentration for injection. The doses of AMPH (2.5 mg/kg, i.p.) and MK-801 (0.15 mg/kg, i.p.) were selected based on our previous studies showing that these drug doses do not produce any ceiling effects on locomotor activity enhancement *(*Meyer et al., 2008*)*. This readily facilitates the detection of modulatory effects of the prenatal and postnatal manipulations on AMPH- and/or MK-801-induced changes in locomotor activity. The volume of injection was 5 ml/kg for both solutions. All solutions were freshly prepared on the day of testing.

Preparation of plasma samples and hippocampal homogenates for cytokine and corticosterone analyses

For blood and brain specimen collection, mice were killed by decapitation, and trunk blood was collected into Microvette (CB300 LH, SARSTEDT) coated with lithium heparin and centrifuged on 2000 x g for 5 minutes. Aliquots of plasma samples were stored at −80°C until later analyses. The brains were extracted from the skull and placed ventral side up on an ice-chilled plate for extraction of the left and right hippocampi. The hippocampi were weighed, placed in 1-ml Eppendorf tubes and stored at −80°C until further processing. Frozen hippocampal samples were placed in 300 µl lysis buffer containing 50 mM Tris-HCl (pH 7.4), 0.6 M NaCl, 0.2% Triton X-100, 0.5% bovine serum

albumin, and protease inhibitors (1 mM benzamidine, 0.1 mM benzethonium chloride and 0.1 mM phenylmethylsulfonyl fluoride). Samples were allowed to thaw and were then homogenized (TissueTearor; BioSpec Products, Bartlesville, OK, USA) for 10 s, sonicated (Vibra Cell; Sonics & Materials, Newtown, CT, USA) for 20 s at 10 mV, and centrifuged as described before (Meyer and Feldon, 2012). The supernatants were aliquoted and frozen at –80°C until the cytokine and PGE2 assays were performed.

Multiplexed particle-based flow cytometric assay

We measured the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α, and the anti-inflammatory cytokine IL-10. In addition, we quantified the cyclooxygenase product PGE2. The selected pro-inflammatory cytokines were chosen because of their relevance to neurodevelopmental psychiatric disorders such as autism and schizophrenia (Patterson, 2009), and because these cytokines have often been associated with altered neuroinflammatory responses in developmental pathogenesis models (Watanabe et al., 2010). IL-10 was selected to ascertain whether the anticipated effects on pro-inflammatory cytokine secretion would be paralleled by concomitant alterations in anti-inflammatory cytokine secretion. PGE_2 was selected because it has been suggested to be an inflammation-related marker relevant to the pathogenesis and (anti-inflammatory) treatment of schizophrenia (Müller and Schwarz., 2010).

Plasma and brain levels of IL-1β, IL-6, IL-10, TNF-α, and PGE_2 were measured using multiplexed particle-based flow cytometric assays (Bio-Plex Pro Mouse Cytokine Assays, Bio-Rad Laboratories, Hercules, CA, USA) as previously described (Meyer et al., 2008). Samples were prepared according to the manufacturer's instructions and were analyzed on a triple-laser FACSCanto II flow cytometer using FACSDiva software (BD Immunocytometry Systems, Heidelberg, Germany). Absolute concentrations were calculated based on the mean fluorescence intensity of cytokine/PGE_2 standards with a 4-parameter logistic curve model using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). For peri-pubertal plasma and lysate samples, the detection limits were 2.1 pg/ml for IL-1β, 0.4 pg/ml for IL-6, 0.3 pg/ml for IL-10, 0.8 pg/ml for TNF-α, and 30.0 pg/ml for PGE_2. For adult plasma and lysate samples, the detection limits were 2.0 pg/ml for IL-1β, 0.6 pg/ml for IL-6, 0.8 pg/ml for IL-10, 0.2 pg/ml for TNF-α, and 35.0 pg/ml for PGE_2. To express hippocampal cytokine/ PGE_2 levels, the cytokine/PGE_2

contents measured in the corresponding hippocampal lysates were normalized to the animal's hippocampal weight measured immediately after hippocampal dissection.

Corticosterone analysis

Plasma corticosterone levels were determined using an enzyme-linked immunosorbent assay (IBL International, Hamburg, Germany) according to the manufacturer's instructions. The cross-reactivity of the anti-corticosterone antibody with other relevant steroids was 7.4% (progesterone), 3.4% (deoxycorticosterone), and 1.6% (11-dehydrocorticosterone). The sensitivity of the assay was 0.6 ng/ml.

Gene expression analysis of contact-dependent neuron-microglia inhibitory signaling pairs by quantitative real-time PCR. Total RNA was isolated by a single step guanidinium isothiocyanate/phenol extraction, using PureZol RNA isolation reagent (Bio-Rad Laboratories s.r.l. Italia) according to the manufacturer's instructions, and quantified by spectrophotometric analysis. Following total RNA extraction, the samples were processed for real-time PCR to assess CD200, CD200R, CD47 and CD172a mRNA levels. An aliquot of each sample was treated with DNase to avoid DNA contamination. RNA was analyzed on a qRT–PCR instrument (CFX384 real-time system, Bio-Rad Laboratories) using the iScript one-step RT–PCR kit for probes (Bio-Rad Laboratories). Samples were run in 384-well formats in triplicates as multiplexed reactions with a normalizing internal control (36B4). Probe and primer sequences were purchased from Eurofins MWG-Operon. Thermal cycling was initiated with incubation at 50°C for 10 min (RNA reverse transcription), and then at 95°C for 5 min (polymerase activation). After this initial step, 39 cycles of PCR were performed. Each PCR cycle consisted of heating the samples at 95°C for 10 s to enable the melting process, and then for 30s at 60°C for the annealing and extension reactions. Relative target gene expression was calculated according to the 2(-Delta Delta C(T)) method (Livak and Schmittgen., 2001).

The following primer and probe sequences were used for the gene expression analysis of neuron-microglia inhibitory signaling pairs:

	FORWARD primer	**REVERSE primer**	**PROBE**
CD200	CTCCACCTACGCCTGATTTG	TCACAATCAAGGGTTCCTGG	AGCACAGCTCAAGTGGAAGTGGT
CD200R	TTTTGGAGAACTTCTGCCCTAG	GTGTTCACTTGTGTCAGAGGA	ACTTGACCCAGCCACAAAGACCC
CD47	AGGAGAAAAGCCCGTGAAG	TGGCAATGGTGAAAGAGGTC	ACAATGAGGCCAAGTCCAGAAGCA
CD172a	TGTGCTTTGCTCGTAGTCC	TCATTTGTGTCCTGGATCTGG	TGTTGACCCCTTGGCTTTCTTCTGT
36B4	AGATGCAGCAGATCCGCAT	GTTCTTGCCCATCAGCACC	CGCTCCGAGGGAAGGCCG

Post-mortem neurochemistry

Animals assigned to the post-mortem neurochemical investigations were killed by decapitation on PND 70. The brains were extracted from the skull within <30 s, snap-frozen in liquid nitrogen, and stored at –80 °C until required. Frozen coronal sections (0.5-mm-thick) were prepared using a cryostat at the following coordinates with respect to Bregma according to The Mouse Brain in Stereotaxic Coordinates by Franklin and Paxinos (Franklin and Paxinos., 2008): AP (+2.3 to +1.3), (+1.3 to +0.3), (–0.1 to –0.6), (–1.2 to –2.2), and (–2.8 to –3.8). Tissue samples from both hemispheres were processed from the mPFC (including anterior cingulate [aCG] and prelimbic [PrL] cortices), nucleus accumbens (NAc; including shell and core subregions), and hippocampus (HPC; including parts of the CA1-3 fields and dentate gyrus) via micropunches of 1 mm diameter as previously described (Meyer et al., 2008, Winter et al., 2009).

Brain samples were homogenized by ultrasonication in 20 vol of 0.1 N perchloric acid at 4°C immediately after collection via micropunches. A total of 100 ml of the homogenate was added to equal volumes of 1 N sodium hydroxide for measurement of protein content. The remaining homogenate was centrifuged at 17 000 g and 4 °C for 10 min. Supernatants were used for immediate measurement of dopamine (DA) and serotonin (5-HT). DA and 5-HT levels were measured by high-performance liquid chromatography (HPLC) with electrochemical detection as previously described *(*Winter et al., 2009*)*. Briefly, the perchloric acid extracts were separated on a column (Prontosil 120-3-C18-SH; length 150 mm, inner diameter 3 mm; Bischoff Analysentechnik GmbH, Leonberg, Germany) at a flow rate of 0.55 ml/min. The mobile phase consisted of 80 mM sodium dihydrogene phosphate, 0.85 mM octane-1-sulfonic acid sodium salt, 0.5 mM

ethylenediaminetetraacetic acid disodium salt, 0.92 mM phosphoric acid, and 4% 2-propanol (all chemicals Merck KGaA, Darmstadt, Germany). DA and 5-HT were detected using an electrochemical detector (41 000, Chromsystems Instruments & Chemicals GmbH, Munich, Germany) at an electrode potential of 0.8 V. For calibration, 0.1 M perchloric acid containing 0.1 µM 5-HT and 1 µM DA was injected into the HPLC system before and after sample analysis. Sample analysis was performed based on peak areas using a computer-based chromatography data system (CSW 1.7, DataApex Ltd, Praha, Czech Republic) in relation to the mean of the applied calibration solutions.

Immunohistochemistry

The animals were deeply anesthetized with an overdose of Nembutal (Abbott Laboratories) and perfused transcardially with 0.9% NaCl, followed by 4% phosphate-buffered paraformaldehyde solution containing 15% picric acid. The dissected brains were postfixed in the same fixative for 6 h and processed for antigen retrieval involving overnight incubation in citric acid buffer (pH = 4.5) followed by a 90 s microwave treatment at 480 W according to protocols established before (Vuillermot et al., 2012). The brains were then cryoprotected using 30% sucrose in PBS, frozen with powdered dry ice, and stored at −80 °C until further processing.

Perfused brain samples were cut coronally at 30 µm thickness from frozen blocks with a sliding microtome. Eight serial sections were prepared for each animal. The sections were rinsed in PBS, and stored at −20 °C in antifreeze solution until further processing. For immunohistochemical staining, the slices were rinsed three times for 10 min in PBS, and blocking was done in PBS, 0.3% Triton X-100, 10% normal serum for 1 h at room temperature. The following primary antibodies were used: Rabbit anti-Iba1 (Wako, CatNo# 019-19741; diluted 1:2000), rat anti-CD68 (AbDSerotec, CatNo# MCA1957; diluted 1:5000), rat anti-CD11b (AbDSerotec, CatNo# MCA74GA; diluted 1:100), rabbit anti-GFAP (DAKO, CatNo# Z0334; diluted 1:5000), and rabbit anti-S100B (NOVUS, CatNo# NB300-670). All antibodies were diluted in PBS containing 0.3% Triton X-100 and 2% normal serum, and the sections were incubated free-floating overnight at room temperature. After three washes with PBS (10 min each), the sections were incubated for 1 h with the biotinylated secondary antibodies diluted 1:500 in PBS containing 2% NGS and 0.3% Triton X-100. Sections were washed again three times for

10 min in PBS and incubated with Vectastain kit (Vector Laboratories) diluted in PBS for 1 h. After three rinses in 0.1 M Tris-HCl, pH 7.4, the sections were stained with 1.25% 3,3-diaminobenzidine and 0.08% H_2O_2 for 10–15 min, rinsed again four times in PBS, dehydrated, and coverslipped with Eukitt (Kindler).

Unbiased stereological estimations

The numbers of Iba1-, CD68, GFAP-, or S100B-immunoreactive cells were determined by unbiased stereological estimations using the optical fractionator method (Gundersen et al., 1988). With the aid of the image analysis computer software Stereo Investigator (version 6.50.1; MicroBrightField), every section of a one-in-eight series was measured, resulting in an average of 5-6 sections per brain sample as previously described (Vuillermot et al., 2012). The following sampling parameters were used: (1) a fixed counting frame with a width of 60 µm and a length of 60 µm; and (2) a sampling grid size of 200 × 150 µm. The counting frames were placed randomly at the intersections of the grid within the outlined structure of interest by the software. The cells were counted following the unbiased sampling rule using the 40× oil lens [numerical aperture (NA), 1.3] and included in the measurement when they came into focus within the optical dissector (Howard and Reed, 2005). All immunohistochemical preparations were quantified in the selected prefrontal and hippocampal areas. Iba1- and CD68-immunoreactive cells were additionally quantified in the secondary motor cortex.

Assessment of microglia morphology

Iba1- immunoreactive microglia were visualized under the 63 x oil lens [numerical aperture (NA), 1.4] using a Zeiss Axiophot microscope. Various parameters of microglia cell morphology were assessed in the II layer of infralimbic cortex, the CA3 region of hippocampus, and II layer of the secondary motor cortex. For each of these regions, a counting frame of 100 µm x 100 µm was randomly placed into three sections of a one-in-eight series. All microglia cells captured by the counting frame were included in the morphological analyses, expect when microglial processes were obscured by either background labeling or other cells. Three to four microglia cells per section were traced using the software Stereo Investigator (version 6.50.1; MicroBrightField), for

which cell soma area, number of branch points, and number of primary processes were estimated giving a total of 9 to 12 cells per region of interest.

Optical Densitometry

Quantification of CD11b immunoreactivity was achieved by means of optical densitometry using NIH ImageJ software as described before *(38)*. Optical densitometry was chosen for the evaluation of CD11b because individual CD11b-immunoreactive cell bodies were often obscured, preventing the detection of isolated CD11b-postive cells using the unbiased sampling rule (i.e., the inclusion of measurements only when cells come into focus within the optical dissector). In addition to the stereological estimation of CD68-positive cells (see above), CD68 staining was additionally quantified using optical densitometry. Digital images were acquired at a magnification of 5.0× using a digital camera (Axiocam MRc5; Zeiss) mounted on a Zeiss Axioplan microscope. Exposure times were set so that pixel brightness was never saturated. Pixel brightness was measured in the respective areas of one randomly selected brain hemisphere. In addition, pixel brightness was measured in non-immunoreactive areas of the forceps minor corpus callosum (for prefrontal cortical measures) and corpus callosum as background measurements. The background-corrected relative optical densities were averaged per brain region and animal. Four to six sections per animal were analyzed for each brain region of interest. All immunohistochemical preparations were quantified in selected prefrontal, hippocampal and secondary motor cortex areas.

Statistical Analyses

All primary data were first analyzed using full-factorial parametric ANOVA to identify the main effects of the prenatal and postnatal manipulations as well as their interactions (Supplementary Table 7-19), followed by Fisher's least significant difference post-hoc comparisons or restricted ANOVAs whenever appropriate. In secondary analyses, a priori comparisons were also conducted for individual gene expression parameters. Statistical significance was set at $P < 0.05$. Plasma cytokine levels and brain monoamine (dopamine and serotonin) were first subjected to logarithmic (base *e*) and square root transformation, respectively, to better conform to the assumption of data homogeneity and normality by parametric ANOVA. For the purpose of stereological, optical densitometric, and morphological analyses, the

experimenter was blind to the animals' treatment conditions and had access only the brain specimen codes (in the form of a number). All statistical analyses were performed using the statistical software StatView (version 5.0) implemented on a PC running the Windows XP operating system.

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Chapter 3

Single and Combined Effects of Prenatal Immune Activation and Peripubertal Stress on Parvalbumin and Reelin Expression in the Hippocampal Formation

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ABSTRACT

Exposure to prenatal infection and traumatizing experiences in peripubertal life are two environmental risk factors for developmental neuropsychiatric disorders. Modelling the cumulative neuronal impact of these factors in a translational animal model has led to the recent identification of pathological interactions between these environmental adversities in the development of adult brain dysfunctions. The present study explored the consequences of combined prenatal immune challenge and peripubertal stress on discrete cellular abnormalities in the γ-aminobutyric acid (GABA) system of the hippocampus. Pregnant mice were treated with the viral mimetic poly(I:C) (= polyriboinosinic-polyribocytidilic acid) or control solution, and offspring born to poly(I:C)-exposed or control mothers were then left undisturbed or subjected to unpredictable sub-chronic stress during peripubertal development. Stereological estimations of parvalbumin-expressing cells revealed a significant reduction of these GABAergic interneurons in the ventral dentate gyrus of adult offspring exposed to combined immune activation and stress. Single exposure to either environmental factor was insufficient to cause similar neuropathology. We further found that peripubertal stress exerted opposite effects on reelin-immunoreactive cells in the dorsal cornu ammonis (CA) region of the hippocampus, with stress increasing and decreasing reelin expression in control offspring and prenatally immune challenged animals, respectively. The present data suggest that the combination of two environmental risk factors, which have each been implicated in the etiology of major neuropsychiatric disease, induces significant but restricted neuropathological effects on hippocampal GABAergic cell populations known to be affected in brain disorders with neurodevelopmental components.

INTRODUCTION

Exposure to prenatal maternal infection and psychological trauma in peripubertal life are two environmental risk factors implicated in the etiology of developmental neuropsychiatric disorders, including schizophrenia (Brown, 2011; van Os et al., 2010), autism (Atladóttir et al., 2012), and bipolar disorder (Parboosing et al., 2013). Epidemiological evidence for the cumulative impact of such prenatal and postnatal environmental challenges on vulnerability to chronic psychiatric disease is still largely missing. However, modelling the consequences of combined exposures to these environmental adversities in mice shows that prenatal immune activation and stress in puberty can indeed interact with each other in the development of adult brain and behavioral dysfunctions (Giovanoli et al., 2013). In this environmental two-hit model, adult offspring subjected to combined prenatal immune challenge and peripubertal stress display numerous behavioral and neurochemical abnormalities that are relevant especially to psychosis-related disease (Giovanoli et al., 2013). These findings support multiple-hit theories of developmental neuropsychiatric disorders (Bayer et al., 1999; Maynard et al., 2001) and further suggest that prenatal immune adversities can function as a neurodevelopmental disease primer, which in turn increases the offspring's vulnerability to the detrimental neuropathological effects of subsequent stress exposure during peripubertal life.

In the present study, we explored the impact of combined prenatal immune challenge and peripubertal stress on cellular abnormalities in the γ-aminobutyric acid (GABA) system of the hippocampus. More specifically, we examined the single and cumulative impact of these environmental adversities on cells expressing parvalbumin (PV) and reelin (RELN), two cellular markers expressed by distinct subpopulations of GABAergic cells. The calcium-binding protein PV is mainly expressed by fast-spiking GABAergic interneurons, which are pivotal for proper neuronal synchronization by providing inhibitory input to axon initial segments (Gonzalez-Burgos and Lewis, 2012). RELN is a glycoprotein that is secreted in the developing and adult nervous system, and it has important functions for proper neural positioning during early brain development (Fatemi, 2005). In the adult brain, RELN is crucially involved in synaptic plasticity and the acquisition, consolidation and expression of distinct forms of memory (Herz and

Chen, 2006). A plethora of neuroanatomical studies has found impaired PV and RELN expression in cortical and hippocampal brain areas of individuals with major neuropsychiatric disease, especially schizophrenia (Gonzalez-Burgos and Lewis, 2012; Volk and Lewis, 2013), autism (Folsom and Fatemi, 2013), and bipolar disorder (Guidotti et al., 2000; Wang et al., 2011). Similar deficits in PV and RELN secretion have also been identified in various developmental animal models relevant to these disorders (Cabungcal et al., 2013; Lodge et al., 2009; Meyer and Feldon, 2010), including models of intense prenatal immune challenge (Fatemi et al., 1999; Harvey and Boksa, 2012; Meyer et al., 2006, 2008; Piontkewitz et al., 2012). Against these backgrounds, we were interested in examining PV and RELN expression in our translational mouse model that combines two etiologically relevant environmental risk factors implicated in developmental neuropsychiatric disease (Giovanoli et al., 2013).

For this purpose, prenatal immune activation was induced by the viral mimetic poly(I:C) (= polyriboinosinic-polyribocytidilic acid), a synthetic analog of double-stranded RNA that induces a cytokine-associated viral-like acute phase response (Meyer et al., 2009). Offspring born to poly(I:C)-exposed or control mothers were then left undisturbed or exposed to variable and unpredictable stress during peripubertal development (Giovanoli et al., 2013). Using this environmental two-hit approach, we performed stereological estimations of PV- and RELN-containing cells in the hippocampal formation in adulthood, which corresponds to the maturational stage at which multiple behavioral abnormalities are functionally manifest following the double-hit exposure (Giovanoli et al., 2013). We focused on the hippocampal formation because it is one of the brain areas strongly affected by the combination of prenatal immune activation and peripubertal stress in terms of developmental neuroinflammation and adult neurochemistry (Giovanoli et al., 2013).

METHODS

Animals

C57BL6/N female and male breeders were obtained from Charles Rivers (Sulzfeld, Germany) at the age of 10-12 weeks. Animals were kept in a temperature- and humidity-controlled (21±1 °C, 55±5 %) holding facility under a reversed light-dark cycle (lights off: 8:00 A.M. to 8:00 P.M.). All animals had ad libitum access to standard rodent chow (Kliba 3430, Klibamuehlen, Kaiseraugst, Switzerland) and water unless specified otherwise. For the purpose of the maternal immunological manipulation during pregnancy, female mice were subjected to a timed-mating procedure as described previously (Meyer et al., 2005).

Prenatal Immune Activation

Pregnant dams on gestation day 9 (GD9) received either a single injection of poly(I:C) (potassium salt; Sigma-Aldrich) or vehicle. The gestational stage (i.e., GD9) was selected based on our previously validated protocols (Giovanoli et al., 2013). The GD9 window in the mouse roughly corresponds to the middle of the first trimester of human pregnancy, which in turn is a gestational stage known to be highly sensitive with respect to increasing the risk of schizophrenia and autism following maternal infection (Brown et al., 2004; Atladóttir et al., 2010). Poly(I:C) (1 mg/kg) was dissolved in sterile pyrogen-free 0.9% NaCl (= vehicle) solution to yield a final concentration of 0.2 mg/ml and was administered via the intravenous (i.v.) route at the tail vein under mild physical constraint (see below). A total of 16 pregnant dams were injected, with half of them receiving poly(I:C) and the other half vehicle treatment. All solutions were freshly prepared at the day of administration and injected with a volume of 5 ml/kg. All animals were returned to their home cages immediately after the injection procedure.

Peripubertal Stress Exposure

Offspring born to poly(I:C)-treated (POL) or vehicle-treated (CON) mothers were weaned on postnatal day (PND) 21 and caged as littermates of 2 animals per cage in order to avoid single caging. They were then left undisturbed (= no stressor; S-) or exposed to variable and unpredictable stress between PND 30 and 40 (S+). For this purpose, all animals of a particular housing cage underwent the same peripubertal

exposure (see below). Whenever possible, littermates were assigned to different treatment conditions in order to minimize potential confounds associated with litter effects. The peripubertal time window (i.e., PND 30 to 40) was selected based on our previous findings showing that it is associated with maximal sensitivity for stress-induced neuropathological changes in prenatally immune-challenged animals (Giovanoli et al., 2013). PND 30-40 in the mouse roughly corresponds to the age of 8 to 14 in humans (Spear, 2000). This period seems particularly critical with respect to the association between exposure to traumatizing experiences and subsequent psychosis-related disease in adulthood (Fisher et al., 2010).

The peripubertal stress procedure was performed according to protocols established before (Giovanoli et al., 2013) and included 5 distinct stressors applied on alternate days between PND 30 and 40 in the following order: 1. Electric foot shock (day 1); 2. Restraint stress (day 3); 3. Water deprivation (day 5); 4. Forced swimming stress (day 7); 5. Multiple changes of home cage embedding (day 9). A one-day resting period followed each stressor day. A detailed description of the peripubertal stress procedure can be found elsewhere (Giovanoli et al., 2013).

Immunohistochemistry

Once the animals reached the adult stage (PND 70), 8 offspring per experimental group were randomly selected from the housing cages, thereby including only 1 offspring per cage for the subsequent immunohistochemcial analyses. Only male animals were included for all analyses. They were deeply anesthetized with an overdose of Nembutal (Abbott Laboratories) and perfused transcardially with 0.9% phosphate buffered saline (PBS), followed by 4% paraformaldehyde solution (in PBS) containing 15% picric acid. The dissected brains were postfixed in the same fixative for 6 h and processed for antigen retrieval involving overnight incubation in citric acid buffer (pH = 4.5) followed by a 90 s microwave treatment at 480 W according to protocols established before (Giovanoli et al. 2013). The brains were then cryoprotected using 30% sucrose in PBS, frozen with powdered dry ice, and stored at −80 °C until further processing.

Perfused brain samples were cut coronally at 30 µm thickness from frozen blocks with a sliding microtome. Eight serial sections were prepared for each animal. The sections were rinsed in PBS, and stored at −20 °C in antifreeze solution (30% glycerol and 30% ethylene glycol in PBS at 25 mM and pH 7.4) until further processing. For immunohistochemical staining, the slices were rinsed 3 times for 10 min in PBS, and blocking was done in PBS, 0.3% Triton X-100, 10% normal serum for 1 h at room temperature. The following primary antibodies were used: mouse-anti RELN (Chemicon, CA, USA; diluted 1:700) and rabbit-anti PV (Swant, Switzerland; 1:10,000). Both primary antibodies were diluted in PBS containing 0.3% Triton X-100 and 2% normal serum. The sections were incubated free-floating overnight at room temperature. After 3 washes with PBS (10 min each), the sections were incubated for 1 h with the biotinylated secondary antibodies diluted 1:500 in PBS containing 2% NGS and 0.3% Triton X-100. Sections were washed again 3 times for 10 min in PBS and incubated with Vectastain kit (Vector Laboratories) diluted in PBS for 1 h. After 3 rinses in 0.1 M Tris-HCl, pH 7.4, the sections were stained with 1.25% 3,3-diaminobenzidine and 0.08% H2O2 for 10–15 min, rinsed again 4 times in PBS, dehydrated, and coverslipped with Eukitt (Kindler).

Stereological Estimation of PV- and RELN- Immunoreactive Cells

The number of PV- and RELN-immunoreactive cells was determined by unbiased stereological estimations using the optical fractionator method (Gundersen et al., 1988). With the aid of the image analysis computer software Stereo Investigator (version 6.50.1; MicroBrightField), every section of a one-in-eight series was measured, resulting in an average of 6 sections for each hippocampal sample. The following sampling parameters were used: (1) a fixed counting frame with a width of 60 µm and a length of 60 µm; and (2) a sampling grid size of 200 × 150 µm. The counting frames were placed randomly at the intersections of the grid within the outlined structure of interest by the software. The cells were counted following the unbiased sampling rule using the 40× oil lens [numerical aperture (NA), 1.3] and included in the measurement when they came into focus within the optical dissector (Howard and Reed, 2005). PV- and RELN-immunoreactive cells were counted in the dorsal cornu ammonis area 1-3 (= dCA1-CA3), ventral cornu ammonis area 1-3 (= vCA1-CA3), dorsal dentate gyrus (dDG), and ventral dentate gyrus (= vDG). Dorsal and ventral parts of the hippocampal formation were

dissociated from each other because of previous findings showing that intense prenatal immune activation by poly(I:C) induces differential effects on GABAergic cells in the dorsal and ventral parts (Meyer et al., 2008). The regions of interest were delineated according to the coordinates provided by the *The Mouse Brain in Stereotaxic Coordinates* by Franklin and Paxinos (2008) and described in detail elsewhere (Giovanoli et al., 2013). Dorsal and ventral regions ranged from Bregma -1.46 to -2.06 mm and from and Bregma -2.92 to -3.52 mm, respectively. All stereological estimates were acquired by an experimenter who was blind to the animals' treatment conditions.

Statistical Analyses

Stereological estimates of PV- and RELN-immunoreactive cells were analysed using 2 × 2 (prenatal immune treatment × peri-pubertal stress) ANOVAs, followed by Fisher's least significant difference (LSD) post-hoc comparisons whenever appropriate. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using the statistical software StatView (version 5.0) implemented on a PC running the Windows XP operating system.

