



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

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Science **339**, 1095 (2013);
DOI: 10.1126/science.1228261

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and downstream inflammatory cascade play a role in trigeminal activation. The effect of CBX was not due to a direct vascular action or blockade of gap junctions because CSD-induced CBF changes, early MMA dilation, and Ca^{+2} rise in astrocytic syncytium were not altered by CBX (Fig. 3, E and F, and fig. S5) (19). Interrupting this signaling cascade with another Panx1 inhibitor, probenecid or HMGB1-shRNA, or NF- κ B activation inhibitor 4-methyl-N1-(3-phenylpropyl) benzene-1,2-diamine suppressed the late MMA dilation (Fig. 3, G and H). Naproxen (40 mg/kg intraperitoneally), a prostaglandin synthase inhibitor, also suppressed the MMA response. Moreover, CSD-induced dural mast cell degranulation, another manifestation of the trigeminal activation (21, 22), was significantly reduced by CBX and HMGB1-shRNA (Fig. 3, I to L). Last, we assessed the headache-like behavior induced by repeated CSDs (which also induced PI influx and nuclear NF- κ B translocation) (19) with a method based on scoring facial grimace (23). Placement of a KCl-pellet over dura in freely moving mice caused pain-related mimics unlike saline-applied, sham-operated mice. CSD-induced pain was reversed by means of CBX treatment (Fig. 3M).

These data show that CSD opens neuronal Panx1 channels as reported during in vitro ischemia, NMDA over-activation, and aberrant bursting (17). Activation of Panx1 by cellular stressors such as excess potassium or glutamate stimulates the inflammasome complex, subsequent caspase-1 activation, and IL-1 β production (17, 18), suggesting that Panx1 megachannels may play a role as a reporter linking neuronal stress to inflammatory response. Similarly, CSD induced caspase-1 activation and HMGB1 release from neurons whose Panx1 channels were activated.

HMGB1 is a member of the alarmin family, which mediates the communication between injured and surrounding cells (24). HMGB1 is passively released from necrotic cells and actively secreted by cells under distress (25, 26). HMGB1 behaves like a cytokine and promotes inflammation when released (27, 28). Therefore, HMGB1 and IL-1 β released during CSD may take part in initiation of the inflammatory response. Subsequent NF- κ B activation in astrocytes may induce formation of cytokines, prostanoids, and inducible NO synthase-derived NO (as suggested by inhibition of MMA response by naproxen and CSD-induced COX2 and iNOS expression in glia limitans), which may be released to the sub-arachnoid space via glia limitans and, hence, stimulate trigeminal nerve endings around pial vessels (fig. S6). By promoting sustained headache, HMGB1 may thus serve to alarm the organism that the brain parenchyma has been stressed by CSD or CSD-like events. HMGB1 is most likely not the only mediator playing this role; other cytokines as well as cells (such as microglia) may also take part along the course of inflammatory response (29, 30). In contrast to mediators such as potassium and protons that are transiently released during CSD, activation of the parenchymal in-

flammatory pathways may provide the sustained stimulus required for sensitization of trigeminal nerve endings and lasting pain as suggested by suppression of long-lasting MMA vasodilatation, mast cell degranulation, and importantly, headache-like behavior by interrupting the inflammatory cascade at one of the steps (11).

We propose a previously unknown link between a noxious intrinsic brain event and activation of the trigeminal pain fibers, involving the opening of Panx1 megachannels on stressed neurons, subsequent activation of the inflammatory pathways, and transduction of this signal to the trigeminal nerves around pial vessels (fig. S6).

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Acknowledgments: We are grateful to M. A. Moskowitz for helpful discussions, K. Kilic and E. Lule for their expert help with the figures, and to A. Can for his help with confocal microscopy. This work was supported by the Turkish Academy of Sciences (T.D.), Hacettepe University Research Fund 08-D07-101-011 (Y.G.-O.), and the Brain Research Association (H.K.).

