Perinatal exposure of rats to the HIV drug efavirenz affects medial prefrontal cortex cytoarchitecture


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abstract
Efavirenz (EFV) is used for antiretroviral treatment of HIV infection, and successfully inhibits viral replication and mother-to-child transmission of HIV during pregnancy and childbirth. Unfortunately, the drug induces neuropsychiatric symptoms such as anxiety and depressed mood and potentially affects cognitive performance. EFV acts on, among others, the serotonin transporter and serotonin receptors that are expressed in the developing brain. Yet, how perinatal EFV exposure affects brain cytoarchitecture remains unclear. Here, we exposed pregnant and lactating rats to EFV, and examined in the medial prefrontal cortex (mPFC) of their adult offspring the effects of the maternal EFV exposure on cortical architecture. We observed a significant decrease in the number of cells, mainly mature neurons, in the infra/prelimbic and cingulate cortices of adult offspring. Next, we found an altered cortical cytoarchitecture characterized by a significant reduction in deep- and superficial-layer cells. This was accompanied by a sharp increase in programmed cell death, as we identified a significantly higher number of cleaved Caspase-3-positive cells. Finally, the serotonergic and dopaminergic innervation of the mPFC subdomains was increased. Thus, the perinatal exposure to EFV provoked in the mPFC of adult offspring cell death, significant changes in cytoarchitecture, and disturbances in serotonergic and dopaminergic innervation. Our results are important in the light of EFV treatment of HIV-positive pregnant women, and its effect on brain development and cognitive behavior.

1. Introduction
For the treatment of HIV infection, antiretroviral therapy (ART) is used, reducing morbidity and mortality significantly by decreasing retroviral replication [1]. International guidelines for HIV treatment recommend the use of two first-line nucleoside reverse transcriptase inhibitors and a third drug, which should be either an enhanced protease inhibitor, an integrase strand transfer inhibitor, or a non-nucleoside reverse transcriptase inhibitor (NNRTI). Efavirenz (EFV) is a drug that falls within the last category [2–3]. EFV effectively reduces viral replication, displays a low degree of interaction with the other antiretroviral drugs, is low in costs, enables single daily dosing and has moderate side-effects which facilitates adherence to the treatment. Because of these characteristics, EFV is advised to be used by pregnant women to prevent the transfer of HIV from mother to child, decreasing the chance of infection to 5% [1,4–7].

During the prenatal period, EFV crosses the placenta freely [8] reaching the fetal blood. EFV is able to cross the blood-brain barrier [9]...
with potential effects on the developing brain. In the postnatal period, EFV is found in breast milk [10,11], indirectly reaching the fetal blood and brain, to possibly affect brain development as well. The potential teratogenic effects of EFV are still not well-described [2,12]. Prematurity, low birth weight and anomalies in the development of the neural tube have been reported when EFV was used by the mother [13–15]. Preliminary data from a pioneer study conducted in children with fetal EFV exposure showed that these children exhibit poorer school performance than children with fetal exposure to non-EFV-based antiretroviral regimens [16]. In addition, exposure of pregnant cynomolgus monkeys to EFV at the onset of gestation caused anencephaly and palatine cleft in 15% of the animals exposed at therapeutic EFV doses [1]. In rats, perinatal exposure to EFV led in the offspring to a delay in motor development as well as disturbances within the cytoarchitecture of the motor cortex [17].

EFV can bind as an agonist or antagonist to vesicular monoamine transporters, dopamine and serotonin (5-HT) transporters, and glutamate, GABA, muscarinic, and the 5-HT receptors 5-HT_1A, 5-HT_2A, 5-HT_2B, 5-HT_2C and 5-HT_6 [18,19]. EFV’s effect on 5-HT receptors and transporter is of particular relevance for brain development, since 5-HT functions as a neurotrophic factor responsible for controlling proliferation, migration, differentiation and cell death during fetal and early postnatal brain development [20–24]. Before the serotonergic raphe nucleus in the embryonic brain starts to produce 5-HT around embryonic day (E) 10.5, the embryo receives 5-HT from the placenta [25,26]. In rats, the dorsal and medial raphe nucleus, located in the brain stem, start to develop and send their fibers to the forebrain which at around E16.5 reach the medial prefrontal cortex (mPFC). Once the serotonergic fibers are able to supply 5-HT to all brain regions (around E18.5), the placenta stops the production of 5-HT [25,26]. It is known that selective pharmacological or genetic interference with components of the 5-HT signaling pathway in pregnant rodents leads to a variety of behavioural changes, including increased anxiety, depression-related behavior in the context of stress, decreased behavioral flexibility and deficits in social behavior [27–29]. These behavioral outcomes rely on prefrontal cortex functioning [30–32]. Furthermore, a prenatal pharmacological/genetic block of the 5-HT transporter (5-HTT) has been associated with an increase in 5-HT and dopamine innervation of the mPFC of embryos and pups, a disorganization of the cytoarchitecture of cortical layers [21,33], as well as a down/up-regulation of numerous serotonergic receptors in various cortical areas including the mPFC [34–36]. Thus, a critical question is whether perinatal EFV exposure affects the cytoarchitecture of the mPFC as well.