RESULTS

Single and Combined Effects of Prenatal Immune Activation and Peripubertal Stress on Hippocampal Parvalbumin Cells

PV-positive cells were found to be distributed across the entire hippocampal formation and displayed darkly stained cell bodies (Figure 1). PV immunoreactivity was also present, to a somewhat lesser extent, on axonal processes (Figure 1). Neither single nor combined exposure to prenatal immune activation and peri-pubertal stress significantly affected the number of PV-immunoreactive cells in the dorsal or ventral CA regions (i.e., in dCA1-CA3 and vCA1-CA3) or the dDG (Figure 1A). The combination of the two environmental factors, however, led to a marked reduction in the number of PV-positive cells in the vDG (Figure 1A,B). Interestingly, neither prenatal immune activation alone, nor exposure to peri-pubertal stress alone, induced such cellular changes in the vDG, suggesting that the combination of environmental adversities is required for deficient PV expression to develop in this brain region.

Statistical support for these interpretations was obtained by the presence of a significant interaction between prenatal immune activation and peri-pubertal stress in the analysis of vDG PV-immunoreactive cells [$F(1,28) = 5.25$, $p < 0.05$]. Subsequent post-hoc group comparisons confirmed a significant difference between stressed POL offspring and non-stressed POL offspring ($p < 0.001$), stressed CON offspring ($p < 0.05$), and non-stressed CON offspring ($p < 0.01$).

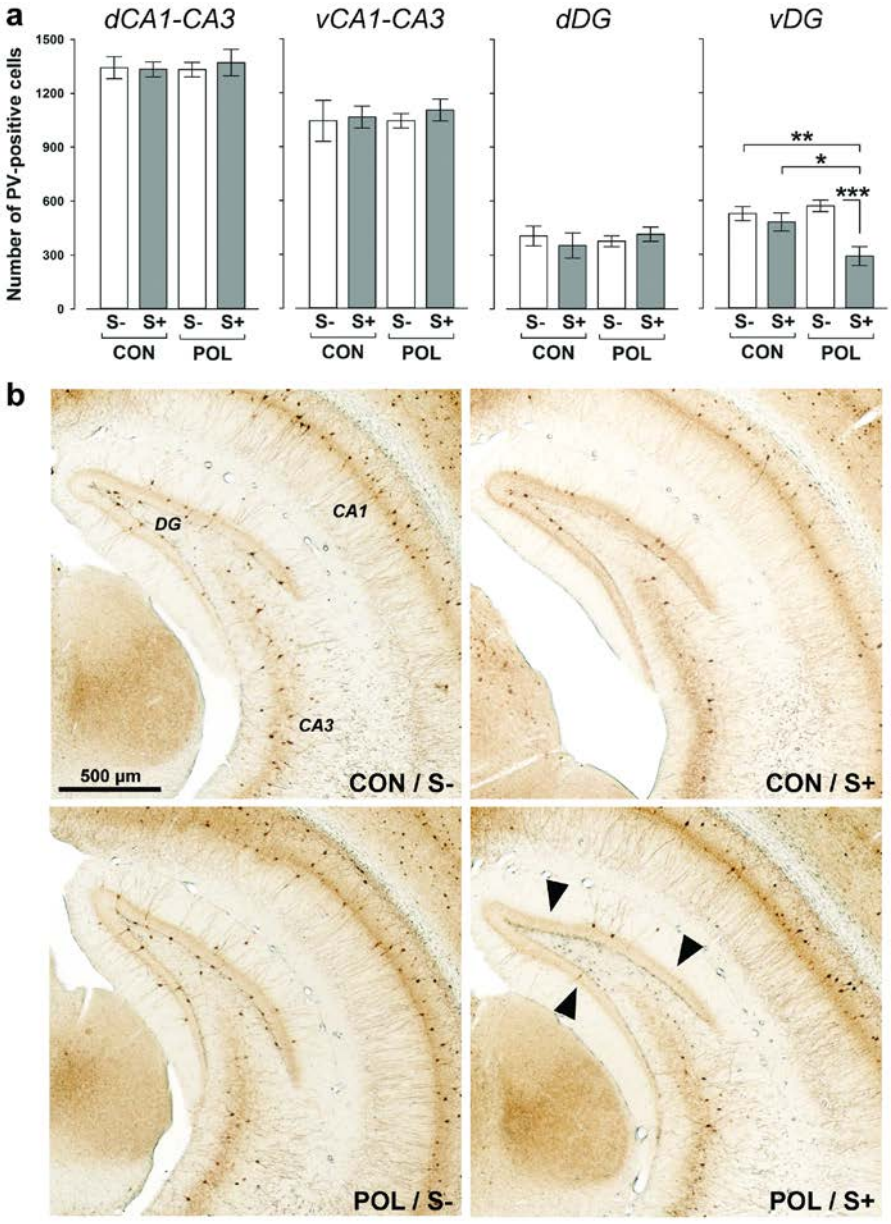

Figure 1. Single and combined effects of prenatal immune activation and peri-pubertal stress on parvalbumin (PV) expression in the hippocampal formation. Pregnant mice were exposed to poly(I:C) (POL) or control (CON) solution, and the resulting offspring were left non-stressed (S-) or subjected to

sub-chronic stress (S+) during peri-pubertal development. (a) The bar plots depict the stereological estimates (cells/mm3) of PV-immunoreactive cells in the dorsal and ventral CA1-CA3 regions (dCA1-CA3 and vCA1-CA3, respectively), and in the dorsal and ventral dentate gyrus (dDG and vDG, respectively). *p < 0.05, **p < 0.01, and ***p < 0.001; *N* = 8 per group. All data are means±SEM. (b) The photomicrographs show representative coronal brain sections at the level of the ventral hippocampus stained with anti-PV antibody. Note the decrease in PV-immunoreactive cells in the vDG of POL/S+ animals (highlighted by the black arrowheads).

Single and Combined Effects of Prenatal Immune Activation and Peripubertal Stress on Hippocampal Reelin Cells

RELN immunoreactivity was present mainly in cell somas of discreet cell populations distributed across the entire hippocampal formation (Figure 2). Both the prenatal and postnatal environmental manipulations affected the expression of RELN in the dCA1-CA3 region: Prenatal poly(I:C) treatment alone was sufficient to increase the number of RELN-positive cells compared to prenatal control treatment, and additional stress exposure in puberty exerted opposite effects on these cells depending on the prenatal manipulation (Figure 2A,B). Indeed, whereas stress exposure markedly enhanced RELN-positive cells in the dCA1-CA3 region of CON offspring, it led to the opposite effect in POL offspring (Figure 2A,B). These effects were supported by the presence of significant interaction between prenatal immune activation and peri-pubertal stress in the analysis of dCA1-CA3 RELN-immunoreactive cells [$F(1,28) = 9.85$, $p < 0.01$]; and by subsequent post-hoc comparisons confirming a significant difference between stressed and non-stressed CON offspring ($p < 0.05$), between stressed and non-stressed POL offspring ($p < 0.05$), and between non-stressed CON and POL offspring ($p < 0.05$). Neither single nor combined exposure to prenatal immune activation and peri-pubertal stress significantly affected the number of RELN-immunoreactive cells in the other hippocampal regions examined (Figure 2A).

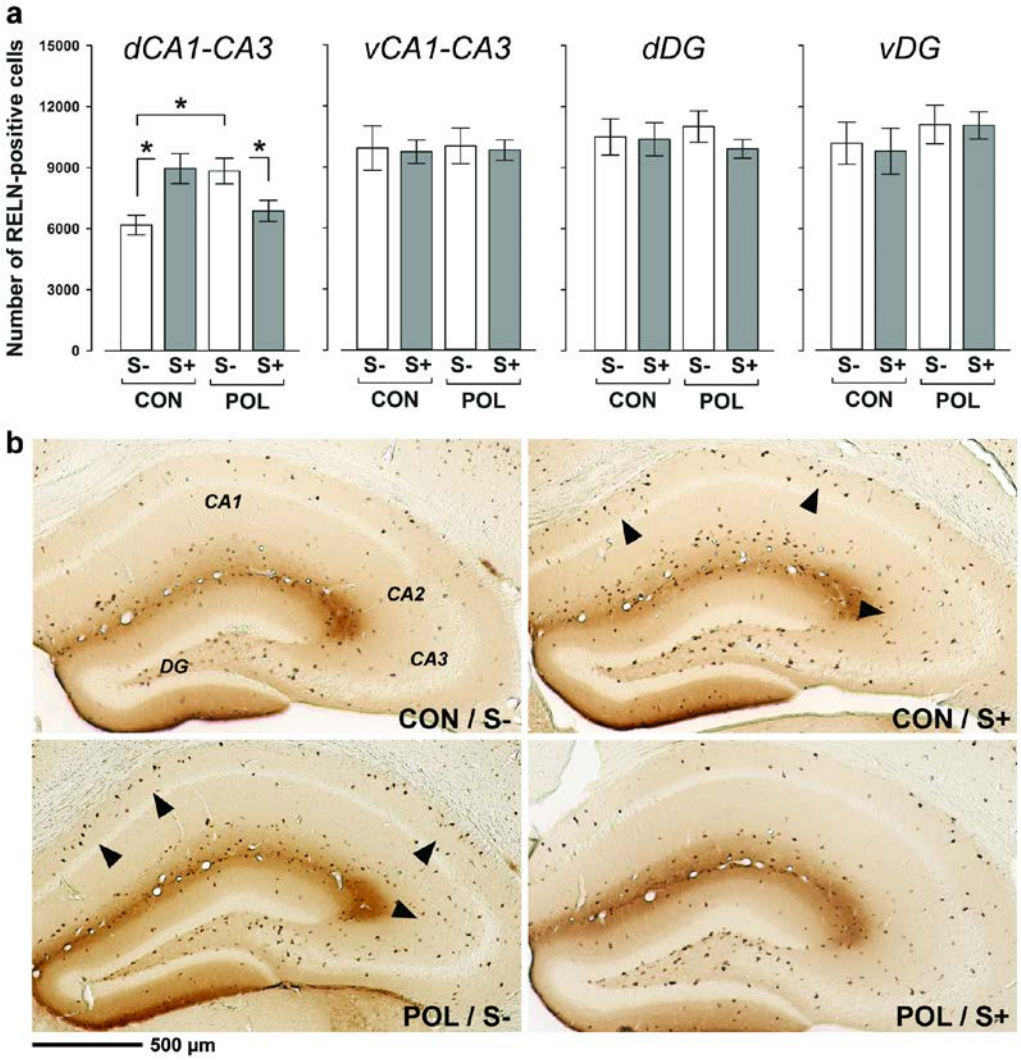

Figure 2. Single and combined effects of prenatal immune activation and peri-pubertal stress on reelin (RELN) expression in the hippocampal formation. Pregnant mice were exposed to poly(I:C) (POL) or control (CON) solution, and the resulting offspring were left non-stressed (S-) or subjected to sub-chronic stress (S+) during peri-pubertal development. (a) The bar plots depict the stereological estimates (cells/mm3) of RELN-immunoreactive cells in the dorsal and ventral CA1-CA3 regions (dCA1-CA3 and vCA1-CA3, respectively), and in the dorsal and ventral dentate gyrus (dDG and vDG, respectively). *$p < 0.05$; $N = 8$ per group. All data are means±SEM. (b) The photomicrographs show representative coronal brain sections at the level of the dorsal hippocampus stained with anti-RELN antibody. Note the increase in RELN-immunoreactive cells in the dCA1-CA3 region of CON/S+ and POL/S- animals (highlighted by the black arrowheads).

DISCUSSION

Based on our previous observations demonstrating synergistic neuropathological interactions between mild prenatal immune activation and peripubertal stress (Giovanoli et al., 2013), the present study explored the single and combined effects of these environmental adversities on two cellular GABAergic markers relevant to neuropathology of developmental neuropsychiatric disorders. We found that immune activation and stress interacted synergistically to cause a deficit in PV expression in the vDG, whilst neither single nor combined exposure to these factors changed PV cell numbers in other regions of the hippocampal formation. Hence, the two environmental adversities investigated here appear to exert a significant but restricted impact on PV abnormalities in the hippocampal formation. Similarly, these manipulations were found to induce significant but limited effects on the number of RELN-expressing cells, which were found to be changed only in the dCA1-CA3 region of the hippocampus. Intriguingly, the direction of stress-induced changes in RELN-expressing cells was dependent on the prenatal treatment histories, suggesting that exposure to the first environmental insult is a developmental determinant of the offspring's pathological reaction to stress-induced changes in RELN expression.

The emergence of deficient PV expression in the vDG following combined immune activation and stress adds to the growing literature suggesting that the ventral part of the hippocampus is more vulnerable than the dorsal part with respect to PV abnormalities following exposure to developmental stressors such as intense prenatal immune challenge (Meyer et al., 2008), inhibition of fetal neurogenesis (Lodge et al., 2009), or increased oxidative stress during peripubertal maturation (Cabungcal et al., 2013; Steullet et al., 2010). The preferential effects of these developmental stressors on PV abnormalities in the ventral hippocampus are consistent with the emerging evidence supporting a pivotal role of ventral hippocampal GABAergic abnormalities in developmental psychiatric disorders, especially with respect to PV-expressing fasts-piking interneurons (Grace, 2010, 2012; Lodge and Grace, 2011). Our findings here suggest that exposure to immune challenge with subthreshold neuronal effects can render the offspring more vulnerable to peripubertal stress-induced changes in ventral hippocampal PV circuitries, suggesting furthermore that both periods are associated

with (latent) susceptibility to long-term GABAergic changes in the ventral hippocampus. Our study thus adds to ongoing attempts to identify such susceptibility periods in the development GABAergic disturbances as seen in schizophrenia and related disorders (Volk and Lewis, 2013).

The observed pattern of RELN changes appears more complex as compared with the effects on PV cells. Stressed offspring that were born to control mothers showed a significant increase in the numbers of RELN-immunoreactive neurons. This effect is consistent with previous observations in other rodent models showing increased hippocampal RELN expression following exposure to neonatal stress (Gross et al., 2012). Interestingly, mild prenatal immune activation (in the absence of additional stress in peripuberty) was sufficient to similarly increase the number of RELN cells in the dCA1-CA3 region. This effect contrasts the long-term consequences of prenatal exposure to intense immune challenge, which are characterized by an impairment of RELN-secreting cells in hippocampal (and cortical) areas (Fatemi et al., 1999; Harvey and Boksa, 2012; Meyer et al., 2006, 2008; Nouel et al., 2012). We do not have a parsimonious explanation for the present observation of increased RELN expression following prenatal exposure to milder forms of maternal immune challenge. In view of the described effects of neonatal stress on RELN hypersecretion (Gross et al., 2012), however, it may be hypothesized that mild prenatal immune activation could represent a certain from of early-life stress that is similarly capable of inducing long-term increases in dorsal hippocampal RELN expression. Interestingly, recent studies using genetically modified mice have demonstrated that forebrain-specific overexpression of reelin prevents the manifestation of behavioral phenotypes related to schizophrenia and bipolar disorder (Texeira et al., 2011). Given that exposure to mild prenatal immune activation alone, or peripubertal stress alone, induces only limited long-term effects on behavioral functions (Giovanoli et al., 2013), one interesting hypothesis would be that the upregulation of reelin seen in the poly(I:C)-alone and stress-alone conditions may be protective against the development of multiple behavioral dysfunctions.

One clear limitation of the present study is that we did not attempt to identify the upstream events leading to altered PV and RELN expression in our model. Hence, we do not know at the present stage whether the numeric changes in PV- and RELN-positive

cells are accounted for by altered gene transcription and subsequent protein synthesis, or, alternatively, whether these effects can be explained by actions on (programmed) cell death. It may also be possible that the selective PV deficit in the vDG might be related to reduced neurogenesis in this brain area. This possibility appears feasible in view of previous findings showing reduced postnatal hippocampal neurogenesis following intense prenatal immune activation in rodents (Meyer et al., 2006; Piontkewitz et al., 2012), so that future investigations towards this direction are therefore clearly warranted. However, if the selective PV deficit in the ventral DG might indeed be accounted for by reduced neurogenesis, one would also expect reduced numbers of other cell populations in the vDG, which was not the case at least with respect to reelin-positive cells.

Future studies will also be needed to explore the contribution of these GABAergic changes to behavioral and cognitive functions. We have already identified a number of behavioral abnormalities after combined exposure to prenatal immune challenge and peripubertal stress (Giovanoli et al., 2013), but the behavioral characterization of this two-hit model is far from complete. Given that both PV and RELN play a prominent role in numerous forms of learning and memory (Fatemi, 2005; Herz and Chen, 2006; Gonzalez-Burgos and Lewis, 2012; Lewis and Moghaddam, 2006), it would be intriguing to explore the cognitive effects of single and combined exposure to prenatal immune activation and peripubertal stress in future studies. Based on our data, however, it may be speculated that animals with the double-hit would only show a limited spectrum of impaired cognitive functions that involve altered GABAergic signaling in the hippocampus. PV is known to be pivotal for proper neuronal synchronization by providing inhibitory input to axon initial segments, and altered neuronal synchronization (in the hippocampus) has often been linked to (hippocampus-regulated) cognitive abnormalities (Gonzalez-Burgos and Lewis, 2012), especially in the domains of spatial learning and memory (Lu et al., 2011). Since the combined effects of prenatal immune activation and peripubertal stress were limited with respect to PV cells in the hippocampus, we would predict that abnormalities in such cognitive domains would rather be mild in offspring with the double-hit exposure.

In conclusion, our stereological investigations show that the combination of two environmental risk factors, which have each been implicated in the etiology of major neuropsychiatric disease, induces significant but restricted neuropathological effects on hippocampal GABAergic cells. These findings support the hypothesis that exposure to these environmental adversities can change the developmental trajectories of hippocampal GABAergic circuitries. At the same time, however, our data suggest that the present two-hit model does not capture the full-spectrum of altered PV and RELN expression as typically seen following intense prenatal immune challenge or in post-mortem brains of individuals with major neuropsychiatric disease.

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Chapter 4

Minocycline Prevents the Neuropathological Interactions Between Prenatal Immune Activation and Peripubertal Stress Exposure

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ABSTRACT

Background: Prenatal exposure to infection and experience of stressful life events in pubescence have both been implicated in the etiology of developmental neuropsychiatric disorders. Recent attempts to assess the cumulative impact of these risk factors in translational animal models have identified pathological effects between these environmental exposures on neuroinflammatory changes during peri-pubertal maturation and brain dysfunctions in adulthood.

Methods: Using an environmental two-hit model in mice, we examined whether the tetracycline antibiotic minocycline given in the event of stress exposure may prevent the subsequent emergence of neuroinflammation and behavioral abnormalities in offspring exposed to combined prenatal immune activation and peri-pubertal stress.

Results: We found that combined exposure to the two environmental factors interacted with each other to cause prepulse inhibition deficits and increased sensitivity to the psychotomimetic drugs amphetamine and dizocilpine in adulthood. Preventive minocycline treatment blocked the emergence of these behavioral dysfunctions. In offspring born to immune-challenged mothers, the antibiotic treatment also prevented abnormal microglia activation and interleukin-1β secretion in response to peri-pubertal stress.

Conclusions: Our findings provide evidence suggesting that minocycline exhibits preventive effects against adult behavioral abnormalities in a developmental two-hit model that combines relevant environmental risk factors and captures neuroinflammatory processes implicated in multifactorial neuropsychiatric disorders. Our preclinical data may encourage attempts to explore the use of minocycline for preventive reasons, especially in brain disorders that are characterized by a delayed onset in adulthood.

INTRODUCTION

Converging evidence implicates an important role of the immune system in normal and pathological brain development (Deverman and Patterson, 2009; McAllister, 2014). The antenatal period is highly sensitive to the damaging effects induced by environmental insults, and therefore, considerable efforts have been made to explore the impact of immune-mediated adversities such as prenatal infection in neuropsychiatric and neurological disorders with developmental components (Meyer et al., 2007). Besides schizophrenia (Brown and Derkits, 2010), maternal exposure to infection and/or inflammatory processes is associated with increased risk of bipolar disorder (Parboosing et al., 2013), autism (Atladóttir et al., 2012), mental retardation (Johnson et al., 2012), and cerebral palsy (Dammann and Leviton, 1997). Such prenatal immune adversities may represent a general vulnerability factor for neurodevelopmental brain disorders, in which the specificity of subsequent disease or symptoms is likely to be influenced by the genetic and environmental context in which the prenatal process occurs (Harvey and Boksa, 2012; Meyer, 2014).

Exposure to psychological trauma during sensitive periods of peri-pubertal maturation is another environmental factor implicated in the etiology of major psychotic and affective disorders (Larsson et al., 2013; Varese et al., 2012). Using a translational model system in mice, we have recently shown that combined exposure to prenatal immune challenge and peri-pubertal stress induces synergistic pathological effects on adult behavioral functions and neurochemistry (Giovanoli et al., 2013). These findings are consistent with the notion that prenatal immune adversities can function as a neurodevelopmental disease primer that increases the offspring's vulnerability to the detrimental neuropathological effects of subsequent stress exposure during peri-pubertal life. In this environmental two-hit model, offspring subjected to combined prenatal immune challenge and peri-pubertal stress also showed marked signs of neuroinflammation that was characterized by microglia overactivation and hypersecretion of brain inflammatory cytokines (Giovanoli et al., 2013). Intriguingly, these neuroinflammatory abnormalities preceded the emergence of behavioral dysfunctions, the latter of which showed a delayed onset in adulthood (Giovanoli et al., 2013). In view of these findings, we have speculated that the induction of transient

neuroinflammation in pubescence may interfere with the processes of neuronal maturation, thereby contributing to the delayed emergence of behavioral dysfunctions in adulthood (Giovanoli et al., 2013). If this scenario holds true, it should be possible to prevent the adult onset of behavioral pathologies by interventions targeting acute neuroinflammation in the event of stress exposure in peri-pubertal life.

The present study was designed to test this hypothesis by exploring whether minocycline (MINO) administration during peri-pubertal stress exposure would be effective in preventing the acute induction of neuroinflammation and subsequent emergence of behavioral pathologies typically seen in adult offspring exposed to combined prenatal immune activation and peri-pubertal stress. MINO is a broad-spectrum antibiotic of the tetracycline family that is believed to act through inhibition of secreted matrix metalloproteinase-9 (Bilousova et al., 2009). It effectively crosses the blood-brain-barrier upon systemic administration (Lee et al., 2006). Importantly, MINO was found to be neuroprotective and highly effective in inhibiting microglia activation and associated neuroinflammation in numerous pathological conditions (Henry et al., 2008; Wixey et al., 2011; Siller et al., 2012), including developmental neuroinflammation models relevant to schizophrenia and related disorders (Mattei et al., 2014). Therefore, we deemed MINO a suitable pharmacological agent to test our hypothesis that reducing stress-induced neuroinflammation in prenatally primed offspring may prevent the subsequent emergence of behavioral dysfunctions in adulthood.

Based on our previous investigations (Giovanoli et al., 2013), prenatal immune activation was induced by the viral mimetic poly(I:C) (= *polyriboinosinic-polyribocytidilic acid*), a synthetic analog of double-stranded RNA that induces a cytokine-associated viral-like acute phase response (Meyer et al., 2009). Offspring born to poly(I:C)-exposed or control mothers were then left undisturbed or exposed to variable and unpredictable stress during peri-pubertal development (Giovanoli et al., 2013). Using this environmental two-hit model, we tested whether MINO treatment during the course of peri-pubertal stress exposure would prevent the adult emergence of behavioral abnormalities identified previously (Giovanoli et al., 2013), including increased anxiety-like behavior, impaired sensorimotor gating, and potentiated sensitivity to psychotomimetic drugs. Furthermore, we ascertained whether this pharmacological

intervention may block the induction of stress-induced microglia activation and inflammatory cytokine expression in prenatally primed offspring and controls.

MATERIAL AND METHODS

Animals

C57BL6/N mice (Charles River, Sulzfeld, Germany) were used throughout the study. A description of the animal housing and maintenance is provided in Supplementary Methods. All procedures described in the present study were approved by the Cantonal Veterinarian's Office of Zurich, Switzerland. All efforts were made to minimize the number of animals used and their suffering.

Prenatal Immune Activation

Pregnant dams on gestation day 9 (GD9) received either a single injection of poly(I:C) (potassium salt; Sigma-Aldrich) at a dose of 1 mg/kg or vehicle (sterile pyrogen-free 0.9% NaCl) as fully described in Supplementary Methods. The dose of poly(I:C) was chosen based on our previous findings showing that this immunological manipulation leads to modest and transient cytokine elevations in the maternal host (Giovanoli et al., 2013). Here, we cofirmed the transient induction of one key inflammatory cytokine, namely interleukin (IL)-6, in the maternal host following treatment with the chosen dose of poly(I:C) (Supplementary Figure 1). The gestational stage (i.e., GD9) roughly corresponds to the first trimester of human pregnancy with respect to fetal developmental biology and was chosen based on our previous findings (Giovanoli et al., 2013).

Peripubertal Stress Exposure

Offspring born to poly(I:C)-treated (POL) or vehicle-treated (CON) mothers were weaned on postnatal day (PND) 21 and caged as littermates of 2-3 animals per cage. They were then left undisturbed (= no stressor; S-) or exposed to variable and unpredictable stress between PND 30 and 40 (S+). For this purpose, all animals of a particular housing cage underwent the same peri-pubertal exposure and preventive treatment (see below). Whenever possible, littermates were assigned to different treatment conditions in order to minimize potential confounds associated with litter

effects. The peri-pubertal time window (i.e., PND 30 to 40) was selected based on our previous findings showing its association with maximal sensitivity for stress-induced neuropathological changes in prenatally immune-challenged animals (Giovanoli et al., 2013). According to protocols established before (Giovanoli et al., 2013), the stress procedure included exposure to 5 distinct stressors (1. electric foot shock; 2. restraint stress; 3. swimming stress; 4. food deprivation; 5. repeated home cage changes) applied on alternate days. A detailed description of the peri-pubertal stress protocol is given in Supplementary Methods.

Minocycline Treatment

Minocycline hydrochloride (MINO; Sigma-Aldrich, Switzerland) was dissolved in regular tap water and provided via regular drinking bottles to avoid additional peri-pubertal stress exposure resulting from injections. Vehicle (VEH)-exposed animals received tap water only. MINO was administered during the course of peri-pubertal stress exposure in order to study its preventive effects against the development of neuroinflammatory and behavioral abnormalities in response to stress. More specifically, MINO treatment started 24 h before exposure to the first stressor on PND 30 and ended after completion of the stress procedure on PND 40. It was administered at a dose of 30 mg/kg/day (per os in drinking water) based on our pilot studies showing that MINO at this dose is highly effective in preventing the up- and down-regulation of pro-inflammatory cytokine expression and neuron-microglia inhibitory signaling typically emerging in the brains of prenatally immune challenged offspring exposed to acute stress in puberty (see Supplementary Figure 2). For each cage, the dose of MINO was calculated based on the average liquid consumption and body weight per cage; this was adjusted every second day based on the liquid consumption and body weight assessed on the preceding two days.

Behavioral Analyses

Behavioral tests in MINO- or VEH-treated offspring subjected to single or combined prenatal immune activation and peri-pubertal stress started 4 weeks after exposure to the last stressor, i.e. between PND 70 and PND 90. This testing age was selected because it corresponds to the adult stage of maturation when the combined effects of prenatal immune activation and peri-pubertal stress become behaviorally

manifested (Giovanoli et al., 2013). In a first cohort of animals, MINO or VEH was given to CON and POL offspring with (S+) or without (S-) peri-pubertal stress exposure. The inclusion of the latter non-stress condition served to ascertain whether the MINO administration regimen might be associated with unwanted side effects on adult behavioral functions in non-stressed animals. Animals from the first cohort were tested in the elevated plus maze test to measure innate anxiety-like behavior, and in the test of prepulse inhibition (PPI) of the acoustic startle reflex to assess sensorimotor gating. A second cohort of animals was used to explore the beneficial effects of MINO against hypersensitivity to the psychotomimetic drugs amphetamine (AMPH) and dizocilpine (MK-801). For this purpose, we only included stressed (S+) POL and CON offspring (with or without MINO treatment) because the preceding tests using the first cohort of animals did not reveal any significant behavioral effects of MINO in non-stressed (S-) animals. The tests assessing AMPH and MK-801 sensitivity were evaluated in terms of drug-induced changes in locomotor activity and were conducted in two independent batches of animals in order to avoid repeated exposure to psychotomimetic drugs. A detailed description of the behavioral test apparatuses and procedure is provided in Supplementary Methods.