Supplementary Materials

www.sciencemag.org/cgi/content/full/339/6123/1092/DC1
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23 October 2012; accepted 30 January 2013
10.1126/science.1231897

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

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Prenatal infection and exposure to traumatizing experiences during peripuberty have each been associated with increased risk for neuropsychiatric disorders. Evidence is lacking for the cumulative impact of such prenatal and postnatal environmental challenges on brain functions and vulnerability to psychiatric disease. Here, we show in a translational mouse model that combined exposure to prenatal immune challenge and peripubertal 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 theories that mental illnesses with delayed onsets involve multiple environmental hits.

Prenatal maternal infection and postnatal exposure to psychological trauma are two environmental risk factors for developmental psychiatric disorders, including autism, schizophrenia, and bipolar disorder (1–4). In spite of their relatively frequent occurrence (5–7), both factors seem to have rather modest effect sizes

in large populations (4, 8, 9). 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 (9, 10). It has therefore been proposed that developmental stressors, such as

infection or traumatizing experiences, may unfold their neuropathological impact primarily in genetically predisposed subjects (11). 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 (12, 13). 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 (14). 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) (14). Offspring born to poly(I:C)-exposed or control mothers were then left undisturbed or exposed to variable and unpredictable stress during peripubertal development, a maturational period known to be highly sensitive to the disrupting effects of traumatizing events relevant to psychosis-related disease (15, 16). The stress protocol included five distinct stressors: (i) electric foot shock, (ii) restraint stress, (iii) swimming stress, (iv) water deprivation, or (v) repeated home cage 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 (14) (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 peripubertal 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 peripubertal 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.

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 peripubertal 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 (17). 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.

The adult behavioral abnormalities emerging after prenatal immune activation and peripubertal stress exposure are unlikely to be associated with

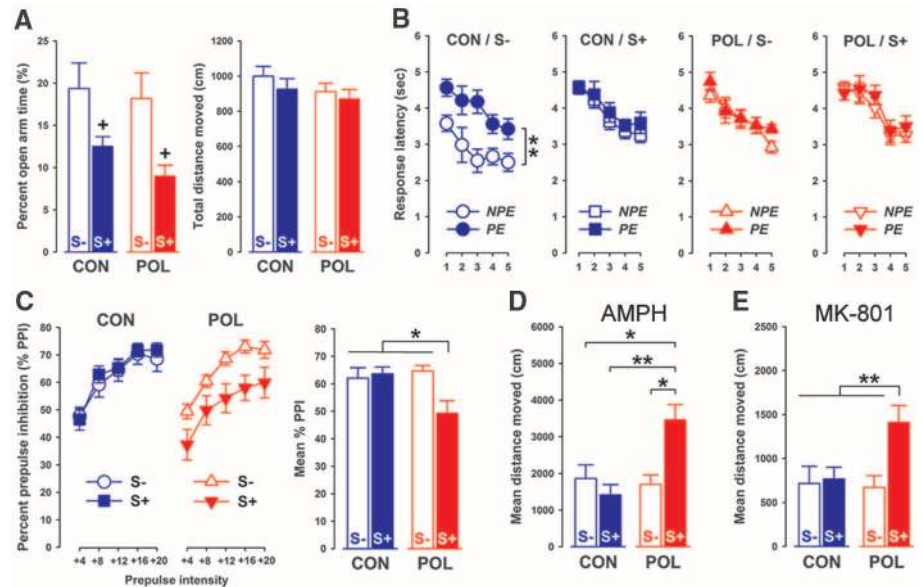


Fig. 1. Prenatal immune activation and peripubertal stress cause independent and synergistic pathological effects on adult behavioral functions. (A) Adult mice subjected to peripubertal stress (S+) display enhanced anxiety-like behavior in the elevated plus maze test (as indexed by the reduced time spent 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 peripubertal 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-), peripubertal 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 \pm SEM.