Immunohistochemical Analyses

To ascertain the effects of MINO on microglia activation, we performed immunohistochemical analyses of ionized calcium binding adaptor molecule 1 (Iba1) and cluster of differentiation 68 (CD68), two cellular markers expressed by the entire (non-activated and activated) and primarily activated microglia population, respectively (Ransohoff and Perry, 2009). In addition, we explored the effects of MINO treatment on proinflammatory cytokine expression by immunohistochemical evaluations of IL-1β protein. For all immunohistochemical evaluations, MINO or VEH was given to CON and POL offspring during the course of the peri-pubertal stress exposure as described above, and the animals were then sacrificed 24 h after exposure to the last stressor on PND 41. The age of sacrifice was chosen based on our previous findings showing that the neuroinflammatory consequences of combined exposure to the two environmental insults are transient and are primarily manifested in the event of and/or shortly after experience of the second environmental hit in puberty (Giovanoli et al., 2013). A detailed

description of the methods used for the immunohistochemical analyses is provided in Supplementary Methods.

Iba1-, CD68-, and IL-1β-positive cells were counted in the hippocampus using unbiased stereological estimations as fully described in Supplementary Methods. In addition to the stereological estimates, we characterized microglia morphology by quantifying the cell soma area, number of branch points, and number of primary processes of Iba1-positive microglia cells in the hippocampus. The methods used for the assessment of microglia morphology are also fully described in Supplementary Methods. All quantifications were performed in the hippocampus because it is one of the brain regions that is highly sensitive to stress-induced neuroinflammatory abnormalities emerging especially in prenatally immune-challenged offspring (Giovanoli et al., 2013). Hippocampal areas were delineated according to The Mouse Brain in Stereotaxic Coordinates by Franklin and Paxinos and as fully described in Supplementary Methods.

Statistical Analyses

All data were analyzed using analysis of variance (ANOVA) to identify the main effects of prenatal immune treatment, postnatal stress treatment, and preventive MINO treatment, as well as their interactions. The individual ANOVAs used for each test are outlined in Supplementary Table 1-3, which also summarize the statistical outcomes obtained by ANOVA. In a first statistical model used for the behavioral analyses, the number of individual offspring in each treatment condition was used as the sample sizes in each ANOVA (see Supplementary Table 1). In a second statistical model (see Supplementary Table 2), we used the number of litters (CON or POL) as sample size and pooled the cages, which contained 2-3 animals, undergoing the same stress procedure and pharmacological treatment. The second model was used to examine whether the statistical outcomes of the first model, in which the behavioral measures were analyzed based on the number of individual offspring rather than litters, would be compromised by artificial inflation resulting from the inclusion of littermates, which in turn are sometimes considered “technical replicates” in rodent developmental biology (Giovanoli and Meyer, 2013). The two models provided highly comparable statistical outcomes (see Supplementary Methods), so that the statistics of the first model were used for the presentation of the data herein. All immunohistochemical data were analyzed using the

first model because they included samples obtained from 1 offspring per litter and cage only (see Supplementary Table 3). Fisher's least significant difference post-hoc tests were used whenever significant interactions were obtained by the initial ANOVAs. Statistical significance was set at $P < 0.05$. All statistical analyses were performed using the statistical software StatView (version 5.0) implemented on a PC running the Windows XP operating system.

RESULTS

Minocycline Fails to Prevent the Emergence of Increased Anxiety-Like Behavior Induced by Peripubertal Stress

First, we evaluated the efficacy of MINO given during the course of peri-pubertal stress experience to block the emergence of increased anxiety-like behavior in animals exposed to such stress (Giovanoli et al., 2013). Consistent with our previous findings (Giovanoli et al., 2013), we found that peri-pubertal stress exposure increased anxiety-like behavior in the elevated plus maze test regardless of the prenatal immune histories. Hence, stressed offspring displayed a significant reduction in the frequency to enter the open arms compared to non-stressed animals, and this effect similarly emerged in the CON and POL conditions (see Figure 1A). The stress-induced changes in open arm frequencies were not accompanied by concomitant alterations in basal locomotor activity as indexed by the total distance moved on the elevated plus maze (Figure 1B). Notably, peri-pubertal MINO administration failed to correct the stress-induced changes in anxiety-like behavior: A comparable reduction in open arm frequencies was observed in stressed animals regardless of whether they received MINO or VEH (Figure 1A). MINO did also not affect the animals' basal locomotor activity scores as measured by the distance moved during the elevated plus maze test (Figure 1B).

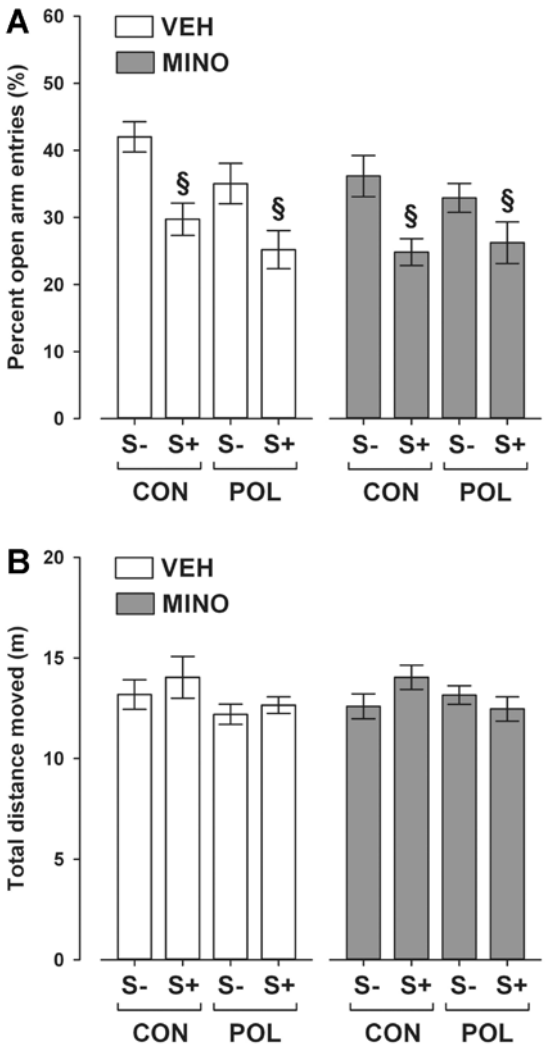


Figure 1. Elevated plus maze performance in adult offspring exposed to single or combined prenatal immune activation and peri-pubertal stress with or without preventive minocycline treatment. Pregnant mice were exposed to poly(I:C) (POL) or control (CON) solution, and the resulting offspring were subjected to sub-chronic stress (S+) or left undisturbed (S-) during peri-pubertal development. During the stress procedure, half of the animals received minocycline (MINO) treatment, and the other half vehicle (VEH = regular tap water) treatment. (A) The bar plot depicts percent open arm entries (%). $^{\S}P < 0.001$, reflecting the significant main effect of peri-pubertal stress. N = 12-18 per group. (B) The bar plot shows the total distance moved (cm) during the entire test period. N = 12-18 per group. All data are means±SEM.

Minocycline Prevents the Development of Sensorimotor Gating Deficiency Following Combined Prenatal Immune Activation and Peripubertal Stress

In a next step, we tested whether MINO treatment during stress exposure may be effective in preventing the adult emergence of sensorimotor gating deficiency following combined prenatal immune activation and peri-pubertal stress (Giovanoli et al., 2013).

Sensorimotor gating was evaluated using the paradigm of PPI of the acoustic startle reflex. In accordance with our previous findings (Giovanoli et al., 2013), we revealed synergistic effects between the two environmental manipulations in the disruption of adult PPI. As summarized in Figure 2, neither prenatal immune activation alone, nor stress alone, was sufficient to significantly affect PPI in the VEH condition, but only the combination of the two factors resulted in a significant attenuation of PPI (Figure 2). Peripubertal MINO administration prevented the disruption of PPI normally seen after combined exposure to prenatal immune activation and stress in puberty (Figure 2). Indeed, MINO treatment in stressed POL offspring significantly elevated PPI scores to levels found in VEH-treated CON offspring with or without additional stress exposure (Figure 2). MINO administration also increased PPI levels in non-stressed offspring, but this effect only emerged in the prenatal CON condition (Figure 2).

Single or combined exposure to prenatal immune activation and stress did not affect the reactivity to pulse-alone trials or prepulse alone trials (Supplementary Table 4). Peripubertal MINO treatment did also not influence these dependent variables, suggesting that the beneficial effects of the drug on PPI scores in stressed POL offspring are dissociable from possible influences on startle reactivity and prepulse-induced reactivity.

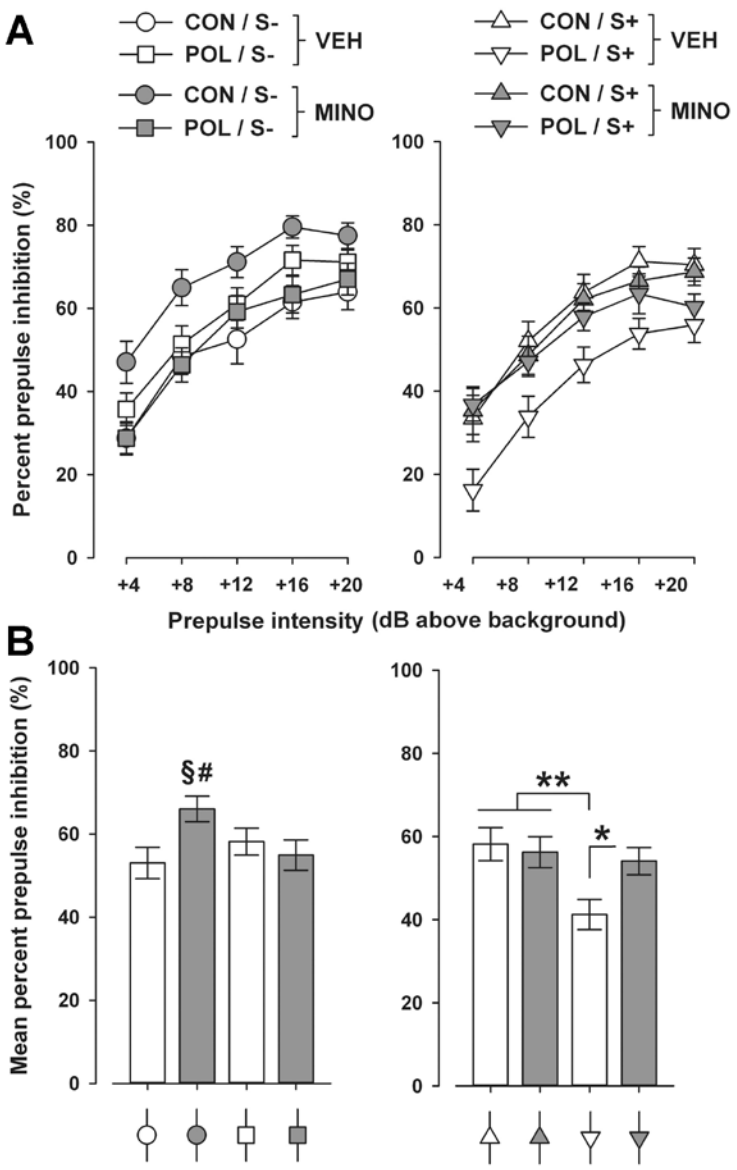


Figure 2. Prepulse inhibition of the acoustic startle reflex in adult offspring exposed to single or combined prenatal immune activation and peri-pubertal stress with or without preventive minocycline treatment. Pregnant mice were exposed to poly(I:C) (POL) or control (CON) solution, and the resulting offspring were subjected to sub-chronic stress (S+) or left undisturbed (S-) during peri-pubertal development. During the stress procedure, half of the animals received minocycline (MINO) treatment, and the other half vehicle (VEH = regular tap water) treatment. (A) The line plots depict percent prepulse inhibition (%) as a function of increasing prepulse intensities (dB above background of 65 dB). *N* = 12-18 per group. (B) The bar plot shows the mean peercent prepulse inhibition (%) across all 5 prepulse intensities. $^{\S}P < 0.01$, reflecting the significant difference between CON/S-/MINO offspring and CON/S-/VEH offspring; $^{\#}P < 0.05$, reflecting the significant difference between CON/S-/MINO offspring and POL/S-/offspring; $^{*}P < 0.05$ and $^{**}P < 0.05$, reflecting the indicated differences in the S+ groups. *N* = 12-18 per group. All data are means±SEM.

Minocycline Prevents the Emergence of Hypersensitivity to Psychotomimetic Drugs Induced by Combined Prenatal Immune Activation and Peripubertal Stress

Another pathological feature emerging following combined exposure to prenatal immune activation and peri-pubertal stress is the adult onset of increased sensitivity to psychotomimetic drugs (Giovanoli et al., 2013). We have previously revealed synergistic effects between these two environmental manipulations on the development of potentiated locomotor reactions to the indirect dopamine receptor agonist AMPH and the non-competitive NMDA receptor antagonist MK-801 (Giovanoli et al., 2013). Here, we were interested to test whether MINO treatment during peri-pubertal stress exposure may be effective in preventing these abnormalities. Consistent with our previous report (Giovanoli et al., 2013), stress exposure in POL offspring markedly increased AMPH-induced locomotor activity in the open field test compared to stress exposure in CON offspring (see Figure 3A). MINO treatment fully prevented this pathological phenotype in stressed POL offspring without significantly influencing AMPH-induced activity in stressed CON offspring (Figure 3A).

Preventive MINO treatment also blocked the potentiation of MK-801-induced hyperactivity typically emerging in offspring with combined prenatal immune activation and peri-pubertal stress (see Figure 3B). Like in the AMPH sensitivity test (Figure 3A), MINO treatment reduced the MK-801-induced hyperlocomotor responses in stressed POL offspring to levels found in stressed CON offspring that had been treated with VEH or MINO (Figure 3B).

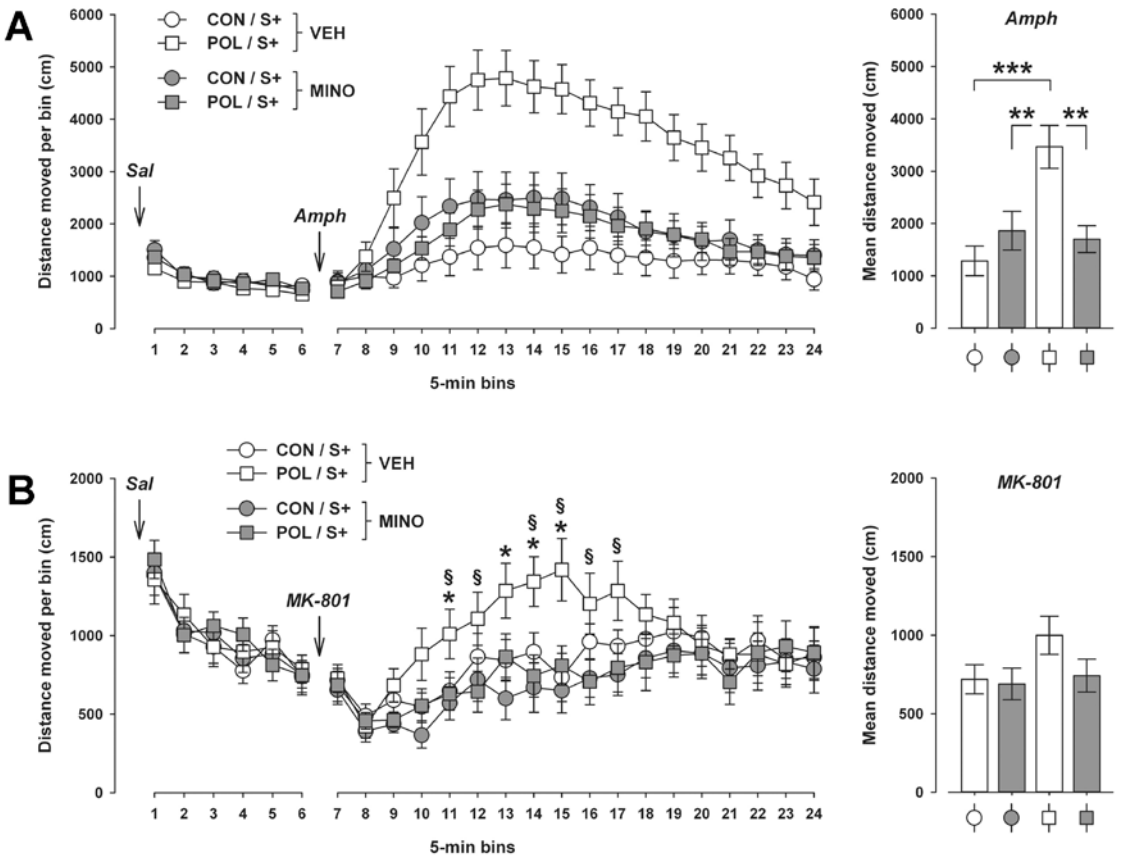


Figure 3. Effects of minocycline on the locomotor responses to acute challenge with psychotomimetic drugs in adult offspring exposed to prenatal control treatment or immune activation with additional stress exposure in puberty. Pregnant mice were exposed to poly(I:C) (POL) or control (CON) solution, and the resulting offspring were subjected to sub-chronic stress (S+) during peri-pubertal development. During the stress procedure, half of the animals received minocycline (MINO) treatment, and the other half vehicle (VEH = regular tap water) treatment. (A) Locomotor reaction to the indirect dopamine receptor agonist amphetamine (Amph; 2.5 mg/kg, i.p.). The line plot depicts the distance moved in a standard open field arena to initial vehicle (saline, Sal) treatment and subsequent Amph treatment as a function of successive 5-min bins. The bar plot depicts the mean distance moved after Amph treatment. $^{**}P < 0.01$ and $^{***}P < 0.001$; N = 12-16 per group. (B) Locomotor reaction to the non-competitive NMDA receptor antagonist dizocilpine (MK-801; 0.15 mg/kg, i.p.). The line plot shows the distance moved in a standard open field arena to initial vehicle (Sal) treatment and subsequent MK-801 treatment as a function of successive 5-min bins. $^{*}P < 0.05$, reflecting the significant difference between VEH-exposed CON/S+ and POL/S+ offspring at individual bins; $^{\S}P < 0.05$, reflecting the significant difference between VEH-exposed POL/S+ and MINO-treated POL/S+ offspring at individual bins. N = 16 per group. All data are means±SEM.

Minocycline Blocks Microglia Activation and IL-1β Secretion in Offspring with Combined Exposure to Prenatal Immune Activation and Stress in Puberty

We have previously demonstrated that offspring born to immune-challenged mothers show an increased sensitivity to stress-induced activation of hippocampal microglia cells (Giovanoli et al., 2013). Intriguingly, such microglia abnormalities in prenatally immunechallenged offspring were clearly manifested shortly after exposure to the last stressor in peri-pubertal life, but absent when the offspring reached adulthood (Giovanoli et al., 2013). Hence, prenatal immune activation can prime latent microglia overactivation that precedes the adult onset of multiple behavioral abnormalities in response to additional stress exposure in puberty (Giovanoli et al., 2013). In view of the preventive potential of MINO on adult behavioral dysfunctions (see **Figure 2** and **3**), we were interested to examine whether this pharmacological intervention may be effective in blocking the stress-induced activation of microglia cells in offspring with prenatal inflammatory histories relative to offspring without such prenatal histories.

As summarized in Figure 4, MINO treatment during the course of peri-pubertal stress exposure did not change the number of Iba1-positive cells, suggesting that it did not influence the overall density of the entire (non-activated and activated) microglia population. On the other hand, the pharmacological intervention was effective in correcting morphological and cellular signs of microglia activation in prenatally primed offspring as assessed shortly after stress exposure in puberty: MINO normalized the enlargement of Iba1-positive cell soma areas in stressed POL offspring to levels present in stressed CON offspring (Figure 4), and it fully blocked the induction of CD68 expression typically seen in offspring with combined exposure to prenatal immune activation and peri-pubertal stress (Figure 5).

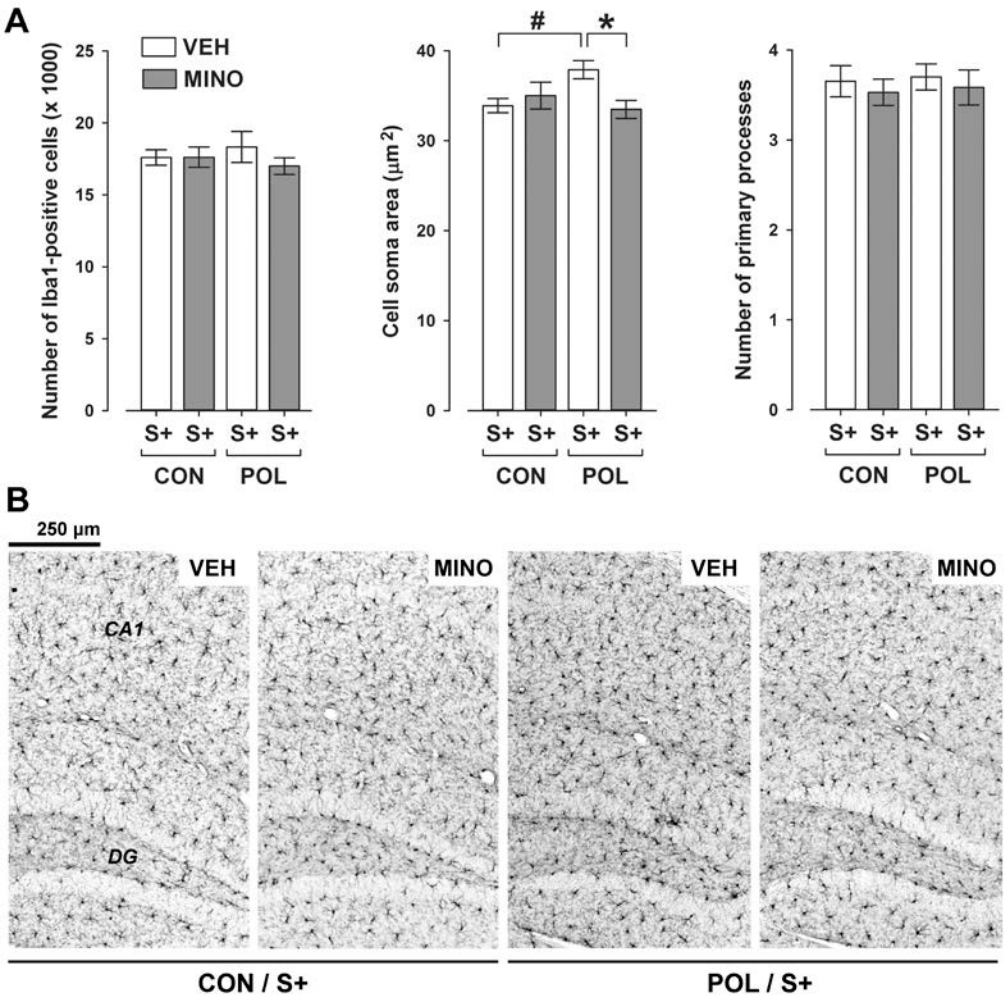


Figure 4. Effects of minocycline on Iba1-positive microglia in the hippocampus of stressed offspring born to control or gestationally immune challenged mothers. Pregnant mice were exposed to poly(I:C) (POL) or control (CON) solution, and the resulting offspring were subjected to sub-chronic stress (S+) during peri-pubertal development. During the stress procedure, half of the animals received minocycline (MINO) treatment, and the other half vehicle (VEH = regular tap water) treatment. **(A)** The bar plots depict the stereological estimates of Iba1-positive cells as well as cell soma area and number of primary processes of Iba1-positive microglia. $^{*}P < 0.05$ and $^{\#}P = 0.07$; $N = 5$ per group. **(B)** The photomicrographs show representative coronal brain sections at the level of the hippocampus (highlighting CA1 and dentate gyrus [DG] regions) stained with anti-Iba1 antibody.

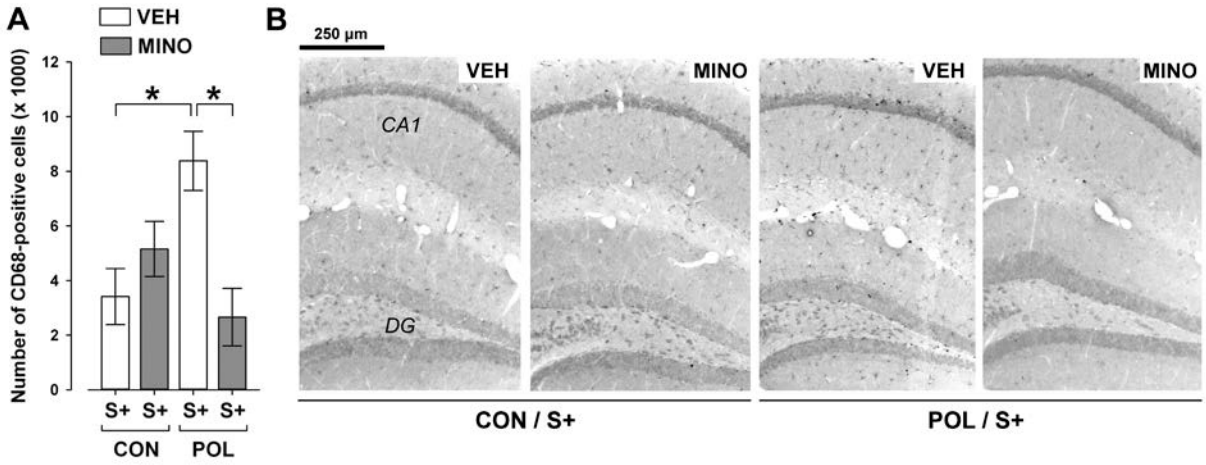


Figure 5. Effects of minocycline on CD68-positive microglia in the hippocampus of stressed offspring born to control or gestationally immune challenged mothers. Pregnant mice were exposed to poly(I:C) (POL) or control (CON) solution, and the resulting offspring were subjected to sub-chronic stress (S+) during peri-pubertal development. During the stress procedure, half of the animals received minocycline (MINO) treatment, and the other half vehicle (VEH = regular tap water) treatment. (A) The bar plot shows the stereological estimates of CD68-positive cells. *P < 0.05, N = 5 per group. (B) The photomicrographs show representative coronal brain sections at the level of the hippocampus (highlighting CA1 and dentate gyrus [DG] regions) stained with anti-CD68 antibody.

Consistent with our previous investigations (Giovanoli et al., 2013), we found that POL offspring exhibited significantly enhanced IL-1β protein expression in response to peri-pubertal stress compared to stress exposure in CON offspring (Figure 6). MINO administration fully normalized this augmentation in inflammatory cytokine expression, so that the numbers of IL-1β-positive cells were highly comparable between VEH-treated CON offspring with stress and MINO-treated POL offspring with stress (Figure 6).

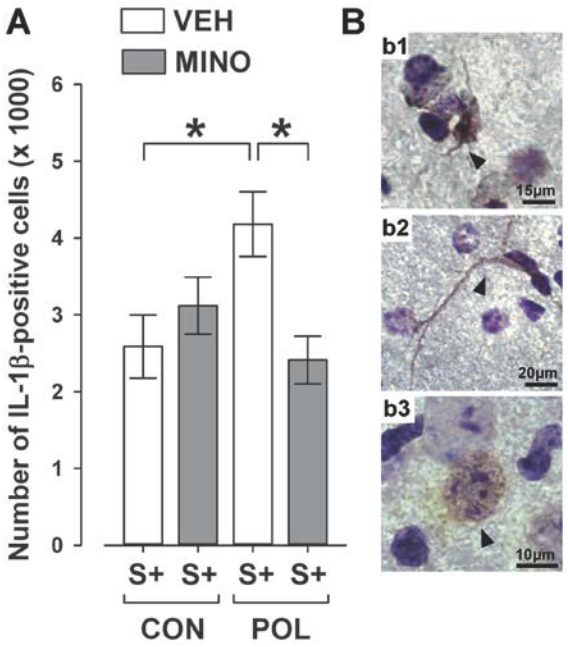


Figure 6. Effects of minocycline on IL-1β-positive cells in the hippocampus of stressed offspring born to control or gestationally immune challenged mothers. Pregnant mice were exposed to poly(I:C) (POL) or control (CON) solution, and the resulting offspring were subjected to sub-chronic stress (S+) during peri-pubertal development. During the stress procedure, half of the animals received minocycline (MINO) treatment, and the other half vehicle (VEH = regular tap water) treatment. (A) The bar plot shows the stereological estimates of IL-1β-positive cells. *P < 0.05, *N* = 5 per group. (B) The photomicrographs b1-b3 show representative IL-1β immunoreactivity (indicated by the black arrowheads) in the hippocampal formation visualized with a Nissl/IL-1β double-staining. Note that IL-1β immunoreactivity was present in cells displaying noticeable immunoreactive processes (b1 and b2) and in cells with no immunoreactive processes (b3).

DISCUSSION

Our study demonstrates that administration of the tetracycline antibiotic MINO during the course of peri-pubertal stress exposure prevents the subsequent emergence of behavioral abnormalities in offspring with a history of prenatal immune activation. In the present environmental two-hit model, combined exposure to prenatal immune challenge and peri-pubertal stress induced synergistic pathological effects on adult behavioral functions, which included impairments in PPI and increased sensitivity to the psychotomimetic drugs AMPH and MK-801. Peripubertal MINO administration was sufficient to block the development of these abnormalities. In contrast, it failed to normalize the emergence of increased anxiety-like behavior, which developed after

experience of peri-pubertal stress regardless of whether the offspring had been exposed to the first environmental hit in prenatal life. These findings indicate that MINO is not simply associated with general protective effects against stress-induced behavioral abnormalities, but rather, it seems to be particularly efficient in preventing pathological effects that require the combination of two environmental adversities, in this case prenatal immune activation followed by peri-pubertal stress exposure.

The beneficial effects of MINO on PPI impairments and hypersensitivity to psychotomimetic drugs as revealed in our two-hit model are consistent with the drug's beneficial effects in other rodent models that capture these aspects of behavioral dysfunctions (Zhang et al., 2007; Levkovitz et al., 2007; Zhu et al., 2014). Notably, our data corroborate the recent findings obtained in a more severe maternal immune activation model, in which prenatal exposure to a high dose of poly(I:C) resulted in persistent microglia activation, increased inflammatory cytokine production, and PPI impairments even in the absence of additional environmental adversities such as stress (Mattei et al., 2014). Consistent with our findings, chronic MINO treatment effectively normalized the neuroinflammatory processes and restored the disruption of PPI in this latter model of prenatal immune challenge (Mattei et al., 2014).

Despite these similarities, there are also important distinctions between the present two-hit model and more severe maternal immune activation models, especially with respect to the postnatal persistence of neuroinflammatory signs. Converging evidence suggests that maternal exposure to intense inflammatory stimuli can lead to long-term neuroinflammatory changes in the offspring, which can persist from juvenile to adult stages of life (Mattei et al., 2014; Borrell et al., 2002; Manitz et al., 2013). These neuroimmune changes contrast the nature of neuroinflammatory signs in our two-hit model, in which increased microglia activation and brain inflammatory cytokine production emerge in prenatally immune challenged offspring only if they are exposed to additional environmental adversities such as peri-pubertal stress. Hence, a second environmental hit is required to unmask latent neuroimmune pathologies following priming by mild prenatal immune activation (Giovanoli et al., 2013). Moreover, these neuroimmune abnormalities in offspring with mild forms of prenatal immune activation are transient and are evident only during the course and shortly after exposure to the

second environmental hit in puberty (Giovanoli et al., 2013). Against these backgrounds, our findings highlight that MINO can exert beneficial effects even if its administration is restricted to a developmental period of transient neuroinflammation that precedes the adult onset of behavioral abnormalities. These data may also encourage clinical attempts to explore the preventive potential of MINO when administered during early or even presymptomatic phases of chronic mental illnesses with delayed onsets in early adulthood, including schizophrenia and related psychotic disorders (Fond et al., 2014). Thus far, MINO has demonstrated positive effects in the treatment of schizophrenia once overt psychopathological symptoms are manifest (Levkovitz et al., 2010; Chaudhry et al., 2012; Khodaie-Ardakani et al., 2014), but its preventive potential still awaits further examination.

The mechanisms through which MINO prevented the subsequent emergence of behavioral abnormalities remain unknown and warrant future investigations. It can be excluded, however, that the drug's beneficial effects are related to a direct action on neurobehavioral processes in adulthood. Indeed, MINO was administered only during a restricted period in peri-pubertal life, which was followed by a wash-out period of more than 4 weeks. It is therefore more likely that MINO may have exerted protective effects in the event of exposures to developmental stressors, thereby preventing abnormal maturation of neuronal substrates involved in adult sensorimotor gating and psychotomimetic drug sensitivity. The peri-pubertal period is arguably a very sensitive and dynamic period of neuronal and hormonal rearrangements that prepare the growing organism to the demands in adult life (Eiland and Romeo, 2013; Schneider, 2013), so that altered neuroinflammatory processes occurring during this period can indeed be expected to have long-lasting consequences on adult brain functions (Meyer, 2013).

It would be tempting to speculate that abnormal microglia activation during peri-pubertal brain maturation may assume a key role in these processes. Microglia play crucial roles in both neuronal protection and pathology, and are often referred to as a "double-edged sword" (Block et al., 2007; Bilbo and Schwarz, 2009). On the one hand, they secrete neurotrophic factors pivotal for cellular repair and recruit immune cells into the brain for clearance of infection or cellular debris. On the other hand, chronic or

exaggerated microglia activation is linked to excessive secretion of proinflammatory factors and has been linked to neurodegenerative processes (Block et al., 2007; Bilbo and Schwarz, 2009). At the present stage, however, the hypothesized mechanistic role of abnormal microglia activation still needs to be met with caution in the present two-hit model. Indeed, we did not establish a direct link between peri-pubertal neuroinflammation and disruption of neuronal substrates across postnatal maturation, nor did we examine alternative mechanism by which MINO could exert beneficial effects in this two-hit model. Despite the converging evidence that MINO can effectively block microglia activation and subsequent inflammatory outputs (Henry et al., 2008; Wixey et al., 2011; Siller et al., 2012; Mattei et al., 2014), it is arguably not a specific microglia inhibitor but is associated with various other pharmacological properties (Bilousova et al., 2009; Block et al., 2007). Despite these limitations, our findings suggest that MINO exerts beneficial effects in a developmental disruption model that bears etiological relevance to brain disorders with delayed onsets in adulthood (Giovanoli et al., 2013).

Another limitation of our study is that we did not attempt to rescue the adult onset of behavioral dysfunctions by post-stress MINO administration. If MINO is effective in preventing such adult dysfunctions even if given shortly after the experience of traumatizing events in puberty, then this would represent a clinically equally or even more relevant approach compared to MINO administration in the event of trauma experience. Furthermore, we did also not test the behavioral effects of adult MINO treatment, that is, when multiple behavioral symptoms have already developed. Our primary interest was to restrict the MINO treatment to a maturational period that is clearly associated with altered microglia activation with the aim to explore the drug's preventive potential in this context. Nevertheless, it would be desirable to examine the therapeutic potential of MINO after the behavioral symptoms have already emerged. If peri-pubertal neuroinflammatory processes were indeed key for mediating the synergistic interactions between prenatal immune activation and stress in pubertal life, then one prediction would be that adult MINO administration might only show limited beneficial effects, at least with respect to the treatment of sensorimotor gating deficits and psychotomimetic drugs hypersensitivity.

In conclusion, our findings provide novel evidence showing that the tetracycline antibiotic MINO exhibits preventive effects against adult behavioral abnormalities in a developmental two-hit model that incorporates two important environmental risk factors and neuroinflammatory processes implicated in multifactorial neuropsychiatric disorders. Our preclinical data may encourage attempts to explore the use of MINO for preventive reasons, especially in brain disorders that are characterized by delayed onsets in adulthood.

SUPPLEMENTARY METHODS

Animals

C57BL6/N female and male breeders were obtained from Charles Rivers (Sulzfeld, Germany) at the age of 10-12 weeks. Animals were kept in a temperature- and humidity-controlled (21±1 °C, 55±5 %) holding facility under a reversed light-dark cycle (lights off: 8:00 A.M. to 8:00 P.M.). All animals had *ad libitum* access to standard rodent chow (Kliba 3430, Klibamuehlen, Kaiseraugst, Switzerland) and water unless specified otherwise. For the purpose of the maternal immunological manipulation during pregnancy, female mice were subjected to a timed-mating procedure as described previously (Meyer et al., 2005).

Prenatal Immune Activation

Pregnant dams on gestation day 9 (GD9) received either a single injection of poly(I:C) (potassium salt; Sigma-Aldrich) or vehicle. The gestational stage (i.e., GD9) was selected based on our previous findings (Giovanoli et al., 2013). GD9 in mice corresponds approximately to the first trimester of human pregnancy with respect to developmental biology and percentage of gestation (Clancy et al., 2001). Poly(I:C) (1 mg/kg) was dissolved in sterile pyrogen-free 0.9 % NaCl (= vehicle) solution to yield a final concentration of 0.2 mg/ml and was administered via the intravenous (i.v.) route at the tail vein under mild physical constraint (see below). All solutions were freshly prepared at the day of administration and injected with a volume of 5 ml/kg. All animals were returned to their home cages immediately after the injection procedure.

For the purpose of the intravenous injections, each animal's tail was first bathed in 40 °C water to better visualize and dilate the tail veins. Subsequently, the animals were mildly restrained using a nonrestrictive Plexiglas restrainer (561-RC, Plas Labs) and the substance was injected either at the right or left tail vein. The success of the injection was verified according to three main criteria: (1) resistance of the injection (this refers to the fact that an incomplete hit of the vein prevents the intravenous administration of the full volume of substance), (2) temporary replacement of blood by the injected substance (this refers to the fact that a successful injection at the tail vein can be visualized by the temporary replacement of blood by the injected substance), and (3) appearance of a blood drop following retraction of the needle (this refers to the fact that following successful intravenous injection, a blood drop appears at the injection site following retraction of the needle). In addition to these criteria, we confirmed the transient induction of one key inflammatory cytokine, namely interleukin (IL)-6, in the maternal host following treatment with the chosen dose of poly(I:C).

Peripubertal Stress Exposure

The peri-pubertal stress procedure was performed according to protocols established before (Giovanoli et al., 2013) and included 5 distinct stressors applied on alternate days between PND 30 and 40 in the following order: 1. Electric foot shock (day 1); 2. Restraint stress (day 3); 3. Food deprivation (day 5); 4. Forced swimming stress (day 7); 5. Multiple changes of home cage embedding (day 9). A one-day resting period followed each stressor day.

- *Exposure to electric foot shock (day 1).* Four identical freezing chambers (Coulbourn Instruments, Allentown, PA) were used, each installed in a sound insulated, wooden cabinet. Each cabinet was equipped with 16 stainless steel bars (diameter: 4 mm) spaced 10 mm apart from centre to centre, through which scrambled electric foot shocks could be delivered by a Coulbourn Precision Regulated Animal Shocker (Model E13-12). Animals were restricted to a semi-circular (19 cm in diameter; 30 cm height) Plexiglas enclosure, which was positioned over the grid. The lights were turned off throughout the entire period, and additional odour stimuli were presented by placing a compressed tissue saturated with SteriliumTM solution (Bode Chemie Hamburg, Germany) into the wooden cabinets. The chambers were cleaned after each run, and

SteriliumTM tissues were saturated again. Each animal was first placed into the chamber for an initial period of 3 min, after which it received 3 mild electric foot shocks (0.25 mA), each lasting 1 sec. The electric foot shocks were separated by intervals of 3 min. The session ended with an additional 3 min period, in which no shocks were delivered.

• *Exposure to restraint stress (day 3).* Each animal assigned to the peri-pubertal stress exposure was kept in a transparent plastic tube (diameter: 3 cm; length: 11.5 cm) for 45 min. The restrainer tubes contained drilled holes (2 mm in diameter) so as to facilitate oxygen supply. The tubes were tapped on a wooden table which was placed in a brightly lit testing room. The animals were immediately returned to their home cages at the end of the restraint stress procedure.

• *Exposure to food deprivation (day 5).* Littermates assigned to peri-pubertal stress were subjected to 20-hrs food deprivation. For this purpose, animals were kept in their homes cages, and the food was removed at 12h00 on day 5 and added again the next day at 08h00. Animals had free access to water during the entire food deprivation period.

• *Exposure to forced swim stress (day 7).* The apparatus used for the forced swim stress was made of a circular white fiberglass tank (diameter: 100 cm, height: 36 cm) filled with water (temperature: 18 °C, depth: 20 cm). The water tank was placed in a brightly lit testing room. Each animal underwent two sessions of forced swimming, each lasting 1 min. The two swimming sessions were separated by a 3-min interval, during which the animals were kept in a waiting box containing sawdust embedding. The animals were dried with a towel and immediately brought back to their home cages after the second swimming session.

• *Exposure to repeated changing of home cages (day 9).* The last stressor was in the form of repeated changing of home cages. For this purpose, the animals were transferred from their original home cages to new cages containing fresh sawdust embedding. Food and water was supplied to the animals immediately after transferring them from one cage to another in order to avoid additional food or water deprivation. This procedure was repeated 5 times at irregular intervals during the dark phase of the light-dark cycle.

Behavioral Analyses

• *Elevated Plus Maze.* The apparatus was made of Plexiglas painted in grey and consisted of 4 equally spaced arms (5 x 30 cm^2) radiating from a square centre (5 × 5 cm^2). One pair of opposing arms was enclosed with opaque walls (height: 15 cm) except for the side adjoining the central square (CZ). The remaining two arms were exposed with a parameter border (height: 3 mm) along the outer edges. The maze was elevated 70 cm above floor level and positioned in a testing room with diffused lighting (approximately 20 Lux in open arm and 10 Lux in closed arm). A digital camera was mounted above the plus maze, captured images at a rate of 5 Hz and transmitted them to a PC running the EthoVision (Noldus Technology, Wageningen, The Netherlands) tracking system. A test session began by placing the animal into the CZ with it facing one of the closed arms. It was then left to explore freely for 5 min before being returned to the home cage. After each trial, the apparatus was cleansed with water and dried before a new trial began. The relative (percent) open arm entries during the entire 5-min test period were analysed in order to index anxiety-related behavior. The relative open arm frequency was calculated using the formula [(open arm entries) / (total arm entries) × 100]. In addition, total distance moved in the entire maze was analysed in order to assess general locomotor activity.

• *Prepulse Inhibition of the Acoustic Startle Reflex.* Sensorimotor gating was assessed using the paradigm of prepulse inhibition (PPI) of the acoustic startle reflex. PPI of the acoustic startle reflex refers to the reduction in startle reaction in response to a startle-eliciting pulse stimulus when it is shortly preceded by a weak prepulse stimulus. The apparatus consisted of four individual startle chambers for mice (San Diego Instruments, San Diego, CA, USA) and has been fully described elsewhere (Meyer et al., 2005). During a 45-min test session, subjects were presented with a series of discrete test trials comprising a mixture of four trial-types as fully described previously (Meyer et al., 2008). In brief, these trial stimuli included pulse-alone, prepulse-plus-pulse, and prepulse-alone trials, as well as no-stimulus trials in which no discrete stimulus other than the constant background noise (65 dB_A) was presented. The startle program consisted of one 40-ms pulse of white noise (120 dB_A) combined with five different pre-pulses. Prepulses were a 20-ms burst of white noise at five different intensities (69, 73, 77, 81, and 85 dB_A, which corresponded to 4, 8, 12, 16, and 20 dB_A

above background). The stimulus onset asynchrony (SOA) of the prepulse and pulse stimuli in prepulse-plus-pulse trials was 100 ms. Following a 2-min acclimatization period, 6 consecutive pulse-alone trials were presented in order to habituate and stabilize the animals' startle response. Subsequent to this startle habituation phase, each trial stimulus was presented 12 times in a pseudorandom order with an average interval between successive trials (ITI) of 15 ± 5 s. The session was concluded with 6 consecutive pulse-alone trials. Boxes were cleansed with warm tap water and dried between each animal. Each box was used by one sex only. For each subject and at each of the five possible prepulse intensities, PPI was indexed by percent inhibition of startle response obtained in the prepulse-plus-trials compared to pulse-alone trials by following expression: [1-(mean reactivity on prepulse-plus-pulse trials / mean reactivity on pulse-alone trials) × 1/100]. The first and last six trials were not included in the calculation and analysis of percent PPI. In addition to PPI, reactivates to pulse-alone trials and prepulse-alone trials were also analysed.

- *Behavioral Sensitivity to Psychotomimetic Drugs.* The sensitivity to psychotomimetic drugs was assessed by measuring drug-induced locomotor activity in an open field apparatus. The apparatus consisted of 4 identical square arenas (40 cm × 40 cm × 35 cm high) made of wood and painted grey as fully described elsewhere (Meyer et al., 2008). First, the animals were intraperitoneally (i.p.) injected with vehicle (isotonic 0.9% NaCl) solution and immediately placed in the apparatus to measure basal locomotor activity for 30 min. Subsequently, the animals were removed from the apparatus and administered with either amphetamine (AMPH; D-amphetamine sulfate, Sigma-Aldrich, Switzerland; 2.5 mg/kg, i.p.) or dizocilpine (MK-801; (+)-MK-801 hydrogen maleate; Sigma-Aldrich, Switzerland; 0.15 mg/kg, i.p.). They were then immediately returned to the same arena, and the locomotor response to the acute drug challenge was monitored for a period of 90 min. Both drugs were dissolved in isotonic 0.9% NaCl solution to achieve the desired concentration for injection. The doses of AMPH (2.5 mg/kg, i.p.) and MK-801 (0.15 mg/kg, i.p.) were selected based on our previous study showing synergistic effects of the combined prenatal and postnatal manipulations on AMPH- and MK-801-induced changes in locomotor activity (Giovanoli et al., 2013). The volume of injection was 5 ml/kg for both solutions. All solutions were freshly prepared on the day of testing.

Immunohistochemical Analyses

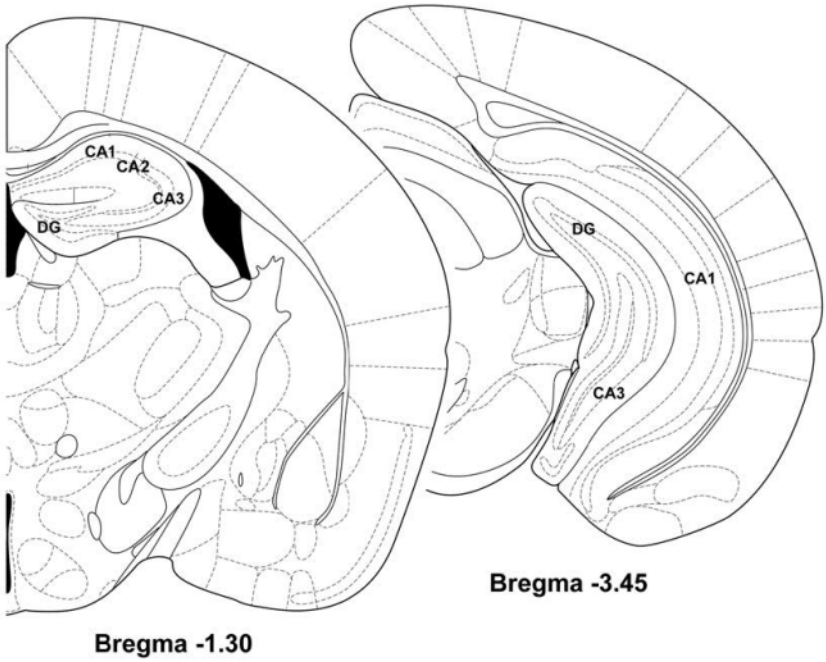
The animals were deeply anesthetized with an overdose of Nembutal (Abbott Laboratories) and perfused transcardially with 0.9 % NaCl, followed by 4 % phosphate-buffered paraformaldehyde solution containing 15 % picric acid. The dissected brains were postfixed in the same fixative for 6 h and processed for antigen retrieval involving overnight incubation in citric acid buffer (pH = 4.5) followed by a 90 s microwave treatment at 480 W according to protocols established before (Vuillermot et al., 2012). The brains were then cryoprotected using 30 % sucrose in phosphate buffered saline (PBS), frozen with powdered dry ice, and stored at −80 °C until further processing.

Perfused brain samples were cut coronally at 30 µm thickness from frozen blocks with a sliding microtome. Eight serial sections were prepared for each animal and, after rinsing in PBS, stored at −20 °C in antifreeze solution (30 % glycerol and 30 % ethylene glycol in PBS at 25 mM and pH 7.4) until further processing. For immunohistochemical staining, the slices were rinsed three times for 10 min in PBS, and blocking was done in PBS, 0.3 % Triton X-100, 10 % normal serum for 1 h at room temperature. The following primary antibodies were used: Rabbit anti-Iba1 (Wako, CatNo# 019-19741; diluted 1:2000), rat anti-CD68 (AbDSerotec, CatNo# MCA1957; diluted 1:5000), and rabbit anti-IL-1β (PeproTech CatNo# 500-P51). All antibodies were diluted in PBS containing 0.3 % Triton X-100 and 2 % normal serum, and the sections were incubated free-floating overnight at room temperature. After three washes with PBS (10 min each), the sections were incubated for 1 h with the biotinylated secondary antibodies diluted 1:500 in PBS containing 2 % NGS and 0.3 % Triton X-100. Sections were washed again three times for 10 min in PBS and incubated with Vectastain kit (Vector Laboratories) diluted in PBS for 1 h. After three rinses in 0.1 M Tris-HCl, pH 7.4, the sections were stained with 1.25 % 3,3-diaminobenzidine and 0.08 % H_2O_2 for 10–15 min, rinsed again four times in PBS, dehydrated, and coverslipped with Eukitt (Kindler). The IL-1β-labelled slices were counterstained with 0.25 % Cresyl Violet (Nissl stain) according to standard protocols (Nyffeler et al., 2008).

Unbiased Stereological Estimations

The numbers of Iba1-, CD68, or IL-1β-immunoreactive cells were determined by unbiased stereological estimations using the optical fractionator method (Gundersen et

al., 1988). With the aid of the image analysis computer software Stereo Investigator (version 6.50.1; MicroBrightField), every section of a one-in-eight series was measured, resulting in an average of 6 hippocampal sections per brain sample as previously described (Giovanoli et al., 2013). The following sampling parameters were used: (1) a fixed counting frame with a width of 60 µm and a length of 60 µm, and (2) a sampling grid size of 200 µm × 150 µm. The counting frames were placed randomly at the intersections of the grid within the outlined structure of interest by the software. The cells were counted following the unbiased sampling rule using the 40× oil lens [numerical aperture (NA), 1.3] and included in the measurement when they came into focus within the optical dissector (Howard and Reed, 2005). All immunohistochemical preparations were quantified in the entire (dorsal and ventral) hippocampus with reference to Bregma [-1.3 to -3.45 mm] and included CA1 to CA3 subfields and dentate gyrus (DG) as outlined below:



Schematic coronal brain sections delineating the hippocampal areas investigated with reference to Bregma [adapted from *The Mouse Brain in Stereotaxic Coordinates* by Franklin and Paxinos (2008)].

Assessment of Microglia Morphology

Iba1-immunoreactive microglia were visualized under the 63× oil lens [numerical aperture (NA), 1.4] using a Zeiss Axiophot microscope. Various parameters of microglia cell morphology were assessed in the CA3 region of the hippocampus by placing a counting frame of 100 µm x 100 µm randomly into three sections of a one-in-eight series. All microglia cells captured by the counting frame were included in the morphological analyses, except when microglial processes were obscured by either background labelling or other cells. Four microglia cells per section were traced using the software Stereo Investigator (version 6.50.1; MicroBrightField), for which cell soma area and number of primary processes were estimated giving a total of 12 cells as described before (Giovanoli et al., 2013).

Maternal Cytokine Measurements

Pregnant mice were killed by decapitation 4 h and 24 h after poly(I:C) or vehicle (saline) administration, and trunk blood was collected into Eppendorf tubes containing appropriate amounts of EDTA (= ethylenediaminetetraacetic acid). The collected blood samples were centrifuged at 2000 x g for 5 min to generate plasma samples, which were stored at −80 °C until later analyses.

Plasma levels of IL-6 were determined using multiplexed particle-based flow cytometric assays (Bio-Plex Pro Mouse Cytokine Assays, Bio-Rad Laboratories, Hercules, CA, USA) as previously described (Giovanoli et al., 2013). Samples were prepared according to the manufacturer's instructions and were analyzed on a triple-laser FACSCanto II flow cytometer using FACSDiva software (BD Immunocytometry Systems, Heidelberg, Germany). Absolute concentrations were calculated based on the mean fluorescence intensity of cytokine standards with a 4-parameter logistic curve model using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). The detection limit for IL-6 was 0.4 pg/ml.

Preparation of Brain Samples for Pilot Gene Expression Analyses

To probe the optimal dosing of the MINO treatment with respect to its influence on inflammatory parameters (see main text), we performed initial gene expression analyses evaluating the effects of acute MINO treatment on transcription of neuron-

microglia inhibitory signalling pairs and IL-1β (see below). Two doses of MINO were included, namely 3 mg/kg or 30 mg/kg (per os, given in regular drinking water across a time span of 24 h), and were compared to vehicle (regular tap water) treatment. The higher dose of MINO (30 mg/kg) was selected based on previous per os dose response studies showing that daily MINO treatment at this dose and administration regime results in efficient diffusion of the drug across the blood-brain-barrier and accumulation of the drug in the brains of C57BL6 mice (Lee et al., 2006), and the lower dose (3 mg/kg/day) was chosen in relation to the typically lower dosages used in human subjects (Levkovitz et al., 2010). The drug was administered on PND 29 for a period of 24 h before the animals were exposed to acute (45-min) restraint stress and immediately sacrificed by decapitation for brain collection. The brains were extracted from the skull and placed ventral side up on an ice-chilled plate for extraction of the left and right hippocampi. The hippocampi were placed in 1-ml Eppendorf tubes and stored at −80 °C until further processing.

Pilot Gene Expression Analyses of Neuron-Microglia Inhibitory Signalling Pairs by Quantitative Real-Time PCR

Total RNA was isolated by a single step guanidinium isothiocyanate/phenol extraction, using PureZol RNA isolation reagent (Bio-Rad Laboratories s.r.l. Italia) according to the manufacturer's instructions, and quantified by spectrophotometric analysis. Following total RNA extraction, the samples were processed for real-time PCR to assess CD200, CD200R, CD47 and CD172a mRNA levels. An aliquot of each sample was treated with DNase to avoid DNA contamination. RNA was analyzed on a qRT–PCR instrument (CFX384 real-time system, Bio-Rad Laboratories) using the iScript one-step RT–PCR kit for probes (Bio-Rad Laboratories). Samples were run in 384-well formats in triplicates as multiplexed reactions with a normalizing internal control (36B4). Probe and primer sequences were purchased from Eurofins MWG-Operon. Thermal cycling was initiated with incubation at 50 °C for 10 min (RNA reverse transcription), and then at 95 °C for 5 min (polymerase activation). After this initial step, 39 cycles of PCR were performed. Each PCR cycle consisted of heating the samples at 95 °C for 10 s to enable the melting process, and then for 30 s at 60 °C for the annealing and extension reactions.