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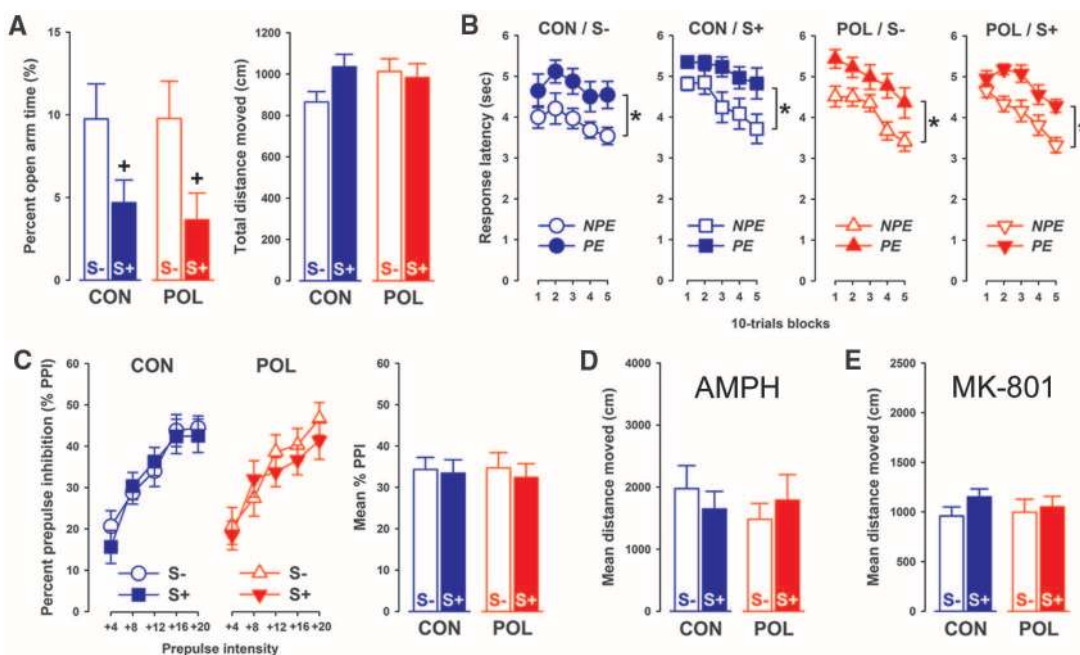
changes in the hypothalamus-pituitary-adrenal (HPA) stress-response system: Neither single nor combined exposure to the environmental adversities affected basal plasma levels of corticosterone (CORT), the main effector hormone of the HPA axis (fig. S6). The developmental stressors also did not affect 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 peripubertal stress: Prenatal immune activation was sufficient to increase the levels of dopamine (DA) in the

nucleus accumbens (NAc) independently of post-natal 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 peripubertal stress (Fig. 3A); this highlighted synergistic effects between the two adverse events in the precipitation of adult hippocampal DA imbalances.

Prenatal immune activation (at high intensity) and chronic stress exposure have individually been linked to the development of immune alter-

tations in the brain and periphery (18–20). Here, we elucidated whether initial exposure to prenatal immune challenge could change the offspring's neuroimmunological responses to peripubertal 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 (21) and show neuroanatomical abnormalities after intense prenatal immune challenge, including CNS immune changes (19). We also included a cortical control region (secondary motor cortex, MC) that is

Fig. 2. Short-term effects of single or combined exposure to prenatal immune activation and peripubertal 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 peripubertal 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 \pm SEM.

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 \pm SEM.