Relative target gene expression was calculated according to the 2(-Delta Delta C(T)) method (Livak and Schmittgen, 2001).

The following primer and probe sequences were used for the gene expression analysis of neuron-microglia inhibitory signaling pairs:

	FORWARD primer	REVERSE primer	PROBE
CD200	CTCCACCTACGCCTGATTTG	TCACAATCAAGGGTTCCTGG	AGCACAGCTCAAGTGGAAGTGGT
CD200R	TTTTGGAGAACTTCTGCCCTAG	GTGTTCACTTGTGTCAGAGGA	ACTTGACCCAGCCACAAAGACCC
CD47	AGGAGAAAAGCCCGTGAAG	TGGCAATGGTGAAAGAGGTC	ACAATGAGGCCAAGTCCAGAAGCA
CD172a	TGTGCTTTGCTCGTAGTCC	TCATTTGTGTCCTGGATCTGG	TGTTGACCCCTTGGCTTTCTTCTGT
36B4	AGATGCAGCAGATCCGCAT	GTTCTTGCCCATCAGCACC	CGCTCCGAGGGAAGGCCG

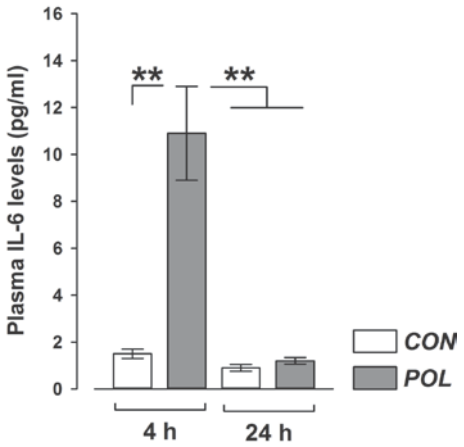
IL-1β Gene Expression Analysis by Quantitative Real-Time PCR

Brain samples were homogenized with a rotor/stator tissue homogenizer (Tissue-Tearor™, BioSpec Products, Bartlesville, OK, USA) in TRI Reagent® (Sigma-Aldrich), and total RNA was isolated according to the manufacturer's instructions. Further purification and DNase I treatment was performed using the RNeasy Micro kit (Qiagen, Hilden, Germany). Concentrations were determined photometrically in a BioPhotometer (Eppendorf, Hamburg, Germany) using a LabelGuard Microliter Cell (Implen, Munich, Germany). First-strand cDNA was synthesized from 1 µg of total RNA using the High Capacitiy cDNA Reverse Transcription Kit (Applied Biosystems). Real-time quantitative PCR was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems) using the FAST qPCR MasterMix Plus Low ROX with UNG (Eurogentec, Seraing, Belgium) and the following cycling conditions: 2 min at 50 °C and 5 min at 95 °C followed by 40 cycles of 3 sec at 95 °C and 40 sec at 60 °C. Cytokine primers and probes were adapted from the RTPrimerDatabase (http://medgen.ugent.be/rtprimerdb/) and were purchased from Eurogentec (Seraing, Belgium). 36B4 was used as reference gene as for above, and relative gene expression was calculated according to the 2(-Delta Delta C(T)) method (Livak and Schmittgen, 2001).

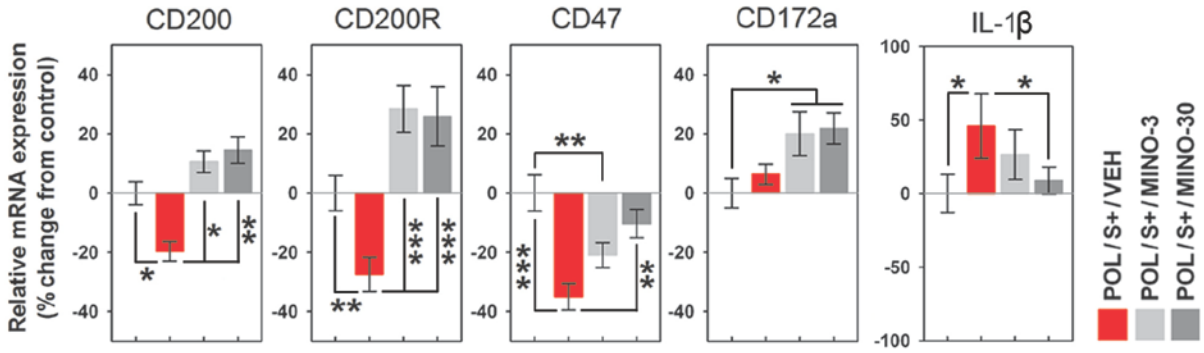
The following primer and probe sequences (5'-3') were used for cytokine gene expression analysis:

	FORWARD primer	REVERSE Primer	PROBE (5'-6-FAM and 3'-BHQ1)
IL-1β	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA	CTGTGTAATGAAAGACGGCACACCCACC
36B4	AGATGCAGCAGATCCGCAT	GTTCTTGCCCATCAGCACC	CGCTCCGAGGGAAGGCCG

SUPPLEMENTERY RESULTS



Supplementary Figure 1. Maternal IL-6 levels at basal conditions and following viral-like immune activation in mid-pregnancy. Pregnant mice were exposed to poly(I:C) (1 mg/kg, i.v. = POL) or control (= CON) vehicle (saline) solution on gestation day 9, and IL-6 plasma levels were measured 4 and 24 h post-treatment. Note that the POL-induced elevation of cytokines was transient and normalized to basal levels within 24 h post-treatment. **P < 0.01, based on post-hoc comparisons, following presence of a significant interaction between maternal treatment and sampling interval ($F(1,16) = 16.27$, $P < 0.01$). *N* = 5 per treatment group/post-injection interval. All data are means±SEM.



Supplementary Figure 2. Effects of minocycline treatment on neuron–microglia inhibitory signalling pairs (CD200, CD200R; CD47, CD172a) and IL-1β gene expression in the hippocampus of offspring exposed to combined prenatal immune activation and peri-pubertal stress relative to control offspring. We have previously documented that the combination of prenatal immune activation by poly(I:C) (= POL) and peri-pubertal stress exposure (= S+) markedly impairs the expression of CD200, CD200R, and CD47, and leads to increased expression of IL-1β (see Giovanoli et al., 2013). Here, we explored whether minocycline (MINO) treatment can restore these deficits. Therefore, POL/S+ offspring were treated with 3 mg/kg/day minocycline (MINO-3), 30 mg/kg/day minocycline (MINO-30), or vehicle (VEH = regular tap water) for 24 hrs prior to stress exposure, and mRNA levels were compared to VEH-treated CON/S- (= control) animals. *P < 0.05, **P < 0.01, and ***P < 0.001, based on student's t test (two-tailed) following one-way ANOVA. *N* = 6-10 males per group. All data are means±SEM.

Test	Dependent Measures	ANOVA	Effects	DF	F-Value	P-Value
Elevated plus maze	Relative open arm entries (%)	2 × 2 × 2 (MIA × Stress × Prevention)	MIA	(1,78)	3.516	0.0645
			Stress	(1,78)	13.838	**0.0004**
			Prevention	(1,78)	0.400	0.5291
			MIA × Stress × Prevention	(1,78)	1.791	0.1847
	Total distance moved (cm)	2 × 2 × 2 (MIA × Stress × Prevention)	MIA	(1,78)	1.461	0.2304
			Stress	(1,78)	1.021	0.3153
			Prevention	(1,78)	0.422	0.5178
			MIA × Stress × Prevention	(1,78)	2.264	0.5178
Prepulse inhibition	Percent PPI	2 × 2 × 5 (MIA × Stress × Prevention × Prepulse)	MIA	(1,111)	7.432	**0.0074**
			Stress	(1,111)	4.365	**0.0390**
			Prevention	(1,111)	4.437	**0.0374**
			MIA × Stress × Prevention	(1,111)	12.213	**0.0007**
			Prepulse	(4,444)	278.009	**<0.0001**
	Startle reactivity (AU)	2 × 2 (MIA × Stress × Prevention)	MIA	(1,111)	2.236	0.1377
			Stress	(1,111)	1.297	0.2572
			Prevention	(1,111)	0.165	0.6855
			MIA × Stress × Prevention	(1,111)	1.371	0.2441
	Prepulse induced reactivity (AU)	2 × 2 × 5 (MIA × Stress × Prevention × Prepulse)	MIA	(1,111)	1.400	0.2392
			Stress	(1,111)	1.130	0.2900
			Prevention	(1,111)	2.329	0.1298
			MIA × Stress × Prevention	(1,111)	2.641	0.1069
			Prepulse	(4,444)	72.392	**<0.0001**
Locomotor response to AMPH	Total distance moved following vehicle (cm)	2 × 2 × 6 (Group × Prevention × Bins)	Group	(1,53)	0.429	0.5151
			Prevention	(1,53)	0.621	0.4342
			Group × Prevention	(1,53)	0.294	0.5899
			Bins	(5,265)	36.876	**<0.0001**
			Bins × Group × Prevention	(5,265)	0.492	0.7819
	Total distance moved following AMPH (cm)	2 × 2 × 18 (Group × Prevention × Bins)	Group	(1,53)	8.737	**0.0046**
			Prevention	(1,53)	3.012	0.0885
			Group × Prevention	(1,53)	11.728	**0.0012**
			Bins	(17,901)	37.582	**<0.0001**
			Bins × Group × Prevention	(17,901)	6.735	**<0.0001**
Locomotor response to MK-801	Total distance moved following vehicle (cm)	2 × 2 × 6 (Group × Prevention × Bins)	Group	(1,60)	0.013	0.9094
			Prevention	(1,60)	1.249	0.2681
			Group × Prevention	(1,60)	0.001	0.9752
			Bins	(5,300)	70.570	**<0.0001**
			Bins × Group × Prevention	(5,300)	0.780	0.5646
	Total distance moved following MK-801 (cm)	2 × 2 × 18 (Group × Prevention × Bins)	Group	(1,60)	1.338	0.2520
			Prevention	(1,60)	1.935	0.1693
			Group × Prevention	(1,60)	0.533	0.4684
			Bins	(17,1020)	15.689	**<0.0001**
			Bins × Group × Prevention	(17,1020)	1.686	**0.0397**

Supplementary Table 1 (previous page). Summary of the statistical tests and outcomes for the behavioral analyses using model 1, in which the number of individual offspring in each treatment condition was used as the sample sizes. The table specifies the dependent measures for each behavioral test and summarizes the main effects of and interactions between the main between-subjects factors (maternal immune activation = MIA; peri-pubertal stress = Stress; minocycline treatment = Prevention), as well as the main effects of and interactions between additional independent factors specific to each behavioral test. Note that the AMPH and MK-801 tests were conducted using the stress condition only, so that the MIA/stress condition appears as "Group". The table also specifies the corresponding degrees of freedom (DF) and F-values. Significant effects ($P < 0.05$) are given in bold font. Both male and female subjects were included in all behavioral analyses; however, initial statistical analyses showed that there were no significant sex-specific effects associated with single or combined exposure to prenatal immune activation and peri-pubertal stress and/or preventive minocycline treatment. Therefore, the data of the two sexes were combined for the final analyses and presentation of the data. AU, arbitrary units; AMPH, amphetamine; MK-801, dizocilpine; PPI, prepulse inhibition.

Test	Dependent measure	ANOVA	Effect	DF	F-Value	P -Value
EPM	Distance moved open arm (cm)	2 × 2 × 2 (MIA × Stress × Prevention)	Stress	(1,53)	18.92	**<0.0001**
			MIA × Stress × Prevention	(1,53)	0.22	0.65
	Relative open arm entries (%)	2 × 2 × 2 (MIA × Stress × Prevention)	Stress	(1,53)	7.80	**0.0072**
			MIA × Stress × Prevention	(1,53)	0.001	0.97
			MIA × Stress × Prevention × Prepulses	(4,212)	274.88	**<0.0001**
PPI	PPI (%)	2 × 2 × 2 × 5 (MIA × Stress × Prevention × Prepulses)	MIA × Stress × Prevention	(1,53)	9.61	**0.003**
			MIA × Stress × Prevention × Prepulses	(4,212)	274.88	**<0.0001**
	Startle reactivity (AU)	2 × 2 × 2 (MIA × Stress × Prevention)	MIA × Stress × Prevention	(1,53)	1.91	0.17
AMPH sensitivity	Total distance moved following vehicle (cm)	2 × 2 × 6 (Group × Prevention × Bins)	Group × Prevention	(1.21)	0.293	0.5943
			Group × Prevention × bins	(5,105)	0.616	0.688
	Total distance moved following AMPH (cm)	2 × 2 × 18 (Group × Prevention × Bins)	Group × Prevention	(121)	13.393	**0.0015**
			Group × Prevention × bins	(17,357)	8.011	**<0.0001**
MK-801 sensitivity	Total distance moved following vehicle (cm)	2 × 2 × 6 (Group × Stress × Prevention × Bins)	Group × Prevention	(1,33)	0.040	0.842
			Group × Prevention × bins	(5,165)	0.695	0.6228
	Total distance moved following MK (cm)	2 × 2 × 18 (Group × Prevention × Bins)	Group × Prevention	(1,33)	0.675	0.5894
			Group × Prevention × bins	(17,561)	1.687	**0.0412**

Supplementary Table 2. Summary of the statistical tests and outcomes for the behavioral analyses using model 2, in which the number of litters/cages were used as the sample size. The table specifies the dependent measures for each behavioral test and summarizes the main effects of and interactions between the main between-subjects factors (maternal immune activation = MIA; peri-pubertal stress = Stress; minocycline treatment = Prevention), as well as the main effects of and interactions between additional independent factors specific to each behavioral test. Note that the AMPH and MK-801 tests

were conducted using combined maternal immune activation and stress, which in the table appears as "Group". The table also specifies the corresponding degrees of freedom (DF) and F-values. Both male and female subjects were included in all behavioral analyses; however, initial statistical analyses showed that there were no significant sex- specific effects associated with single or combined exposure to prenatal immune activation and peri-pubertal stress and/or preventive minocycline treatment. Therefore, the data of the two sexes were combined for the final analyses and presentation of the data. Significant effects ($P < 0.05$) are given in bold font. AU, arbitrary units; AMPH, amphetamine; MK-801, dizocilpine; PPI, prepulse inhibition.

Cellular Marker	Dependent Measures	Effects	DF	F-Value	P-Value
Iba1	Number of cells (cells/mm^3)	Group	(1,16)	0.223	0.6429
		Prevention	(1,16)	1.598	0.2243
		Group × Prevention	(1,16)	1.674	0.2141
Iba1 Cell morphology	Cell soma area (µm^2)	Group	(1,16)	0.002	0.9676
		Prevention	(1,16)	0.202	0.6590
		Group × Prevention	(1,16)	4.516	**0.0495**
	Number of primary processes (primary process/cell)	Group	(1,16)	0.095	0.7617
		Prevention	(1,16)	0.522	0.4805
		Group × Prevention	(1,16)	4, E^{-4}	0.9842
CD68	Number of cells (cells/mm^3)	Group	(1,16)	0.193	0.6660
		Prevention	(1,16)	1.025	0.3264
		Group × Prevention	(1,16)	7.709	**0.0135**
IL-1β	Number of cells (cells/mm^3)	Group	(1,16)	0.352	0.5611
		Prevention	(1,16)	0.900	0.3569
		Group × Prevention	(1,16)	5.918	**0.0271**

Supplementary Table 3. Summary of the statistical tests and outcomes for the immunohistochemcial analyses conducted in peri-pubertal (postnatal day 41) offspring following combined prenatal immune activation and stress with or without preventive minocycline treatment. The table summarizes the main effects of and interactions between the main between-subjects factors (combined maternal immune activation and stress = Group; preventive minocycline administration = Prevention). The table also specifies the corresponding degrees of freedom (DF) and F-values. Only male subjects were included in the immunohistochemcial analyses. Significant effects ($P < 0.05$) are given in bold font.

Group	Mean Startle Reaction to the Pulse (AU) ± S.E.M.	Mean Startle Reaction to the Prepulses (AU) ± S.E.M
SAL/S-/VEH	94.1 ± 11.5	3.7 ± 0.33
SAL/S+/VEH	89.8 ± 10.3	5.9 ± 0.96
SAL/S-/MINO	76.5 ± 16.4	4.9 ± 0.84
SAL/S+/MINO	101.9 ± 11.2	4.7 ± 0.98
POL/S-/VEH	91.2 ± 10.1	5.0 ± 0.44
POL/S+/VEH	106.4 ± 13.8	4.8 ± 0.50
POL/S-/MINO	106.8 ± 11.8	3.7 ± 0.39
POL/S+/MINO	110.6 ± 15.5	3.7 ± 0.44

Supplementary Table 4. Summary of the mean±S.E.M startle reactivity (= reaction to pulse alone trials) and prepulse-induced reactivity (= reaction to prepulse-alone trials). Pregnant mice were exposed to poly(I:C) (POL) or control (CON) solution, and the resulting offspring were subjected to sub-chronic stress (S+) or left undisturbed (S-) during peri-pubertal development. During the stress procedure, half of the animals received minocycline (MINO) treatment, and the other half vehicle (VEH = regular tap water) treatment. No significant group differences were obtained for these measures. *N* = 12-18 per group.

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Chapter 5

General Discussion

Overview of the Main Research Lines and Findings

The main goal of the experimental work presented in this thesis was to evaluate whether the consequences of (mild) prenatal immune challenge may interact synergistically with exposure to peri-pubertal stress in the development of brain and behavioral abnormalities relevant for developmental psychiatric disease. Hence, the work described here is a direct test of the ''two-hit hypothesis" of neurodevelopmental disorders (Keshavan, 1999), in which prenatal immune challenge by poly(I:C) represents the first environmental hit and peri-pubertal exposure to sub-chronic mild stress the second environmental hit. The main underlying hypothesis of the presented work was that an immune-mediated first hit in prenatal life might render the organism more vulnerable to postnatal stress-induced disruption of subsequent brain maturation.

In a first step, we conducted a series of behavioral and neurochemical investigations in order to compare the consequences of single versus combined exposure to prenatal poly(I:C) and peri-pubertal stress (chapter 2). For this purpose, we focused on behavioral abnormalities that are primarily implicated in psychotic and affective disorders with presumed neurodevelopmental origins. The behavioral experiments included tests for anxiety-like behavior on the elevated plus maze (EPM), tests for sensorimotor gating as evaluated using the prepulse inhibition (PPI) paradigm, tests for selective associative learning in an active-avoidance latent inhibition (AA-LI) paradigm, and sensitivity to the indirect dopamine agonist amphetamine (AMPH) and the NMDA - receptor antagonist dizocilpine (MK-801). These investigations revealed that the two environmental insults are indeed able to synergistically interact in causing adult behavioral pathology. Subsequent post-mortem neurochemical investigations further identified that the dopaminergic effects of peri-pubertal stress are magnified by prenatal immune activation, especially with respect to the elevation of dopamine levels in the adult hippocampus (chapter 2). In chapter 3, we performed additional immunohistochemical investigations to explore whether synergistic effects of combined exposure to prenatal immune activation and peri-pubertal stress would also be observed with respect to cellular GABAergic abnormalities implicated in chronic neuropsychiatric disorders. These investigations partly confirmed this hypothesis by

demonstrating reduced numbers of PV-containing interneurons in the ventral DG of offspring with the double-hit exposure (chapter 3).

We further examined whether the precise timing, during which the second environmental hit (stress exposure) is applied, would have an impact on the nature of interactions between prenatal immune activation and postnatal stress. To address this, we exposed prenatally immune-challenged and control offspring to sub-chronic stress at a later maturational period, namely late adolescence, and then again compared the single and combined effects of prenatal immune activation and adolescent stress on behavioral functions in adulthood (chapter 2). The findings from these experimental investigations indeed suggest that the extent to which the first environmental hit (prenatal immune activation) interacts with the second environmental hit (postnatal stress exposure) is critically influenced by the precise timing of stress exposure (chapter 2).

In many neuropsychiatric disorders, including schizophrenia and bipolar disorders, full-blown psychopathological symptoms are often clinically manifest only when the affected individuals reach late adolescence or early adulthood (Keshavan and Hogarty, 1999). To study the onset of behavioral changes in our two-hit model, we conducted an additional behavioral test battery in peri-pubertal offspring shortly after exposure to the last stressor (chapter 2). The findings from these additional behavioral analyses suggest that many of the functional abnormalities, including disruption of PPI and LI as well as psychotomimetic drug hypersensitivity, show a delayed onset and thus only appear in adulthood (chapter 2).

We went on to explore possible mechanisms mediating the pathological interactions between prenatal immune activation and peri-pubertal stress (chapter 2). Thereby, we focused on possible convergence points between the immune- and stress-related manipulations. First, we assessed the consequences of the developmental exposures on basal and stress-induced corticosterone secretion. Corticosterone is one of the main effector hormones secreted by the adrenal cortex in response to activation of the hypothalamus-pituitary-adrenal (HPA) - axis (Sapolsky et al., 2000). Our measurements of corticosterone indicate that the synergistic interactions on brain and behavioral functions are unlikely to be explained by prenatally acquired changes in

stress-induced corticosterone release (Chapter 2). Indeed, immune-challenged and control offspring were found to be highly comparable with respect to basal and peri-pubertal stress-induced corticosterone release. On the other hand, our detailed analysis of microglia and inflammatory markers revealed that prenatal immune activation strongly enhances the offspring's vulnerability to neuroinflammatory changes in response to peri-pubertal stress (chapter 2). Intriguingly, such neuroinflammatory abnormalities in stressed offspring of immune-challenged mothers appeared to be transient and only emerged during and shortly after exposure to stress. Based on these latter findings, we went on to explore possible preventive interventions that would preferentially target the stress-induced activation of microglia and associated inflammatory processes in the maturing brain (chapter 4). For this purpose, the animals were treated with minocycline, a tetracycline with known neuroprotective and immunosuppressive functions (Bilousova et al., 2009; Henry et al., 2008; Wixey et al., 2011), during the course of stress exposure (chapter 4). We found that minocycline was sufficient to block stress-induced microglia over-activation and inflammatory cytokine production in prenatally immune challenged animals. Moreover, the pharmacological intervention was found to be effective in preventing the emergence of behavioral abnormalities typically seen in adult offspring with the double-hit exposure (chapter 4).

Sex Independency of Neuropathologies

Early traumatizing experiences have been described to have differential effects on males and females, and such differences are often explained by differential stress vulnerability in males versus females (DeSantis et al., 2011). Sex steroids such as estrogen and testosterone are known neuromodulators and have also been described to influence the nature and/or severity of behavioral symptoms in developmental psychiatric disorders such as schizophrenia (Hendrick et al., 1996; Romeo, 2003; Rose et al., 2004) and in disease-relevant animal models (Arad and Weiner, 2008, 2010; Thwaites et al. 2014). Our two-hit environmental model, however, did not reveal sex-dependent effects on the main behavioral, neuroanatomical, and neurochemical measures assessed here. Indeed, all the synergistic effects of prenatal immune challenge and stress appeared to be sex-independent (chapter 2). These sex-independent effects may seem surprising in view of the notable differences in hormonal maturation in

females versus males during the course of peri-pubertal development (Hill et al., 2012), which corresponds to the time when sub-chronic stress was applied. According to our findings, one interpretation is that the peri-pubertal hormonal fluctuations in male and female offspring may only play a minor role in determining the extent to which peri-pubertal stress can unmask latent pathologies induced by prenatal immune challenge. One possibility would be that the endocrine and inflammatory responses to the applied stressors might have overridden, and therefore masked, any possible (protective) influences of sex hormones on the subsequent brain maturational processes. An alternative (but not mutually exclusive) possibility would be that the initial prenatal insults might have caused a blunting of the natural hormonal differences between male and female offspring, so that sex hormones would be expected to exert a less prominent impact on the development of sex-specific effects. Interestingly, this interpretation would also be compatible with a plethora of previous findings suggesting that many of the long-term brain and behavioral consequences of intense (poly(I:C)-induced) prenatal immune challenge are similarly seen in both male and female offspring (Meyer et al., 2005; Bitanihirwe et al., 2010).

Puberty and the Vulnerability to Stress-Induced Brain Pathology

One of the key aims of the present thesis was to elucidate the role of the precise postnatal timing, during which the second environmental hit is applied (chapter 2). As discussed in chapter 2, key behavioral abnormalities such as PPI deficits and enhanced sensitivity to psychomimetic drugs did not develop in prenatally immune-challenged animals when stress exposure took place during late adolescence. This suggests that the adolescent stage of maturation is less sensitive than the peri-pubertal stage with respect to stress-induced disruption of adult behavioral functions.

The present thesis did not explicitly explore the mechanisms underlying this differential vulnerability between the peri-pubertal and late adolescent stages. On speculative grounds, however, there are a number of possible factors that could have contributed to the differential effects induced by peri-pubertal versus late adolescent stress in our two-hit model.

Consistent with our findings, numerous previous experimental studies in animals suggest that the early pre-pubertal period is highly vulnerable to adverse environmental exposures (reviewed in Dahl, 2004; de Kloet et al., 2005; Dahl and Gunnar, 2009; Holder and Blaustein, 2013), including sever stress experience (Oztan et al., 2011) and exposure to drugs of abuse (Schneider, 2008). Furthermore, human studies suggest that similar kinds of stressors are subjectively felt to be more severe and induce greater emotional responses when exposed to during puberty as compared to exposures in late adolescence (Gunnar et al., 2009; Silk et al., 2009). Animal models of chronic stress exposure generally confirm these human findings by showing that more prominent neuronal (hypothalamic) and endocrine responses to stressful stimuli when applied in early puberty as compared to late adolescence (Romeo et al., 2006). Pubertal rodents also exhibit relatively slow recovery after single stress exposure and further exhibit sensitization if repeatedly exposed to a stressor (Romeo and McEwen, 2006; Doremus-Fitzwater et al., 2009). In the context of the present two-hit model, these findings raise the question as to whether stress exposure in late adolescence failed to interact with prenatal immune activation because the adolescent offspring were generally less responsive (or susceptible) to the chosen stressors? One clear limitation of the presented work is that this hypothesis was not tested directly, for example by comparing the stress-induced corticosterone responses in peri-pubertal and late adolescent offspring. It needs to be pointed out, however, that stress exposure in peri-puberty and late adolescence was similarly effective in inducing anxiety-like behavior (chapter 2), suggesting that offspring in the later maturational stage were not fully unresponsive to the applied stressors.

The differential vulnerability of the peri-pubertal and late adolescent stage to stress-induced pathological changes in prenatally primed offspring may be further related to maturational neuronal differences. The peri-pubertal stage is arguably a time period characterized by considerable neuroplastic rearrangements, during which neural circuits mature and thereby undergo various structural and functional changes (Gogtay et al., 2004; Gidd, 2004; Paus et al., 2008). Such maturational processes include (activity-dependent) programmed cell death, dendritic and axonal reformation, and increased myelination (Benes et al., 2000; Nunez et al., 2001; Andersen et al., 2002; Dahl, 2004). An important aspect of these maturational processes is called “synaptic pruning”, a term

used to describe processed leading to the elimination or generation and strengthening of synapses (Blinzinger and Kreutzberg 1968). Synaptic pruning is particularly effective during peri-pubertal maturation and in stress-reactive brain areas such as the prefrontal cortex (Gogtay et al., 2004; Gidd, 2004) and hippocampus (Goddings et al., 2013). These brain regions can thus be expected to be particularly vulnerable to environmental adversities such as stress when challenged during sensitive periods such as peri-puberty. As discussed in more detail below (see section "Mediating Mechanisms: An Unresolved Puzzle"), converging evidence suggest that normal synaptic maturation (in prefrontal and hippocampal areas) can be disrupted by developmental neuroinflammatory reactions, similarly to those identified in our two-hit model (Andersen, 2003; Garay and McAllister, 2010). It would therefore be highly interesting to explore in future studies whether altered synaptic maturation my underlie the emergence of long-term brain and behavioral pathology induced by combined prenatal immune challenge and peri-pubertal stress, and whether peri-pubertal offspring may be more sensitive than adolescent ones with respect to such synaptic changes.

Delayed Onset of Stress-Induced Behavioral Pathology

With the exception of increased anxiety-like behavior, the effects of combined exposure to the two environmental insults were behaviorally manifest in adulthood only (chapter 2). The adult emergence of multiple behavioral abnormalities in our two-hit model is similar to the delayed onset of full-blown psychopathological symptoms in schizophrenia and related psychotic disorders (Keshavan and Hogarty, 1999). Our observations thus indicate that the manifestation of multiple behavioral abnormalities is dependent on certain, yet unknown processes occurring between exposure to the last stressor in peri-puberty and the gradual attainment of adulthood. The work presented in this thesis did not aim at identifying these processes, so that they await direct investigation in future studies. In view of the possible role of altered synaptic pruning discussed above, one feasible mechanism underlying the delayed onset of behavioral pathology may involve progressive changes in synaptic functions. If this hypothesis holds true, one would expect the synaptic abnormalities induced by combined immune activation and stress to be more prominent in adulthood as compared to post-acute stages shortly after stress exposure in puberty. As discussed above, synaptic changes in

the prefrontal cortex may be of special interest in this context because (1) it is one of the latest region to fully mature (Giedd, 2004), and (2) it is highly implicated in the pathophysiology of schizophrenia and other psychotic disorders (Weinberger and Lipska, 1995). Similarly, age-specific synaptic changes in the hippocampus might also play role given that this brain area is also known to undergo considerable morphological functional changes across adolescent brain development (Goddings et al., 2013). It is also highly interesting to note that abnormal hippocampal and prefrontal maturation can be induced by a single exposure to prenatal immune challenge, granted that it is intense enough and associated with (or predictive of) behavioral consequences per se (Piontkewitz et al., 2011). Whether or not such changes in brain maturation following intense prenatal immune challenge involve (progressive) synaptic abnormalities still needs to be elaborated.

Mediating Mechanisms: An Unresolved Puzzle

The knowledge acquired through the work presented in this thesis still leaves many questions unanswered, especially with respect to the mediating mechanisms involved in this developmental two-hit model. As illustrated in **Figure 1** ("black boxes"), two main mechanistic aspects warrant particular attention in future studies: (1) What are the (immune-mediated) mechanisms relevant for the priming of altered neuroinflammatory reactions to stress exposure in puberty? (2) What mechanisms are most relevant for mediating the synergism between the two environmental insults?

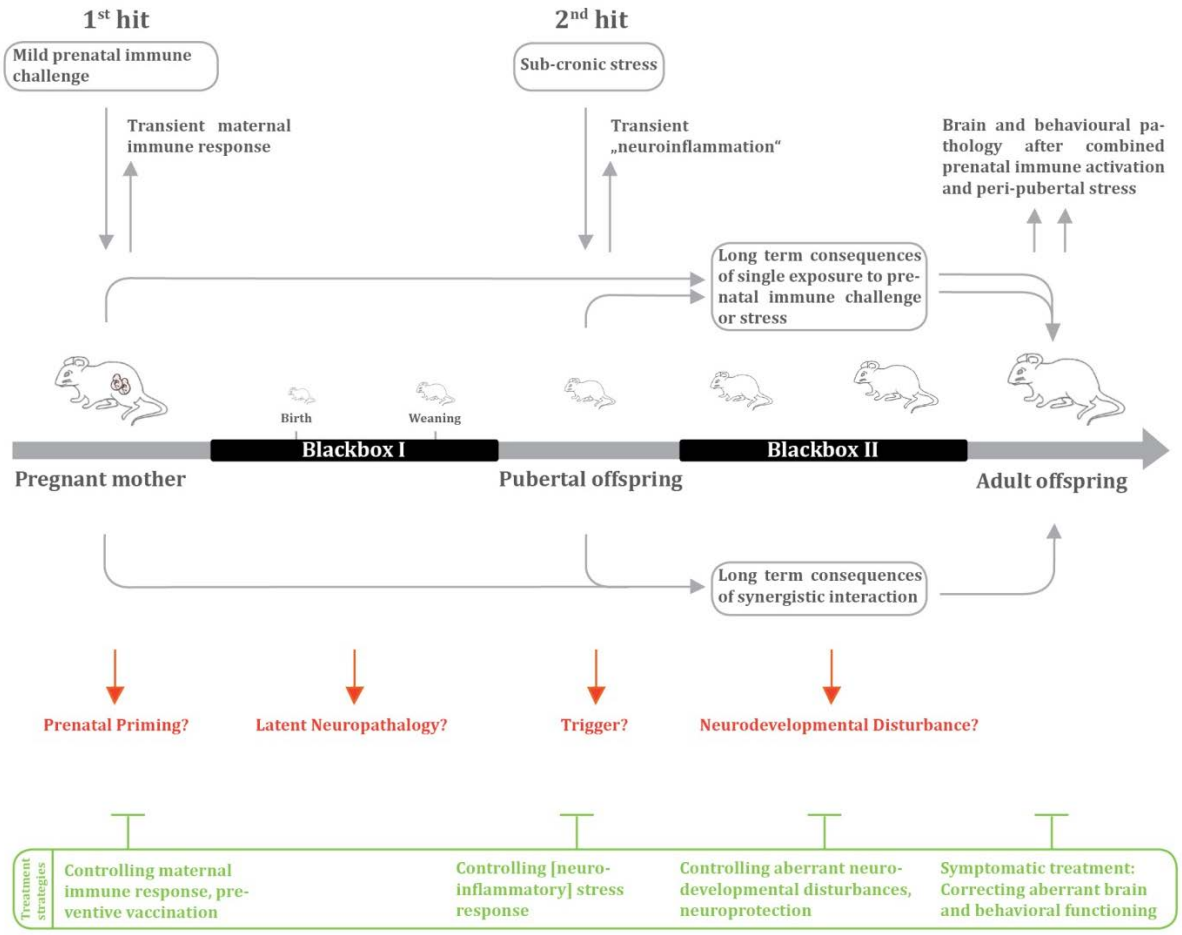

Figure 1. A schematic representation of the environmental two-hit model with the findings presented in this thesis. Prenatal immune activation by sub-threshold poly(I:C) treatment represents a first environmental insult, and sub-chronic stress exposure the second. Prenatal immune activation evoked a transient immune response characterized by secretion of pro- and anti-inflammatory cytokines in the maternal host. This early prenatal insult primed increased neuroinflammatory reactions in response to peri-pubertal stress and further facilitated the development of adult behavioral pathology when combined with the second environmental hit. The current stage of knowledge leaves many questions open, especially with respect to the mediating mechanisms relevant for this two-hit model (highlighted in red color). The illustration also outlines possible interventions that could be used to preventively or symptomatically treat the brain and behavioral pathology associated with combined exposure to prenatal immune activation and peri-pubertal stress.

Prenatal Priming of Latent Neuroinflammation and the Impact of Cytokines [Black Box I]

Previous investigations in rodent models have shown that intense forms of maternal immune activation by poly(I:C) treatment can induce both acute and long-term changes in immune system functioning, the latter of which include increases in inflammatory cytokine secretion and microglia activation during postnatal development (Meyer et al., 2006; Garay et al., 2013; Juckel et al., 2011; Mattei et al., 2014). The present findings suggest that a milder form of prenatal immune activation is insufficient to cause overt neuroinflammatory changes in the offspring (chapter 2). Yet, this initial environmental hit can prime latent neuroinflammatory abnormalities in the offspring, which can be unmasked by a subsequent environmental hit, in this case peri-pubertal stress (chapter 2).

The neuroinflammatory phenotype emerging in offspring exposed to combined immune activation and stress included signs of enhanced microglia activation in the hippocampus and prefrontal cortex (chapter 2 and 4). Microglia are often referred to as "brain macrophages" as they constantly survey the central nervous system and exhibit phagocytic functions (Nimmerjahn et al., 2005). Microglia cells can be activated by various environmental stimuli and, depending on the severity, type and duration of these stimuli, they undergo morphological and functional changes from a resting (non-activated) to an activated state (Nimmerjahn et al., 2005, Czeh et al., 2011). It is important to point out that microglia cells, like many other cells of the immune system, are long-lived and have a certain degree of "immune memory". Such immune memory is often referred to as "microglia priming" and describes the process of (abnormally) enhanced microglia responses to a second environmental stimulus following initial (and often remote) exposure to a first stimulus (Frank et al., 2007; Perry and Teeling, 2013). In our model, the first stimulus would represent prenatal exposure to immune activation, and the second stimulus would represent peri-pubertal exposure to stress. Hence, prenatal immune activation could have primed microglia cells to respond more vigorously to the subsequent peri-pubertal stress stimulus.

One limitation of the work presented in this thesis is that it does not provide insights into the precise mechanisms leading to increases in stress-induced microglia

activation as observed in prenatally immune challenged offspring compared to prenatal control offspring. One possible mechanism for this effect, however, may be related to fetal exposure to pro-inflammatory stimuli such as cytokines. Even though transient in nature, the poly(I:C) treatment caused a significant increase in pro-inflammatory cytokine secretion in the maternal host, which in turn can also be expected to induce subsequent inflammatory reactions in the fetal brain (Meyer et al., 2006). Besides their impact on neuronal development (Deverman and Patterson, 2009; Meyer, 2013), pro-inflammatory cytokines are also known to affect the development of cells of the myeloid lineage, including microglia cells (Bauer et al., 2007). Hence, microglia may acquire a certain postnatal "immune memory" resulting from prenatal exposure to abnormal levels of cytokines, and such mechanisms may be relevant to explain our findings of enhanced stress-induced microglia activation in prenatally primed offspring.

A second plausible mechanism for these abnormal microglia reactions may involve deficient neuron-microglia inhibitory signaling (chapter 2). Converging evidence suggests that neuronal cells exert an inhibitory influence on microglia activation through actions involving contact-dependent neuron-microglia inhibitory signaling pairs (Perry and Teeling, 2013). Two known inhibitory signaling pairs are CD200 (neuron) $\rightarrow$ CD200R (microglia) and CD47 (neuron) $\rightarrow$ CD172a (microglia). Down-regulation of either the neuronal or microglia component of such signaling pairs can strongly facilitate microglia activation (Ransohoff and Cardona, 2010). In chapter 2 and 4, we observed that prenatally immune-challenged offspring display marked impairments in the expression of CD200, CD200R, and CD47 in response to acute stress, indicating that such neuron-microglia inhibitory signaling may be deficient in these animals, especially in the event of stress exposure. Whether or not such deficits in inhibitory signaling might indeed contribute to the abnormally enhanced microglia responses in stressed poly(I:C) offspring needs to be ascertained in future investigations.

The Neuroinflammatory Response to Stress and its Impact on Brain Maturation [Black Box II]

Another unresolved question relates to the nature of neuropathological changes occurring between the experience of peri-pubertal stress and the adult onset of behavioral pathology as seen in offspring with the double-hit exposure. Furthermore, the precise neuroimmune mechanisms mediating such changes remain obscure. Based on the work presented in this thesis, however, it is conceivable that abnormal microglia-associated neuroinflammatory processes may assume a key role in mediating abnormal neuronal maturation and behavioral development. Indeed, we have found that treatment with the broad-spectrum antibiotic drug minocycline effectively prevented peri-pubertal stress-induced microglia activation, cytokine hyper-secretion, and subsequent development of multiple behavioral abnormalities in offspring exposed to the environmental double-hit (chapter 4). As fully discussed in chapter 4, minocycline is arguably not a specific microglia inhibitor, and therefore, alternative mechanisms by which minocycline prevented adult behavioral abnormalities still need to be considered.

Additional indirect evidence supporting the abovementioned "developmental neuroinflammation" hypothesis can be derived by experimental work performed by others in recent years. For example, activated microglia take active roles in the remodeling of synapses, a process that appears to involve synaptic expression of complement proteins (Stephan et al., 2012). Activated microglia recognize these synaptic complement proteins (Schafer et al., 2012). This further leads to the accumulation of activated microglia at synaptic sites and the eventual phagocytosis of synaptic elements by activated microglia (Stephan et al., 2012; Schafer et al., 2012). In view of the present findings of marked (but transient) microglia over-activation in offspring with combined exposure to immune activation and stress, it would be highly interesting to further explore whether combined exposure to these environmental insults would lead to abnormally enhanced synaptic pruning by microglia cells. Such synaptic "over-pruning" may also represent a possible explanation for the delayed onset of behavioral symptoms in adulthood (see above), primarily because microglia-mediated synaptic pruning represents a relatively slow process that can take several days to weeks to occur (Stephan et al., 2012; Schafer et al., 2012).

In addition to its hypothesized effects of synaptic maturation, over-activation of microglia may also cause changes in neuronal apoptosis. For example, we have observed that prenatally poly(I:C)-treated offspring display a marked increase in hippocampal TNF-α secretion in response to peri-pubertal stress (chapter 2). Since TNF-α is a potent inducer of apoptosis (Curfs et al. 1997) it can be expected that abnormally high levels of this pro-inflammatory cytokine during critical stages of brain maturation may exert negative effects of neuronal survival. Taken together, there are several feasible mechanisms whereby the presence of (transient) neuroinflammation during peri-pubertal life could affect normal brain maturation in offspring with combined exposure to prenatal immune activation and stress. The establishment and initial behavioral characterization of this two-hit model as described in this thesis now provide unique opportunities to study such mechanisms in the future investigations.

Relevance of the Two-Hit Model to Human Neuropsychiatric Disease

By combining a mouse model of (mild) prenatal immune challenge with a model of mild sub-chronic stress exposure in peri-pubertal life, we were able to demonstrate that two environmental risk factors implicated in the etiology of major mental disorders have a cumulative impact in the causation of long-term behavioral abnormities. The epidemiological and clinical interest in developmental exposure to environmental adversities is constantly increasing and has attained broad recognition in etiological theories of multi-factorial brain disorders such as schizophrenia (Brown, 2011; van Os et al., 2010), bipolar disorder (Larsson et al., 2013; Parboosing et al., 2013), and autism (Atladóttir et al., 2012; Herbert, 2010). Thus far, epidemiological evidence for the cumulative impact of such prenatal and postnatal environmental challenges on vulnerability to chronic psychiatric disease is still missing. Our findings support the biological plausibility of interactive effects between two relevant environmental risk factors of human neurodevelopmental brain disorders. Furthermore, our data suggest that peri-pubertal stress may be important etiological risk factors for long-term mental illness especially upon combined exposure, given that each individual factor appears to have rather modest effect sizes in large populations with respect to increasing the disease risk (Tsuchiya et al., 2003; Varese et al., 2012; Selten et al. 2010).

Many of the behavioral functions that were affected by the combination of prenatal immune activation and peri-pubertal stress bear relevance to human psychotic disease (Arguello and Gogos, 2006; Meyer and Feldon, 2010). Indeed, impaired sensorimotor gating (as indexed by PPI deficiency), disrupted selective associative learning (as indexed by a loss of LI), and increased sensitivity to the psychotomimetic drugs AMPH and MK-801, are commonly observed in patients with psychotic disorders, including schizophrenia and bipolar disorder (Feldon and Weiner, 1992; Braff et al., 2001; Swerdlow and Geyer, 1998; Laruelle et al., 2003), and to a lesser extent autism (Braff et al., 1992, 2001). Moreover, the single and combined effects of prenatal immune activation and peri-pubertal stress on dopamine levels in the ventral striatum and hippocampus (see chapter 2) also seem highly relevant to human psychotic disease, given that positive symptoms such as paranoia, hallucinations, thought disorders typically involve increased dopamine transmission in subcortical brain areas, including the striatum and, to a lesser extent, the hippocampal formation (Roiser et al., 2013 Winton-Brown et al., 2014; Tamminga et al. 2010). Even though our behavioral measures in mice cannot be directly translated to the human clinical condition, our findings nevertheless suggest that combined exposure to prenatal immune activation and peri-pubertal stress may be especially relevant for the adult emergence of psychotic symptoms. This hypothesis is consistent with the findings from animal models showing that intense prenatal immune challenge can alter dopaminergic development and cause dopamine-associated behavioral abnormalities in adulthood (Zuckerman et al., 2003; Ozawa et al., 2006; Vuillermot et al. 2010). Moreover, epidemiological studies in humans indicate that peri-pubertal exposure to traumatizing evens are particularly relevant in the development of psychotic disturbances (Janssen et al., 2004; Kelleher et al., 2008, van Winkel et al., 2013.). With respect to the latter epidemiological association, our experimental data would suggest that the psychosis-increasing effects of peri-pubertal stress are significantly augmented in offspring with a history of prenatal infection.

It needs to be pointed out, however, that the experimental work presented in this thesis did not aim to establish causal relationships between distinct behavioral and neurochemical abnormalities. Hence, we do not know the neuronal correlates of the adult behavioral phenotype at the present stage of knowledge. Additional studies exploring these relationships are thus clearly warranted in future investigations.

Finally, our two-hit model offers the unique opportunity to study early interventions aiming at attenuating or even preventing the emergence of multiple brain dysfunctions following developmental exposures to environmental adversities (Piontkewitz et al., 2012). The identification of preventive strategies is particularly important for chronic mental illnesses such as schizophrenia, bipolar disorder, and autism with little hope for complete functional recovery once the disorder has developed. One promising strategy would be to advance our understanding of the precise mechanisms whereby latent or post-acute neuroinflammation negatively affects the development and maturation of brain and behavioral functions. The further elucidation of such mechanisms might indeed lead to the establishment of novel immunomodulatory interventions that could help to achieve successful prevention of adult brain pathology in the event of developmental neuroinflammation.

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Giovanoli S, Meyer U. (2013) Response to comment on "Stress in puberty unmasks latent neuropathological consequences of prenatal immune activation in mice". *Science*. 340:811.

Accepted Manuscripts

Giovanoli S, Weber L, Meyer U (2014) Single and Combined Effects of Prenatal Immune Activation and Peripubertal Stress on Parvalbumin and Reelin Expression in the Hippocampal Formation. Brain, Behavior, and Immunity, accepted for publication.

Appendix 1

Response to Comment on "Stress in Puberty Unmasks Latent Neuropathological Consequences of Prenatal Immune Activation in Mice"

Sandra Giovanoli, Urs Meyer

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ABSTRACT

Lazic criticizes the statistical analyses used to support the conclusions in our mouse model. His theory-biased criticism is disproportionate in view of the robustness of our findings (even if different statistical methods are applied) and falls short in explaining the postpubertal onset of effects.

MAIN TEXT

In his Technical Comment, Lazic raises an essential but not novel issue related to the (in)appropriateness of experimental designs and statistics in rodent developmental biology (Lazic, 2013). Most rodents, including mice and rats, are multiparous species and normally produce litters of up to 8 to 10 offspring. Compared with pups from other litters, littermates share similar, but not identical, antenatal (e.g., in utero physiology) and postnatal (e.g., maternal physiology and behavior) environments. Therefore, littermates are to some degree interdependent, a situation that can produce pervasive and persistent litter effects. In an influential article by Zorrilla (Zorrilla, 1997), which was surprisingly not cited in Lazic's Comment, a number of solutions have been presented as to how one can best avoid false interpretations when using multiparous species in developmental biology. Some of these issues are reiterated here against the background of our recent study (Giovanoli et al., 2013).

We developed an environmental two-hit model in mice, in which the first experimental manipulation targeted pregnant dams, whereas the second manipulation was given to the resulting offspring (Giovanoli et al., 2013). In brief, the first environmental hit was composed of prenatal viral-like immune activation induced by maternal administration of the synthetic double-stranded RNA poly(I:C) (polyriboinosinic-polyribocytidilic acid) during mid-gestation. Pregnant control mothers received corresponding vehicle (physiological pyrogen-free saline) solution. Offspring born to poly(I:C)–treated or vehicle-treated mothers were weaned on postnatal day (PND) 21. They were then left undisturbed (i.e., nonstressed) or exposed to variable and unpredictable stress between PND 30 and 40, the latter of which corresponded to the second environmental hit (Giovanoli et al., 2013). As correctly pointed out by Lazic, littermates were housed two to three animals per cage and were then randomly assigned on a per cage basis to a stress or no-stress control condition. We have used two to three littermates per cage to avoid the negative influences of social isolation by single caging, which are well known to affect brain and behavioral development, especially in rodents (Lukkes et al., 2009).

One might argue now that the effects of prenatal poly(I:C) versus control treatment are best captured when using one offspring per litter only. In this case, the

number of offspring per prenatal treatment condition [poly(I:C) or vehicle] would be identical to the number of treated mothers in each experimental group, and the offspring can easily be treated as independent observations in the statistical analyses. However, given the aforementioned problems associated with social isolation by single caging, we feel that this is not an appropriate solution here. Another possibility would be to use group caging, in which one littermate is picked out for the subsequent stress procedure. It is self-explanatory, however, that the latter experimental design would drastically inflate the number of laboratory animals to be used, and consequently, it is not an ethically feasible solution.

Contrary to the five anonymous experts who have reviewed our manuscript, Lazic suggests that the number of litters and not the number of offspring is the sample size when comparing the effect of (prenatal) poly(I:C) versus control (treatment). From a biological point of view, this argument holds true only partially in the present context because littermates from one specific litter cannot simply be considered as "technical replicates." Indeed, upon maternal administration of poly(I:C) during pregnancy, each individual fetus reacts to the maternal immune challenge by turning on or shutting down its innate gene expression machinery (Meyer et al., 2008; Garbett et al., 2012). This fetal response is not only dependent on the maternal manipulation but is further regulated to a great extent by the individual fetal system, including the surrounding amniotic fluid (Elovitz et al., 2011; Salminen et al., 2008). It is therefore not surprising that the precise fetal (immune) response to maternal poly(I:C) administration varies significantly between individual fetuses coexisting in a single uterine horn (Meyer et al., 2008; Salminen et al., 2008; Elovitz et al., 2011; Garbett et al., 2012). Thus, the individual offspring has a biological meaning even for multiparous species, and it is indeed the individual offspring that attracts the primary scientific interest as compared to the outcomes analyzed for whole litters.

Even if one were to analyze the data according to Lazic's theory-biased recommendations, the reported findings and conclusions drawn from our study remain unchanged. In what follows, we present an example of the effects of prenatal immune activation and peri-pubertal stress on sensorimotor gating in the form of prepulse inhibition (PPI). Even though significant, the reported changes in PPI after immune challenge and stress were arguably statistically weaker compared with the outcomes in

many other behavioral and neuroanatomical tests. Therefore, the PPI data set represents a valid example to test whether our findings might have been compromised by false positive outcomes. Following Lazic, one should consider the number of litters (and not the number of offspring) as the sample size and pool the cages. This statistical exercise yields a significant interaction between prenatal immune activation and stress [$F(1,25) = 5.309$, $P = 0.032$, $N = 7$ to 8] on percent PPI in adulthood, consistent with our original analysis using the number of offspring as the sample size [$F(1,65) = 4.233$, $P = 0.044$, $N = 16$ to 19] [see figure 1C and table S8 in (Giovanoli et al., 2013)]. The equivalence of these statistical outcomes is not surprising given that our study included large numbers of offspring originating from an appreciable number of multiple independent litters.

We would also like to emphasize that the experimental manipulations of interest led to significant changes in several behavioral functions only when the offspring reached the adult stage of development, whether or not the data are analyzed using Lazic's suggestions. Indeed, the same manipulations did not induce multiple behavioral abnormalities in pubescence despite the fact that we used (i) an identical experimental design, (ii) identical statistical methods, and (iii) comparable group and litter sizes. To our surprise, a critical evaluation of these age-dependent effects is missing in Lazic's Comment, perhaps because his criticism falls short in explaining the post-pubertal onset of the behavioral changes.

In conclusion, we feel that Lazic's theory-biased criticism is disproportionate in view of the robustness of our findings and falls short in explaining the age-dependent manifestation of effects

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Appendix 2

Altered GSK3β Signaling in an Infection-based Mouse Model of Developmental Neuropsychiatric Disease.

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ABSTRACT

Protein kinase B (AKT) and glycogen synthase kinase 3 beta (GSK3β) are two protein kinases involved in dopaminergic signaling. Dopamine-associated neuropsychiatric illnesses such as schizophrenia and bipolar disorder seem to be characterized by impairments in the AKT/GSK3β network. Here, we sought evidence for the presence of molecular and functional changes in the AKT/GSK3β pathway using an established infection-based mouse model of developmental neuropsychiatric disease that is based on prenatal administration of the viral mimetic poly(I:C) (=polyriboinosinic-polyribocytidilic acid). We found that adult offspring of poly(I:C)-exposed mothers displayed decreased total levels of AKT protein and reduced phosphorylation at AKT threonine residues in the medial prefrontal cortex. Prenatally immune challenged offspring also exhibited increased GSK3β protein expression and activation status, the latter of which was evidenced by a decrease in the ratio between phosphorylated and total GSK3β protein in the medial prefrontal cortex. These molecular changes were not associated with overt signs of inflammatory processes in the adult brain. We further found that acute pre-treatment with the selective GSK3β inhibitor TDZD-8 dose-dependently normalized aberrant behavior typically emerging following prenatal immune activation, including deficient spontaneous alternation in the Y-maze and increased locomotor responses to systemic amphetamine treatment. Taken together, the present mouse model demonstrates that prenatal exposure to viral-like immune activation leads to long-term alterations in GSK3β signaling, some of which are critically implicated in schizophrenia and bipolar disorder.

INTRODUCTION

The intracellular signaling pathways targeted by dopamine involve multiple molecular substrates (Boyd and Mailman, 2012). In addition to the canonical pathways involving G protein-mediated signaling and regulation of cyclic adenosine monophosphate (cAMP), recent interest has been centered upon cAMP-independent mechanisms of dopaminergic signaling (Beaulieu et al., 2009; Beaulieu et al., 2011). The latter involves the actions of protein kinase B (AKT) and glycogen synthase kinase 3 beta (GSK3β), two protein kinases that phosphorylate substrates on specific serine and threonine residues. AKT itself is activated through phosphorylation by intracellular kinases, and once active, it phosphorylates a number of down-stream targets such as GSK3β (Beaulieu et al., 2009; Beaulieu et al., 2011). GSK3β is biologically active in the unphosphorylated form but inactivated upon serine phosphorylation at the N-terminus. GSK3β acts on a wide range of down-stream targets involved in various biological processes such as development, growth and survival, immunity, and endocrinology (Sutherland, 2011).

In the context of dopaminergic signaling, AKT/GSK3β seems to mediate dopamine actions especially through dopamine D2-like receptors (D2Rs): Activation of D2Rs inhibits AKT activity through arrestin-dependent dephosphorylation processes, and such reduction in AKT activity can further lead to decreased phosphorylation of the GSK3β target (Beaulieu et al., 2009; Beaulieu et al., 2011). Alterations in the AKT/GSK3β signaling pathway have been implicated in dopamine-associated neuropsychiatric disease, especially schizophrenia (Freyberg et al., 2010) and bipolar disorder (Gould and Manji, 2005). Patients with schizophrenia display reduced protein and/or mRNA expression of AKT and parallel reductions in phosphorylation of GSK3β in cortical and subcortical brain areas (Emamian et al., 2004; Thiselton et al., 2008). The total levels of GSK3β were found to be increased in peripheral blood mononuclear cells of bipolar patients with acute manic episodes (Li et al., 2010). Furthermore, genetic variations in AKT and/or GSK3β have been associated with both schizophrenia (Emamian et al., 2004; Norton et al., 2007; Tan et al., 2008; Thiselton et al., 2008; Benedetti et al., 2010;) and bipolar disorder (Karege et al., 2012; Benedetti et al., 2013). It has therefore been hypothesized that impaired AKT/GSK3β signaling may provide an important molecular

mechanism contributing to the pathology of schizophrenia, bipolar disorder, and related dopamine-associated neuropsychiatric illnesses (Rowe et al., 2007; Emamian, 2012).