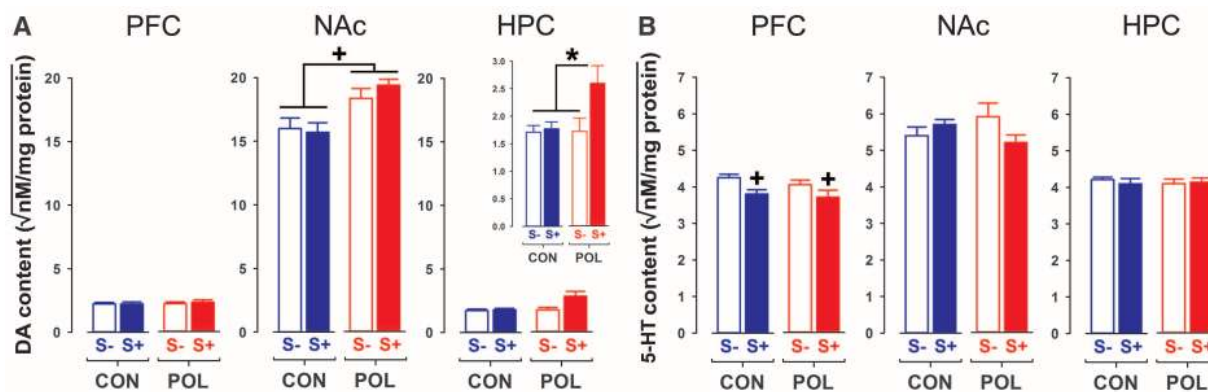


Fig. 3. Neurochemical parameters in adult offspring exposed to single or combined prenatal immune activation and peripubertal stress. **(A)** DA contents (nM/mg protein, square root-transformed) in the medial PFC, NAc, and HPC of adult (PND 70) offspring under the conditions described above. * $P < 0.05$, 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 \pm SEM.

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 (22). Unbiased stereological estimations of microglia immunoreactive for the calcium-binding protein Iba1 revealed that peripubertal 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 (22). Likewise, the two environmental factors did not change the hippocampal levels of the inflammatory molecules interleukin-1 β (IL-1 β), tumor

necrosis factor- α (TNF- α), and prostaglandin E₂ (PGE₂) in adulthood (fig. S8). Thus, single or combined exposure to prenatal immune activation and peripubertal 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 peripubertal 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-1 β 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 peripubertal age.

We performed additional molecular analyses to explore whether the transient microglia changes