In the present study, we sought experimental evidence for this hypothesis by exploring the AKT/GSK3β pathway in a well-established neurodevelopmental mouse model known to capture relevant aspects of dopamine-associated neuropsychiatric illnesses such as schizophrenia and bipolar disorder. The model is based on prenatal maternal exposure to the viral mimetic poly(I:C) (=polyriboinosinic–polyribocytidilic acid), which stimulates a cytokine-associated viral-like acute phase response in maternal and fetal units (Meyer et al., 2009). The prenatal poly(I:C) model has been established based on epidemiological evidence demonstrating enhanced risk of schizophrenia and related disorders following prenatal maternal infection (Brown and Derkits, 2010). As extensively reviewed elsewhere (Meyer et al., 2009; Boksa, 2010; Meyer and Feldon, 2010; Meyer and Feldon, 2012), prenatal poly(I:C) treatment in mice leads to numerous psychosis-like behavioral and cognitive dysfunctions, some of which can be restored by preventive or symptomatic antipsychotic drug administration. Furthermore, these functional deficits are accompanied by a number of disease-relevant neuroanatomical and neurochemical changes, including prefrontal and striatal dopaminergic alterations (Meyer et al., 2008; Winter et al., 2009; Bitanihirwe et al., 2010a; Bitanihirwe et al., 2010b; Vuillermot et al., 2010). The mouse prenatal poly(I:C) model is thus ideally suited to explore possible changes in intracellular dopamine pathways pertinent to developmental neuropsychiatric disorders such as schizophrenia and bipolar disorder.

Here, we used Western blot techniques to quantify the levels of unphosphorylated and phosphorylated AKT and GSK3β in the medial prefrontal cortex (mPFC) and striatum (STR) of adult offspring born to immune-challenged mothers and controls. These molecular investigations were complemented by pharmacological studies assessing whether the behavioral deficits induced by prenatal immune activation can be normalized by acute treatment with TDZD-8 (=4-benzyl-2-methyl-1,2,4,-thiadiazolidine-3,5-dione), a highly selective inhibitor of GSK3β (Kalinichev and Dawson, 2011; Lipina et al., 2011). We also assessed whether the anticipated effects of prenatal immune activation on the AKT/GSK3β signaling network would be associated with persistent inflammatory processes in the brain. GSK3β is a key regulator of the

inflammatory response (Wang et al., 2011), and inflammatory cytokines such as interleukin (IL)-1β are known to increase GSK3β activity by reducing the amounts of GSK3β phosphorylation (Green and Nolan, 2012). Therefore, we quantified the expression of hallmark inflammatory cytokine genes (IL-1β, IL-6, and tumor necrosis factor (TNF)-α) and further explored the activation of microglia, a population of immunocompetent cells in the brain capable of secreting inflammatory factors (Ransohoff and Perry, 2009).

MATERIALS AND METHODS

Animals

C57BL/6 mice were used throughout the study. Female and male breeders were obtained from our in-house specific pathogen free colony at the age of 10–14 weeks. Breeding began after 2 weeks of acclimatization to the new animal holding room, which was a temperature and humidity controlled (21 ± 1 °C, 55 ± 5%) holding facility under a reversed light–dark cycle (lights off: 08:00–20:00). All animals had ad libitum access to food (Kliba 3430, Klibamühlen, Kaiseraugst, Switzerland) and water unless specified otherwise. All procedures described in the present study had been previously approved by the Cantonal Veterinarian's Office of Zurich and are in agreement with the principles of laboratory animal care in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication, 8th edition 2011.).

Maternal poly(I:C) exposure during pregnancy

For the purpose of the maternal immunological manipulations during pregnancy, female mice were subjected to a timed mating procedure as described previously (Meyer et al., 2005). Pregnant dams on gestation day 9 (GD9) received either a single injection of poly(I:C) (potassium salt; Sigma–Aldrich) at a dose of 5 mg/kg or vehicle (sterile pyrogen-free 0.9% NaCl) according to dose-responses and protocols established before (Meyer et al., 2005). The gestational stage (i.e., GD9) was selected because of previous findings showing that poly(I:C) treatment on GD9 in C57BL/6 mice is capable of inducing widespread neuroanatomical and neurochemical alterations in the prefrontal and striatal dopaminergic systems (Meyer et al., 2008; Winter et al., 2009; Vuillermot et al., 2010). Poly(I:C) was dissolved in sterile pyrogen-free 0.9% NaCl (=vehicle) solution to yield a final concentration of 1.0 mg/ml and was administered via

the intravenous (i.v.) route at the tail vein under mild physical constraint. All solutions were freshly prepared at the day of administration and injected with a volume of 5 ml/kg. The effectiveness of this poly(I:C) administration regimen to induce cytokine-associated fetal brain inflammation has been confirmed in previous studies (e.g., Meyer et al., 2006). Consistent with previous investigations (Meyer et al., 2005), there were no significant effects of the prenatal manipulation on litter size, sex ratio, and length of gestation (data not shown).

Allocation and testing of offspring

All offspring were weaned and sexed on postnatal day (PND) 21. Littermates of the same sex were caged separately and maintained in groups of 2–4 animals per cage as described above. Only male subjects were included in all tests to circumvent bias arising from sexual dimorphism. All molecular, immunohistochemical, and behavioral analyses were performed when the offspring reached adulthood (i.e., between PND 85 and PND 100). Separate cohorts of behaviorally naïve male animals were used for all individual analyses in order to avoid possible confounds and carry-over effects arising from prior testing. Hence, the molecular and immunohistochemical analyses were conducted using brain samples derived behaviorally naïve animals that had not undergone behavioral testing. To avoid possible confounds arising from litter effects (Meyer et al., 2009), each cohort of animals assigned to the molecular or behavioral analyses included 1–2 males that stemmed from multiple independent litters (N = 12 for poly(I:C) and N = 14 for vehicle control litters). All behavioral testing was carried out during the dark phase of the reversed light–dark cycle.

Collection of brain samples for Western blot and RT-PCR analyses

Following decapitation of the animals, the brains were immediately extracted from the skull and placed dorsal side up on an ice-chilled plate. This was directly followed by preparing 1-mm coronal brain sections using razorblade cuts and subsequent micro-dissection of the medial prefrontal cortex (mPFC) and striatum (STR) as established before and fully described elsewhere (Bitanihirwe et al., 2010a). These two brain regions were selected because of previous findings showing that prenatal viral-like immune activation affects dopaminergic parameters in the mPFC and STR of adult offspring (Meyer et al., 2008; Winter et al., 2009; Bitanihirwe et al., 2010a;

Bitanihirwe et al., 2010b; Vuillermot et al., 2010). The brain samples were then immediately placed into Eppendorf tubes and either snap-frozen in liquid nitrogen for subsequent Western blot analyses, or placed into Eppendorf tubes containing RNAlater RNA Stabilization Reagent (Qiagen, Hombrechtikon, Switzerland) for subsequent RNA purification and real-time RT-PCR. The latter samples were incubated in RNAlater solution at room temperature for 4 h and then stored at −80 °C until further processing. Snap-frozen samples were also stored at −80 °C after collection.

All protein and gene expression analyses in the mPFC (Bregma: +2.3 to +1.3) were performed on tissue that included anterior cingulate (aCG), prelimbic (PrL) and infralimbic (IL) subregions, and the STR samples (Bregma: +1.1 to +0.1) included the caudate putamen region (Bitanihirwe et al., 2010a). Both left and right brain hemispheres were collected and were either used for gene or protein expression analyses, with right and left hemispheres being counterbalanced across the different molecular analyses (Western blot and real-time PCR analyses, see below) and prenatal treatment groups (control versus poly(I:C) offspring).

Protein extraction and Western blot analysis

PFC samples were homogenized in hypotonic lysis buffer (0.3 M sucrose, 4 mM HEPES, 1 mM EDTA, 1 mM EGTA and protease inhibitors [Roche, Basel, Switzerland]) using a Precellys tissue lyser (Biolabo, Châtel-st-Denis, Switzerland) according to the manufacturer's instructions. Protein concentration was determined using BCA assay (Pierce, Rockford, IL, USA) following the manufacturer's instructions and eluted to equal concentration in lysis buffer with NuPAGE LDS sample buffer (Invitrogen, Carlsbad, CA, USA). Proteins were separated by Criterion XT Precast Gels (Bio-Rad, Reinach, Switzerland) and transferred onto nitrocellulose membranes. After blocking for 2 h at room-temperature in blocking buffer (LI-COR, Bad Homburg, Germany), membranes were incubated overnight at 4 °C with the following primary antibodies: anti-Akt (1:1000; Cell Signaling, Danvers, MA, USA), anti-phospho(Thr308)-Akt (1:500; Cell Signaling, Danvers, MA, USA), anti-phospho(Ser473)-Akt (1:1000; Cell Signaling, Danvers, MA, USA), anti-GSK3β (1:1000; Cell Signaling, Danvers, MA, USA), anti-phospho(Ser9)-GSK3β (1:500; Cell Signaling, Danvers, MA, USA), and anti-β-actin (1:10,000; Millipore, Zug, Switzerland). After extensive washing with PBS 0.2% Tween 20, the proteins were visualized with a dual wavelength detection system (Odyssey

Imager; LI-COR, Bad Homburg, Germany) following the manufacturer's protocols using appropriate IRDye conjugated secondary antibodies (Rockland, Gilbertsville, PA, USA). Quantification of protein bands was carried out using Image Studio software (LI-COR, Bad Homburg, Germany); the protein expression ratio was calculated after normalization to β-actin. Total GSK3β and phospho-GSK3β was run on the same gel in order to be able to express the GSK3β activation status in terms of the phospho-GSK3β/total GSK3β ratio. The same principle was applied to total and phosphorylated AKT.

RNA purification and real-time RT-PCR

Cytokine levels in mPFC and STR were assessed using real-time RT-PCR. We favored this method over measurements of cytokine proteins using Western blot techniques because real-time RT-PCR has been shown to be capable of measuring even relatively small amounts of brain cytokines, which are typically expressed at very low levels under non-infectious conditions (Garay et al., 2012). Total RNA was extracted and processed for PCR analysis using the RNeasy Plus Micro Kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's instructions. The concentration of each total RNA sample was determined by spectrophotometry (NanoDrop ND-1000, Witec, Littau, Switzerland) and RNA quality was evaluated using the Experion Automated Electrophoresis System (Bio-Rad, Reinach, Switzerland). Real-time RT-PCRs were performed on the LightCycler 480 Real-Time PCR System (Roche Diagnostics, Rotkreuz, Switzerland) using 384-well plates, the QuantiTect Probe RT-PCR Kit (Qiagen, Hombrechtikon, Switzerland), and Taqman gene expression assays (Applied Biosystems, Rotkreuz, Switzerland). The total volume of each reaction was 10 µl, containing 4 ng of corresponding total RNA. All reactions were carried out in triplicate. The protocol consisted of reverse transcription for 20 min at 50 °C, initial activation for 15 min at 95 °C, followed by 40 cycles of 1 s at 95 °C and 60 s at 60 °C. The following Taqman gene expression assays were applied: GADPH (Mm99999915_g1), IL-1β (Mm01336189_m1), IL-6 (Mm00446190_m1), and TNF-α (Mm00443258_m1). The amount of target genes was obtained by the $2-\Delta\Delta Ct$ method (Livak and Schmittgen, 2001) after normalization against the internal control (GADPH) and expressed as fold changes of the target genes of interest.

Immunohistochemistry

The animals were deeply anesthetized with an overdose of Nembutal (Abbott Laboratories) and perfused transcardially with 0.9% NaCl, followed by 4% phosphate-buffered paraformaldehyde solution containing 15% picric acid. The dissected brains were postfixed in the same fixative for 6 h and processed for antigen retrieval involving overnight incubation in citric acid buffer (pH = 4.5) followed by a 90 s microwave treatment at 480 W according to protocols established before (Meyer et al., 2008). The brains were then cryoprotected using 30% sucrose in PBS, frozen with powdered dry ice, and stored at −80 °C until further processing.

Perfused brain samples were cut coronally at 30 µm thickness from frozen blocks with a sliding microtome. Eight serial sections were prepared for each animal. The sections were rinsed in PBS, and stored at −20 °C in antifreeze solution until further processing. For immunohistochemical staining, the slices were rinsed three times for 10 min in PBS, and blocking was done in PBS, 0.3% Triton X-100, 10% normal serum for 1 h at room temperature. The following primary antibodies were used: Rabbit anti-Iba1 (Wako, CatNo# 019-19741; diluted 1:2000) and rat anti-CD68 (Serotec, CatNo# MCA1957; diluted 1:5000). All antibodies were diluted in PBS containing 0.3% Triton X-100 and 2% normal serum, and the sections were incubated free-floating overnight at room temperature. After three washes with PBS (10 min each), the sections were incubated for 1 h with the biotinylated secondary antibodies diluted 1:500 in PBS containing 2% NGS and 0.3% Triton X-100. Sections were washed again three times for 10 min in PBS and incubated with Vectastain kit (Vector Laboratories) diluted in PBS for 1 h. After three rinses in 0.1 M Tris–HCl, pH 7.4, the sections were stained with 1.25% 3,3-diaminobenzidine and 0.08% H2O2 for 10–15 min, rinsed again four times in PBS, dehydrated, and coverslipped with Eukitt (Kindler).

Unbiased stereological estimations

The numbers of Iba1- or CD68-immunoreactive cells were determined by unbiased stereological estimations using the optical fractionator method (Gundersen et al., 1988). With the aid of the image analysis computer software Stereo Investigator (version 6.50.1; MicroBrightField), every section of a one-in-eight series was measured, resulting in an average of 4–5 sections per brain sample. The following sampling

parameters were used: (1) a fixed counting frame with a width of 60 μm and a length of 60 μm; and (2) a sampling grid size of 200 × 150 μm. The counting frames were placed randomly at the intersections of the grid within the outlined structure of interest by the software. The cells were counted following the unbiased sampling rule using the 40× oil lens [numerical aperture (NA), 1.3] and included in the measurement when they came into focus within the optical dissector (Howard and Reed, 2005). All immunohistochemical preparations were quantified in the mPFC (including aCG, PrL, and IL regions from Bregma: +2.3 to +1.3) and STR (including CPu region from Bregma: +1.1 to +0.1) as outlined above.

Spontaneous alternation in the Y-maze

A spontaneous alternation task in the Y-maze was used to assess cognitive performance in vehicle- and TDZD-8-treated poly(I:C) and control offspring. This task is dependent on the integrity of the mPFC (Lalonde, 2002) and is based on the innate tendency of rodents to explore novel environments, that is, their preference to investigate a new arm of the maze rather than returning to one that was previously visited.

The Y-maze apparatus was made of transparent Plexiglas and consisted of three identical arms (500 mm long × 90 mm wide) surrounded by transparent Plexiglas walls 100 mm in height. The three arms radiated from a central triangle (80 mm on each side) and were spaced 120° from each other. The floor of the maze was covered with saw dust bedding, which was changed between each test run. The maze was elevated 900 mm above the floor, and was positioned in a well-lit room enriched with distal spatial cues. A digital camera was mounted above the Y-maze apparatus. Images were captured at a rate of 5 Hz, and transmitted to a PC running the EthoVision tracking system (Noldus Information Technology), which calculated the total distance moved and the number of entries into the three arms and the center zone of the Y-maze.

Mice were administered with TDZD-8 or corresponding vehicle solution (see below). The animals were then placed in appropriate waiting boxes containing saw dust embedding for 30 min before they were placed in the center of the Y maze. An observer in an adjacent room who was blind to the treatment conditions viewed the mice through a video camera, and recorded the number and sequence of arm entries (defined as entry

of the whole body into an arm) during a period of 5 min. Alternation was defined as entry into the three arms in any non-repeating order (for example, ABC, BAC, CBA). The percentage alternation was calculated as the total number of alternations divided by the possible alternations given the number of arm entries (total number of arm entries-2). In addition to the analysis of percentage alternation, the total distance moved and the total number of arm entries were recorded and analyzed to assess general activity during the 5-min test period.

Amphetamine-induced locomotor hyperactivity

An amphetamine (AMPH)-induced locomotor hyperactivity test was used to assess the animals' behavioral response to acute dopaminergic stimulation. This test was carried out in four identical open-field arenas (40 × 40 × 35 cm high) made of wood and painted gray as described before (Meyer et al., 2005; Meyer et al., 2008). They were located in a testing room under dim diffused lighting (~35 lux as measured in the center of the arenas). A digital camera was mounted directly above the four arenas. Images were captured at a rate of 5 Hz and transmitted to a PC running the Ethovision (Noldus) tracking system.

To acclimatize the animals to the open field, they were placed in the center of the arena and allowed to explore freely for 20 min. At the end of this time period, the animals were removed from the apparatus and injected with sterile 0.9% NaCl (saline) solution. They were then immediately returned to the same arenas and allowed to explore for another 20 min in order to gauge the animal's locomotor reactions to injection stress. Subsequently, the animals were briefly removed from the apparatus once more and administered with TDZD-8 or corresponding vehicle solution (see below). The animals were then placed in appropriate waiting boxes containing saw dust embedding for 15 min before they were injected with AMPH solution (see below). The animals were then returned to the same open field arenas again, and the locomotor responses were then monitored for a period of 80 min.

Drugs

AMPH (Sigma–Aldrich) was dissolved in isotonic 0.9% NaCl solution to achieve the desired concentration for injection. AMPH was administered via the intraperitoneal (i.p.) route at a dose of 2.5 mg/kg. The dose was selected based on our previous studies

showing that GD9 poly(I:C) offspring display enhanced locomotor responses to AMPH at this dose (e.g., Meyer et al., 2005, 2008). TDZD-8 (Calbiochem) was dissolved in saline containing 0.3% Tween-20 (=vehicle). It was administered at a dose of 1 or 10 mg/kg (i.p.) according to previous dose response studies (Kalinichev and Dawson, 2011; Lipina et al., 2011). TDZD-8 or vehicle solution was given 30 min prior testing in the Y-maze spontaneous alternation test, and 15 min prior to acute AMPH administration in the AMPH-induced locomotor hyperactivity test. The volume of injection was 5 ml/kg for all solutions. All solutions were freshly prepared on the day of testing.

Statistical analysis

All Western blot, RT-PCR, and immunohistochemical data were analyzed using independent Student's t tests (two-tailed). The dependent measures obtained in the Y-maze spontaneous alternation test (% alternation, number of arm entries, and total distance moved) were analyzed using a 2 × 3 (prenatal treatment × drug) ANOVA, followed by Fisher's least significant difference (LSD) post-hoc comparisons. In the AMPH sensitivity test, the locomotor activity (indexed by the distance traveled in the entire open field) was expressed as a function of 5-min bins and analyzed using a 2 × 3 × 4 (prenatal treatment × drug × bins) repeated-measures ANOVA for the initial acclimatization and saline treatment phases, and by a 2 × 3 × 16 (prenatal treatment × drug × bins) repeated-measures ANOVA for the AMPH treatment phase. Locomotor activity scores (cm) were first square-root (sqrt) transformed in order to better conform to the assumption of data homogeneity and normality by parametric ANOVA. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using the statistical software StatView (version 5.0) implemented on a PC running the Windows 7 operating system.

RESULTS

Prenatal immune activation alters prefrontal GSK3β expression and activation

First, we evaluated the effects of prenatal poly(I:C)-induced immune activation on the expression of total and phosphorylated GSK3β protein in the mPFC and STR using Western blot techniques. As shown in Fig. 1A, offspring of immune-challenged mothers

displayed a ~20% (t23 = 2.64, p < 0.05) increase in total GSK3β levels compared to control offspring in the mPFC region. No significant group differences were found with respect to the total GSK3β protein levels in the STR (Fig. 1A).

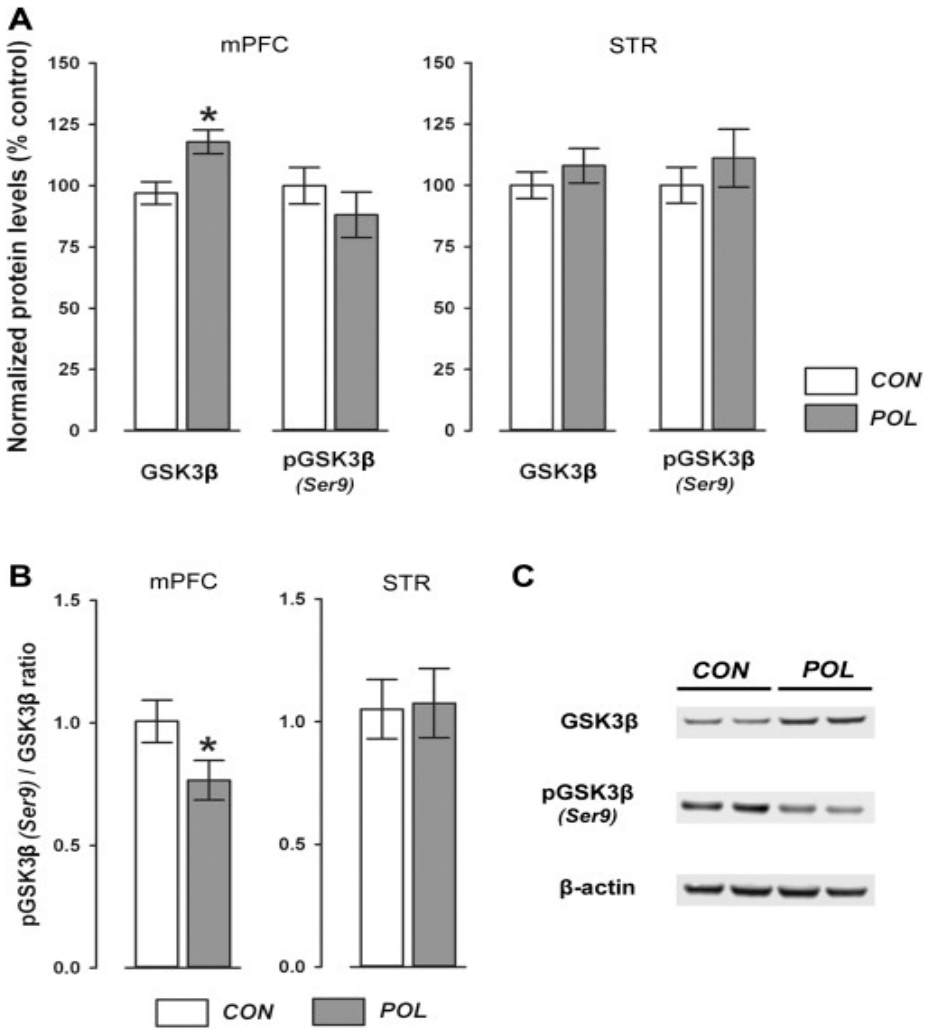

Figure1. Effects of prenatal immune activation on GSK3β expression and phosphorylation. Densitometric analysis of Western blots was used to measure total GSK3β protein and GSK3β phosphorylation at serine (Ser9) residues in the medial prefrontal cortex (mPFC) and striatum (STR) of adult offspring born to poly(I:C)-exposed (POL) or control (CON) mothers. (A) Total GSK3β and Ser9-phosporlyated GSK3β levels shown as percent expression in CON offspring. *p < 0.05; *N* (CON) = 12, N(POL) = 13. All values are means ± SEM. (B) Ratio between Ser9-phosporylated and total GSK3β. *p < 0.05; N(CON) = 12, N(POL) = 13. All values are means ± SEM. (C) The photomicrographs show Western blot samples for total GSK3β and Ser9-phosporylated GSK3β in the mPFC of two CON and POL offspring. β-Actin is shown as control for comparison.

We also explored whether prenatal immune activation may alter the activation status of GSK3β, which becomes deactivated upon phosphorylation of serine (Ser9)

residues at the N-terminus (Beaulieu et al., 2009; Beaulieu et al., 2011). The absolute levels of serine-phosphorylated GSK3β were not significantly different between poly(I:C) and control offspring, neither in the mPFC nor the STR (Fig. 1A). However, an additional analysis of the ratio between phosphorylated and total GSK3β protein demonstrated a ~25% ($t_{23} = 2.52$, $p < 0.05$) decrease in the relative GSK3β phosphorylation levels in the mPFC but not the STR of poly(I:C) offspring (Fig. 1B).

Prenatal immune activation alters prefrontal AKT expression

In a next step, we explored the effects of prenatal immune activation on prefrontal and striatal AKT protein expression and phosphorylation using Western blot analyses. AKT is a key up-stream signaling partner of GSK3β: It is activated upon phosphorylation at serine (Ser473) and threonine (Thr308) residues, and once active phosphorylates down-stream targets such as GSK3β (Beaulieu et al., 2009, 2011). Prenatal poly(I:C)-induced immune challenge reduced the total levels of AKT by ~20% in the mPFC ($t_{23} = 3.23$, $p < 0.01$; Fig. 2A). In addition, the prenatal insult decreased the levels of threonine-phosphorylated AKT in the mPFC by ~25% ($t_{23} = 2.10$, $p < 0.05$; Fig. 2A). In contrast to these prefrontal effects, prenatal immune activation did not significantly alter the expression of total or phosphorylated AKT in the STR (Fig. 2A). There were also no significant group differences with respect to the ratio between phosphorylated and total AKT protein, neither in the mPFC nor the STR (Fig. 2B,C).

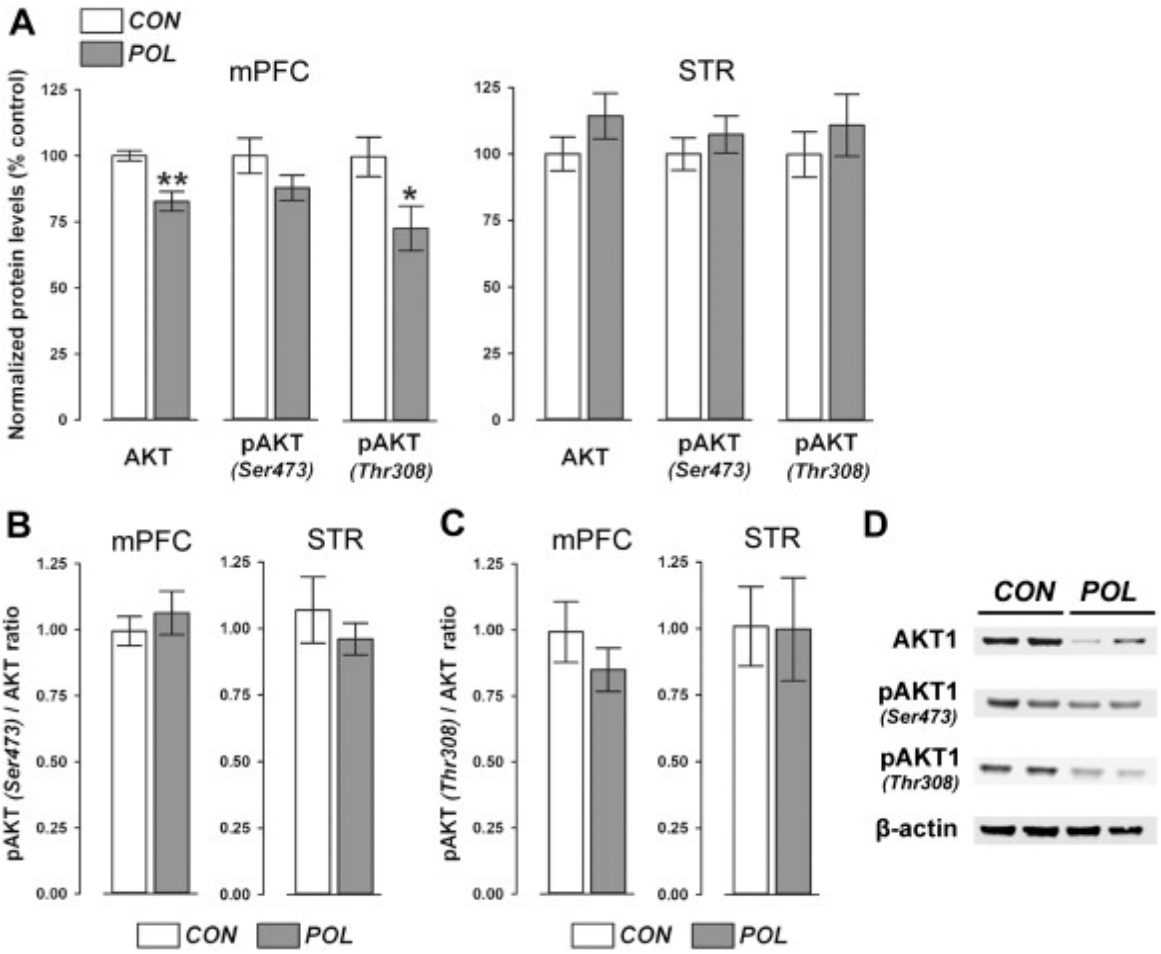

Figure 2. Effects of prenatal immune activation on AKT expression and phosphorylation. Densitometric analysis of Western blots was used to measure total AKT protein and AKT phosphorylation at serine (Ser473) and threonine (Thr308) residues in the medial prefrontal cortex (mPFC) and striatum (STR) of adult offspring born to poly(I:C)-exposed (POL) or control (CON) mothers. (A) Levels of total AKT, Ser473-phosporlyated AKT, and Thr308-phosporlyated AKT shown as percent expression in CON offspring. *$p < 0.05$ and **$p < 0.01$; N(CON) = 12, N(POL) = 13. All values are means ± SEM. (B) Ratio between Ser473-phosporlyated and total AKT. N(CON) = 12, N(POL) = 13. (C) Ratio between Thr308-phosporlyated and total AKT. N(CON) = 12, N(POL) = 13. (D) The photomicrographs show Western blot samples for total AKT, Ser473-phosporlyated AKT, and Thr308-phosporlyated AKT in the mPFC of two CON and POL offspring. β-Actin is shown as control for comparison.