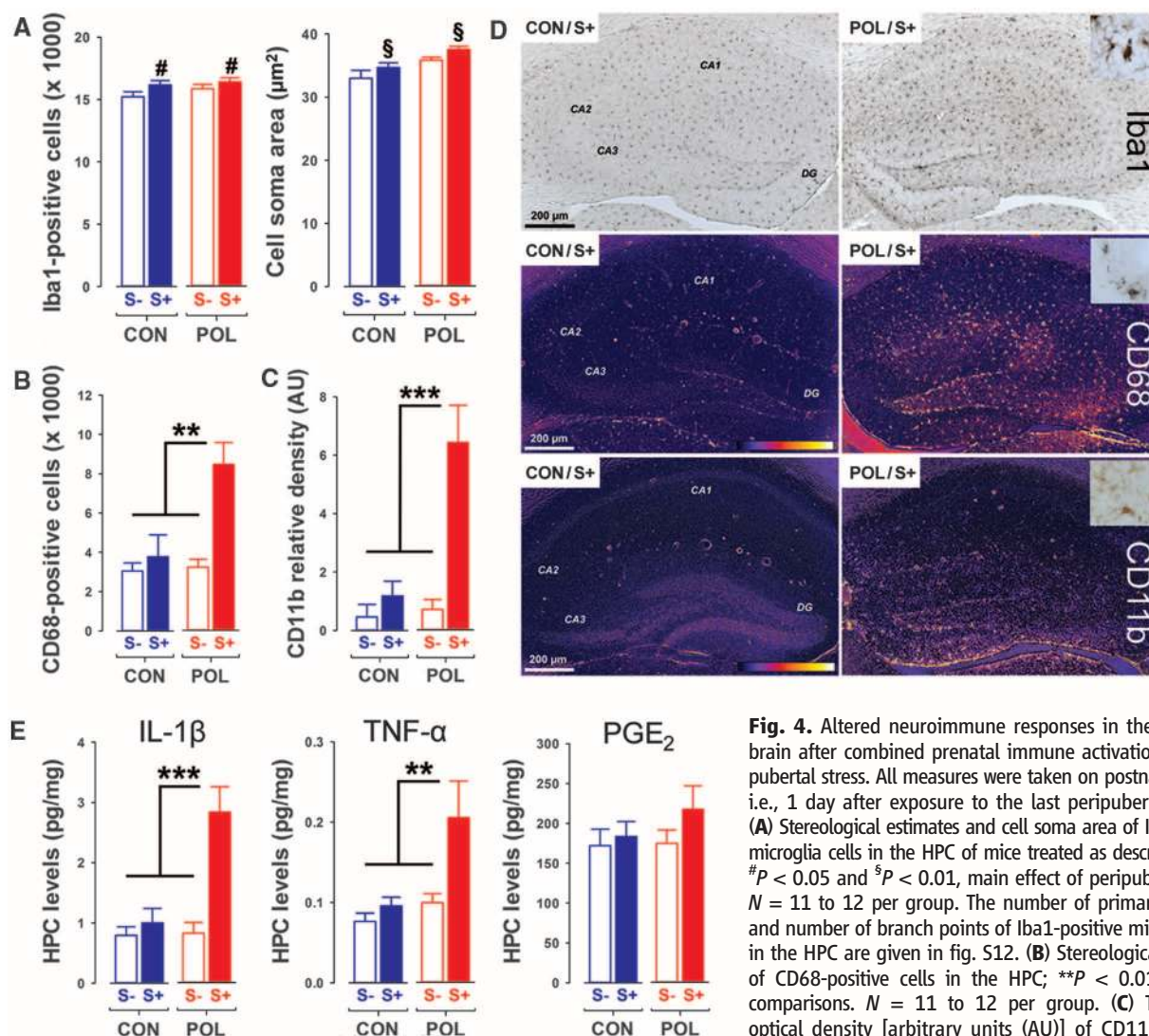


Fig. 4. Altered neuroimmune responses in the pubescent brain after combined prenatal immune activation and peripubertal stress. All measures were taken on postnatal day 41, i.e., 1 day after exposure to the last peripubertal stressor. (A) Stereological estimates and cell soma area of Iba1-positive microglia cells in the HPC of mice treated as described above. [#] $P < 0.05$ and [§] $P < 0.01$, main effect of peripubertal 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. (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-1 β , 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 \pm SEM.

in peripuberty would be associated with dysfunctional neuron-microglia inhibitory signaling, which, under nonpathological conditions, aims to restrain microglia from activation (23). 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 (23). Previous investigations in rats have shown that exposure to severe stress impairs the CD200 expression in the HPC (20). 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 peripubertal life. Prenatal

infection and peripubertal 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 (24). The transient neuroimmunological changes emerging in peripubertal 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 peripubertal brain maturation could offer a valuable strategy to prevent possible neuronal maladaptations and subsequent psychopathology after exposure to multiple environmental adversities.

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Acknowledgments: We thank L. Weber and T. Wirth for their technical assistance in the immunohistochemical and flow cytometric analyses; U. Stadlbauer and M. Labouesse for assisting behavioral experimentation and animal care; and the laboratory animal technician team, Schwerzenbach, for animal husbandry. We also thank J. Feldon for his past mentoring and W. Langhans for the continuous infrastructural support. This work was supported by the European Union Seventh Framework Programme (FP7/2007–2011) under Grant Agreement no. 259679 awarded to U.M., and by the Lundbeck Foundation, the Stanley Medical Research Institute, and a European Research Council Advanced Grant awarded to P.B.M., and partially by the German Research Foundation (FOR 1581, SCHE 341/19-1; FOR 751, EN 814/1-2). All authors declare that they have no conflicts of interest to disclose. R.W. is employed by F. Hoffmann–La Roche, Ltd.

Supplementary Materials

www.sciencemag.org/cgi/content/full/339/6123/1095/DC1
Materials and Methods
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1 August 2012; accepted 16 January 2013
10.1126/science.1228261