Prenatal immune activation does not alter inflammatory cytokine gene expression in the adult brain

To assess whether the identified molecular alterations in the AKT/GSK3β network would be associated with persistent signs of neuroinflammation, we measured

the mRNA levels of the prototypical inflammatory cytokines IL-1β, IL-6, and TNF-α in the mPFC and STR of prenatally poly(I:C)-exposed and control offspring. As shown in Fig. 3A, the expression of none of the cytokines was significantly different between poly(I:C)-treated offspring and controls in either brain area of interest. There were also no group differences with respect to the entire (activated and non-activated) microglia cell numbers in the mPFC and STR as verified by stereological estimations of Iba1-positive cells (Fig. 3B). Furthermore, poly(I:C) and control offspring did not differ in the expression of CD68 (Fig. 3B), a cellular marker typically expressed by activated microglia cells (Ransohoff and Perry, 2009). Taken together, these findings suggest that prenatal immune activation by poly(I:C) does not lead to overt signs of inflammation in the mPFC or STR at adult age.

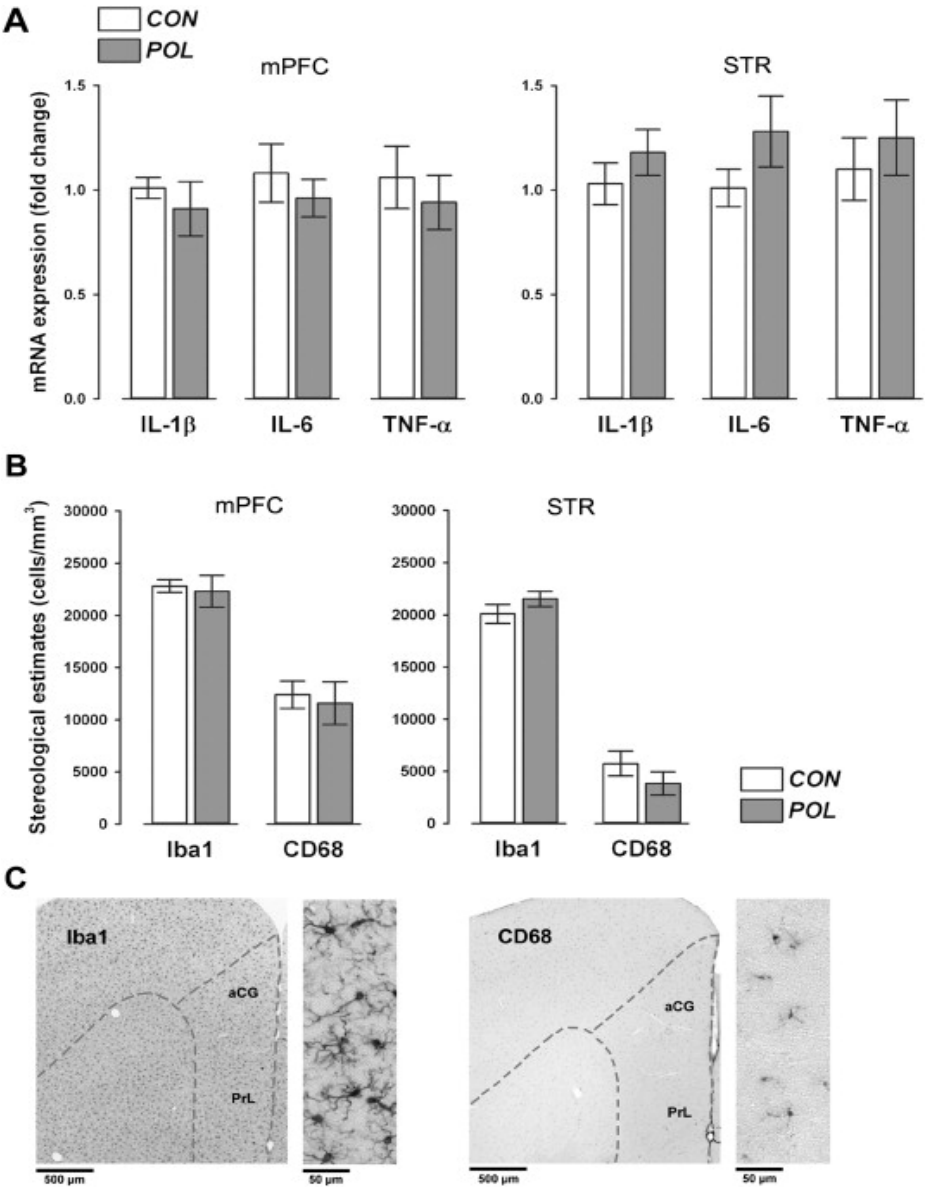

Figure 3 (previous page). Effects of prenatal immune activation on inflammatory cytokine expression and microglia activation in the medial prefrontal cortex (mPFC) and striatum (STR) of adult offspring born to poly(I:C)-exposed (POL) or control (CON) mothers. (A) Levels of interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α mRNA expression as assessed using quantitative real-time PCR. N(CON) = 12, N(POL) = 13. All values are means ± SEM. (B) Stereological estimates of Iba1- and CD68-immunoreactive microglia cell numbers. N(CON) = 8, N(POL) = 7. All values are means ± SEM. (C) The photomicrographs show examples of coronal brain sections at the level of the mPFC (highlighting anterior cingulate (aCG) and prelimbic (PrL) cortices) stained with anti-Iba1 or CD68 antibody at low and high resolution.

The selective GSK3β inhibitor TDZD-8 normalizes deficient spontaneous alternation induced by prenatal immune challenge

We ascertained the potential consequences of altered (prefrontal) GSK3β activation on behavior by treating poly(I:C) and control offspring with TDZD-8 (0, 1, or 10 mg/kg, i.p.), a selective inhibitor of GSK3β (Kalinichev and Dawson, 2011; Lipina et al., 2011). First, we used a Y-maze spontaneous alternation task because performance in this test is dependent on the integrity of the mPFC (Lalonde, 2002). As shown in Fig. 4A, prenatal immune activation fully disrupted spontaneous alternation as indexed by the percent score of arm alternation: Whilst vehicle-treated control offspring exhibited an alternation score of ~70%, the alternation score of vehicle-treated poly(I:C) offspring fell below chance level (50%). TDZD-8 administration normalized the poly(I:C)-induced spontaneous alternation deficits in a dose-dependent manner: Poly(I:C)-exposed offspring treated with the high (10 mg/kg) but not low (1 mg/kg) dose of TDZD-8 showed an alternation score of ~70% similarly to control offspring (Fig. 4A). TDZD-8 treatment did not alter behavioral performance in control offspring (Fig. 4A).

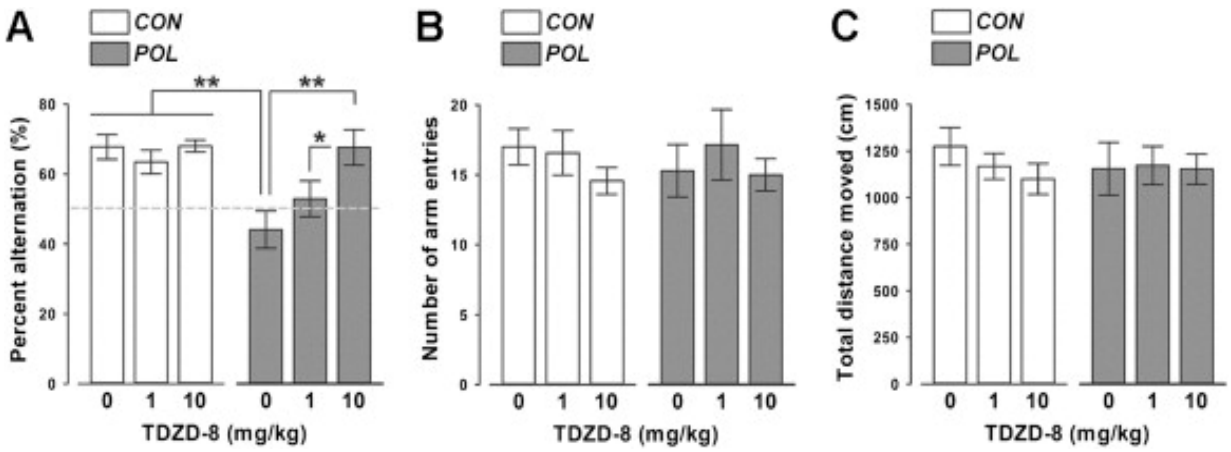

Figure 4 (previous page). Effects of pre-treatment with the selective GSK3β inhibitor TDZD-8 (0, 1, or 10 mg/kg) on spontaneous alternation. Adult offspring from poly(I:C)-exposed (PON) and control (CON) mothers were tested in a Y-maze spontaneous alternation tests. (A) Means ± SEM of percent alternation (%) scores.*$p < 0.05$ and **$p < 0.01$. (B) Means ± SEM of total number or arm entries. (C) Means ± SEM of total distance moved (cm). *N* = 7 in each prenatal treatment and drug treatment condition.

Statistical support for these impressions was obtained by ANOVA yielding a significant main effect of prenatal treatment ($F(1,36) = 9.94$, $p < 0.01$), drug ($F(2,36) = 4.26$, $p < 0.05$), and their interaction ($F(2,36) = 3.91$, $p < 0.05$). Subsequent post-hoc comparisons confirmed the significant difference between vehicle- or TDZD-8-treated control offspring and vehicle-treated poly(I:C) offspring (all $p < 0.01$) and between the different TDZD-8 treatment conditions amongst the poly(I:C) offspring ($p < 0.05$ or $p < 0.01$, see Fig. 4A). Neither prenatal immune activation nor TDZD-8 administration affected the number or total arm entries (Fig. 4B) or total distance moved (Fig. 4C) in the Y-maze spontaneous alternation test. Hence, the poly(I:C)-induced deficits in alternation and its normalization by TDZD-8 reflect genuine effects on spontaneous alternation.

The selective GSK3β inhibitor TDZD-8 reduces AMPH-induced hyperactivity

We extended our behavioral analyses to an AMPH-induced locomotor hyperactivity test in the open field. No significant group differences were detected during the initial 20-min habituation phase of the test, during which locomotor activity decreased as a function of 5-min bins in all groups (main effect of bins: $F(3,99) = 73.82$, $p < 0.001$; Fig. 5A). Similarly, no significant group differences were detected in the subsequent saline-treatment phase (Fig. 5A). Acute AMPH treatment generally led to an increase in locomotor activity, as indicated by the significant main effect of bins ($F(15,495) = 43.55$, $p < 0.001$; Fig. 5A). The locomotor-enhancing effects of AMPH treatment were significantly increased in poly(I:C) offspring compared to control offspring (Fig. 5B), leading to a significant main effect of prenatal treatment ($F(1,33) = 10.68$, $p < 0.01$) and its interaction with bins ($F(15,495) = 2.12$, $p < 0.01$). Pre-treatment with TDZD-8 dose-dependently attenuated the AMPH-induced hyperactivity response, both in poly(I:C) and control offspring (Fig. 5B). This impression was statistically supported by the presence of a significant main effect of drug ($F(2,33) = 7.04$, $p < 0.01$) and its interaction with bins ($F(30,495) = 2.67$, $p < 0.001$), and by the subsequent post-

hoc analysis verifying a significant difference between animals pre-treated with the high dose of TDZD-8 (10 mg/kg) and animals pre-treated with either the low dose of TDZD-8 (1 mg/kg) or vehicle (all $p < 0.01$; Fig. 5B).

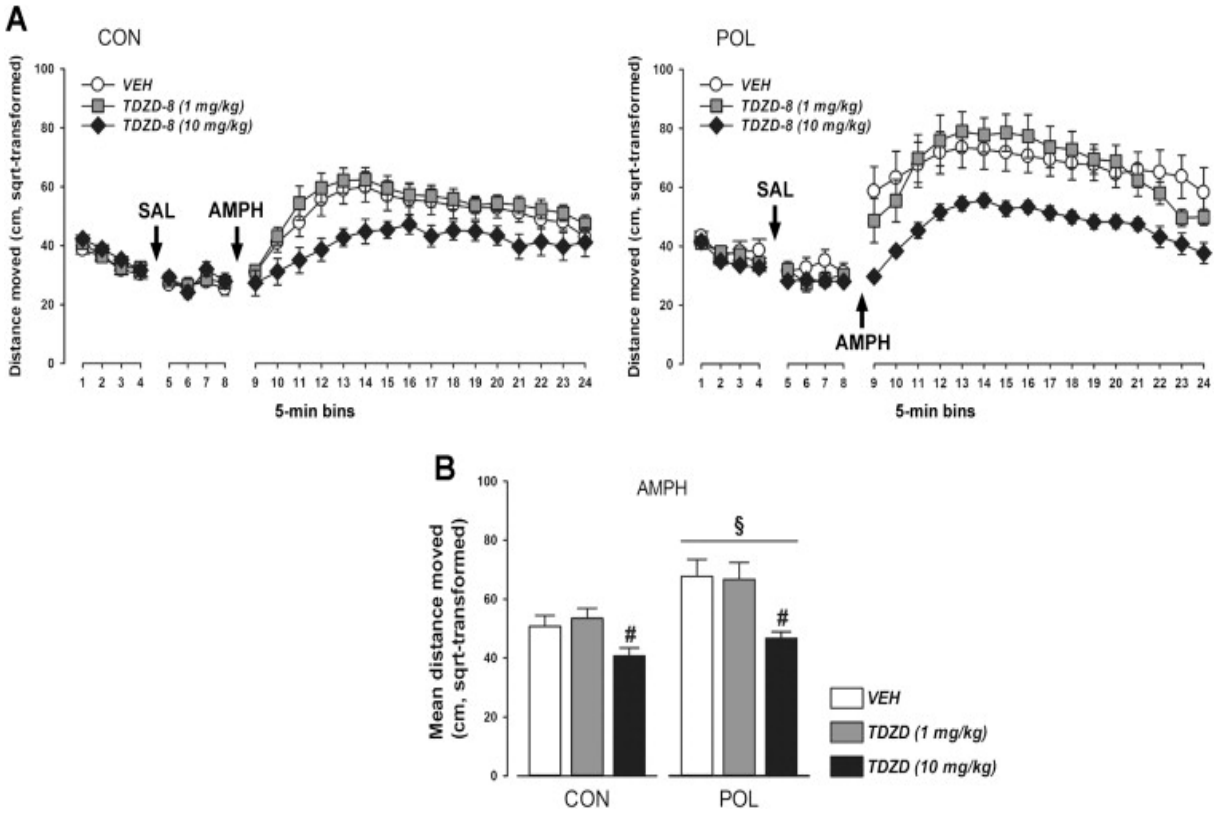

Figure 5. Effects of pre-treatment with the selective GSK3β inhibitor TDZD-8 (0, 1, or 10 mg/kg) on amphetamine (AMPH)-induced hyperactivity. Locomotor activity displayed by adult offspring from poly(I:C)-exposed (PON) and control (CON) mothers was measured in a standard open filed during an initial habituation phase, following saline (SAL) treatment, and following acute AMPH (2.5 mg/kg) treatment. CON and POL offspring received vehicle (VEH) or TDZD-8 (1 or 10 mg/kg) 15 min prior to AMPH administration. (A) Locomotor activity in the open field expressed as distance moved (cm, sqrt-transformed) per 5-min bin during the initial habituation phase and the subsequent SAL and AMPH treatment phases. The data are depicted separately for CON and POL offspring to facilitate visualization. (B) Mean distance moved (cm, sqrt-transformed) during the AMPH treatment phase. §$p < 0.01$, reflecting the significant increase in POL offspring; #$p < 0.01$, reflecting the significant decrease in animals pre-treated with the high dose (10 mg/kg) of TDZD-8 relative to animals treated with VEH or the low dose (1 mg/kg) of TDZD-8. N = 6–7 in each prenatal treatment and drug treatment condition.

DISCUSSION

The present mouse model demonstrates that prenatal exposure to viral-like immune activation by maternal poly(I:C) treatment leads to long-term molecular alterations in the AKT/GSK3β signaling network. These changes include long-term increases in total GSK3β levels and decreased ratios between phosphorylated and total GSK3β protein in the mPFC. GSK3β activity is tightly regulated by (AKT-induced) phosphorylation: It is biologically active in the unphosphorylated form but inactivated upon serine phosphorylation at the N-terminus (Beaulieu et al., 2009, 2011). Overall, the GSK3β activation status is governed by the relative expression of the phosphorylated (=non-activated) form in the entire pool of GSK3β protein (Kaidanovich-Beilin and Woodgett, 2011). Therefore, our findings here can be readily interpreted as a molecular sign of increased prefrontal GSK3β activation status following prenatal immune activation. These data extend previous investigations in mice showing increased cortical GSK3β activation status (indexed by a decrease in the ratio between phosphorylated and total GSK3β protein) in neonatal mice exposed to prenatal poly(I:C)-induced immune challenge (Abazyan et al., 2010).

We also found that prenatal viral-like immune challenge decreased the total levels of AKT and reduced phosphorylation at AKT threonine residues in the offspring's mPFC. The former effects corroborates and extends the recent findings by Bitanihirwe et al. (2010b) who demonstrated reduced AKT protein levels in the adult mPFC of prenatally poly(I:C)-exposed mice using immunohistochemical techniques. However, it needs to be emphasized that the AKT activations status (as indexed by the ratio between phosphorylated and total AKT protein) was not significantly affected by the prenatal manipulation. Together, these findings suggest that prenatal viral-like immune robustly decreases prefrontal AKT expression as such but does not further lead to changes in AKT activity.

Interestingly, significant long-term changes in the AKT/GSK3β signaling network were only clearly manifested in the mPFC but not in the STR. Even though we cannot rule out the possibility of other effects in additional brain areas, our data highlight a

certain degree of region-specificity with respect to the negative influence of prenatal immune activation on AKT/GSK3β. Indeed, the mPFC appears to be more vulnerable than the STR in terms of altered AKT/GSK3β signaling following prenatal immune challenge. The precise mechanisms determining such region-specificity remain uncharted by the present study. On speculative grounds, however, the extent to which prenatal immune activation affects or spares AKT/GSK3β signaling in distinct brain areas may be related to differential effects of the prenatal insult on signaling partners acting up-stream of the AKT/GSK3β network. For example, it is known that (chronic) activation of D2Rs inhibits AKT activity through dephosphorylation processes, and such reduction in AKT activity can further lead to decreased phosphorylation of the GSK3β target (Beaulieu et al., 2009; Beaulieu et al., 2011). Winter et al. (2009) have previously found that prenatal poly(I:C)-induced immune challenge on GD9 leads to permanent increases in basal dopamine levels in the mPFC but not in the STR, and these effects are accompanied by a compensatory decrease in prefrontal but not striatal D2R expression (Meyer et al., 2008). Hence, there seem to be similar brain region-specific consequences of prenatal immune activation in terms of critical dopamine-associated pathways acting up-stream of the AKT/GSK3β network. An alternative (but not mutually exclusive) mechanism for the presence of long-lasting changes in the AKT/GSK3β network may include epigenetic modifications triggered by prenatal infection. This possibility seems indeed feasible in view of the recent findings showing that prenatal poly(I:C)-induced immune activation in mice induces permanent epigenetic modifications at multiple gene promoters (Tang et al., 2013). Future research will be needed, however, to ascertain possible epigenetic effects of prenatal immune challenge on the AKT/GSK3β network.

We further found that acute pre-treatment with the selective GSK3β inhibitor TDZD-8 normalized aberrant behavior in a dose-dependent manner, providing additional functional support for the presence of increased GSK3β activity following prenatal immune challenge. Most notably, administration of the higher dose of TDZD-8 (10 mg/kg) fully corrected the poly(I:C)-induced deficits in spontaneous alternation in the Y-maze without affecting performance in control offspring. This suggests that the beneficial effects of TDZD-8 treatment on spontaneous alternation are selective to animals with a neurodevelopmental predisposition to such behavioral abnormalities. TDZD-8 administration also dose-dependently reduced the AMPH-induced locomotor

responses in the open field. Consistent with numerous previous investigations, prenatal poly(I:C) exposure potentiated the locomotor response to systemic AMPH treatment (e.g., Zuckerman et al., 2003; Meyer et al., 2005, 2008; Vuillermot et al., 2010). Pre-treatment with the high dose of TDZD-8 (10 mg/kg) in poly(I:C) offspring led to locomotor activity scores comparable to those seen in AMPH-exposed control offspring that were pre-treated with vehicle solution. However, it should be noted that the high dose of TDZD-8 also reduced AMPH-induced activity in control offspring similarly to what has been reported in a recent dose response studies (Kalinichev and Dawson, 2011). Hence, the drugs' efficacy to suppress AMPH-induced locomotor activity is not specific to offspring with neurodevelopmentally precipitated AMPH hypersensitivity but is similarly operational in otherwise behaviorally intact animals.

To what extent may the selective (spontaneous alternation) versus non-selective (AMPH-induced locomotion) behavioral effects of TDZD-8 be explained by brain region-specific molecular changes in the AKT/GSK3β network? The present data does not readily allow to provide a solid answer to this question because we did not further evaluate possible region-specific effects of TDZD-8 on aberrant behavior using selective intra-mPFC or intra-STR drug infusions. On speculative grounds, however, the mPFC is more directly engaged in the behavioral/cognitive processes regulating spontaneous alternation in the Y-maze compared to the STR (Lalonde, 2002). For example, it has been established before that T- or Y-maze alternation (and the underlying cognitive processes such as working memory) are critically dependent on prefrontal dopamine signaling: Both under- and overstimulation of dopamine receptors in the mPFC disrupts performance in such tasks according to an inverted-U mechanism of prefrontal dopamine signaling (Dent and Neill, 2012; Williams and Castner, 2006). On the other hand, the striatal dopamine system is arguably more strongly involved in the precipitation of AMPH-induced locomotor hyperactivity compared to the prefrontal dopamine network (David et al., 2005). Since prenatal immune activation clearly affected GSK3β in the mPFC but not STR, one possibility would be that the group-selective effects of GSK3β-inhibiting drugs such as TDZD-8 are more readily evident in behavioral tasks that involve more strongly the prefrontal than the striatal dopamine network. However, this interpretation still needs to be met with caution because spontaneous alternation is not exclusively regulated by the mPFC but also underlies

modulation by other brain areas, including the hippocampus and cerebellum (Lalonde, 2002). Related to this, another limitation of the present study is that only one specific GSK3β inhibitor (TDZD-8) was used to explore potential beneficial effects of pharmacological GSK3β inhibition on aberrant behavior. It seems therefore warranted to extend our study by testing other GSK3β-inhibiting drugs in order to ascertain the generality of the present findings. Furthermore, it remains uncharted here whether the observed effects on AKT and GSK3β signaling would be similarly manifest in both male and female offspring, given that our study included male mice only.

In addition to its modulation by dopaminergic signals, the AKT/GSK3β signaling pathways is also regulated by various immune factors, including inflammatory cytokines. For example, both IL-1β and TNF-α have been shown to increase GSK3β expression and/or activity both in the periphery and the central nervous system (Park et al., 2011; Green and Nolan, 2012). Here, we failed to find significant signs of on-going inflammatory processes in the brains of adult poly(I:C)-exposed offspring relative to controls: Neither the expression of three prototypical inflammatory cytokines (IL-1β, IL-6, and TNF-α) nor the activation status of microglia cells were found to be altered following prenatal immune activation. It seems therefore unlikely that the identified changes in the prefrontal AKT/GSK3β network result from on-going inflammatory processes in the adult brain (Wang et al., 2011). Nevertheless, it remains possible that the changes in the expression of total AKT and GSK3β protein following prenatal immune activation could arise from inflammatory processes taking place during early brain development. Indeed, maternal administration of poly(I:C) in mice has repeatedly shown to lead to marked but transient increases in inflammatory cytokine expression in the maternal and fetal compartments, including the fetal brain (Meyer et al., 2006; Smith et al., 2007; Abazyan et al., 2010; Vuillermot et al., 2012). It would be desirable to further address this issue by longitudinal studies, in which the association between inflammatory responses and altered AKT/GSK3β are investigated at distinct maturational time points.

With respect to human pathological condition, our findings resemble studies demonstrating reductions in total AKT protein and decreased ratios between phosphorylated and total GSK3β protein in cortical tissue of schizophrenic patients (Emamian et al., 2004). Our data also matches the findings of decreased AKT and

increased GSK3β activity in the PFC of subjects with major depression (Karege et al., 2007). Finally, it is also of interest to point out some subsidiary similarities between the increased total GSK3β protein levels in our model system and those found in peripheral blood mononuclear cells of bipolar manic patients (Li et al., 2010). As extensively reviewed elsewhere (Boksa, 2010; Meyer and Feldon, 2010; Meyer and Feldon, 2012), prenatal poly(I:C) exposure in rodents has been widely used to experimentally induce and mimic brain and behavioral pathologies relevant to developmental neuropsychiatric disorders, including schizophrenia, bipolar disorder, and autism. Therefore, we believe that our data can be taken to experimentally support the emerging role of altered AKT/GSK3β signaling in such neurodevelopmental psychiatric disorders. However, our findings should be extended using other behavioral measures to further consolidate their relevance to schizophrenia, bipolar, and related disorders. Even though both working memory deficiency and sensitized AMPH responses are implicated in these mental illnesses (Peleg-Raibstein et al., 2012), they are arguable not specific to schizophrenia or bipolar disorder. Hence, our behavioral data should be bolstered by other translational paradigms relevant to schizophrenia or bipolar disorder, including tests for sensorimotor gating, selective attention, and cognitive flexibility (Peleg-Raibstein et al., 2012).

In conclusion, the present study documents for the first time the presence of long-term alterations in the prefrontal AKT/GSK3β network following immune-mediated neurodevelopmental disruption. The identified beneficial effects of pharmacological GSK3β inhibition on neurodevelopmentally acquired behavioral abnormalities suggest that these molecular alterations are functionally relevant to at least certain aspects of behavioral dysfunctions. The present model system may thus be helpful to further explore the relevance of altered intracellular dopamine signaling in the mPFC in relation to the prefrontal dopaminergic pathology associated with schizophrenia (Knable and Weinberger, 1997) and bipolar disorder (Gamo and Arnsten, 2011), and to the behavioral and cognitive consequences of such prefrontal deficits.

Disclosure and Acknowledgments

All authors declare that they have no conflicts of interest to disclose. R.W. and A.H. are employed by F. Hoffmann-La Roche Ltd. The present work is purely academic.

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