Here, our objective was to explore the effects of EFV-exposure throughout the gestational period and during the first postnatal week on the cytoarchitecture of the infralimbic, prelimbic and cingulate cortices of the rat mPFC, and to determine if any changes were maintained during adult life. We found that perinatal EFV exposure decreased the number of cells, in particular mature neurons, in all mPFC subareas studied and increased the expression of the apoptotic marker cleaved Caspase-3. We furthermore observed differences in the expression of layer markers, and serotonergic and dopaminergic innervation of the mPFC. Because EFV causes significant disturbances in cortical cytoarchitecture of the mPFC subareas, our results are important when considering EFV treatment of HIV-positive women during their pregnancy and lactating period.

2. Material and methods

2.1. Animals

All experiments were approved by the Animal Experimentation Committee of the Radboud University Medical Center Nijmegen, The Netherlands (ref no. 2012-236) and carried out in accordance with the Directive of the Council of European Communities (2010/63/EU). Male and female nulliparous Wistar rats weighing 185–215 g (Charles River, Cologne, Germany) were acclimated and housed together. After detection of the vaginal plug, at the first gestational day (GD1), pregnant females were randomly housed in pairs in Macrolon® type 3 standard cages in temperature-controlled rooms (21 °C ± 1 °C) under a 12-hour standard light/dark cycle (lights on at 7:00 a.m.) with food and water available ad libitum.

2.2. Drug treatment

Pregnant rats were randomly assigned to daily treatment with either EFV or vehicle from GD1 until postnatal day 7 (PND7). The drug solution was prepared by diluting the oral suspension of EFV (Stocrin suspension 30 mg/ml, Merck Sharp & Dohme, Haarlem, The Netherlands) with distilled water. As a vehicle, we used a 1% cellulose suspension (Genfarma BV, Maarsen, The Netherlands), enriched with additives from the EFV solution, consisting of medium chain triglyceride oil (Newpharma, Liège, Belgium) and strawberry and mint flavors (Lecoq NV/SA, Zonhoven, Belgium). The EFV or vehicle was given blindly by oral gavage in a volume of 5 ml/kg. A dose of 100 mg/kg was used, based on unpublished pilot work and previous work demonstrating plasma levels within the human therapeutic range (1.0–4.0 mg/l) [2]. Plasma EFV levels were measured on the fourth gestational day (GD4) 90 min after drug administration in a blood sample obtained via the tail and collected in Microvette CB 300 tubes containing EDTA (Sarstedt, Germany), and the sample was processed as described by Van de Wijer et al. [17]. At PND70, male offspring were sacrificed, and their brains removed for immunohistochemical studies.

2.3. Immunohistochemistry

The animals received a single intraperitoneal (IP) injection of sodium pentobarbital (200 mg/kg; Sigma-Aldrich, St Louis, Missouri, USA), causing deep anesthesia. They were perfused transcardially with cold phosphate buffered saline (PBS, pH 7.4), followed by 4% paraformaldehyde (PFA; Sigma-Aldrich) in PBS. Brains were rapidly removed and immersed in 4% PFA in PBS for 48 h at 4 °C. After 2 days, the brains were immersed in a 30% sucrose (Sigma-Aldrich) solution in PBS until they sunk, frozen on dry ice and stored at −80 °C. 16 μm sections were cut on a Microm Cryostat, mounted on Superfrost Plus slides (Thermo Fisher Scientific, Waltham, MA, USA), air dried and stored desiccated at −20 °C. After one hour in blocking buffer (BB; 1.7% normal goat serum (NGS; Thermo Fisher Scientific), 1.7% normal donkey serum (NDS; Thermo Fisher Scientific), 1.7% normal horse serum (NHS; Thermo Fisher Scientific), 0.1% l-lysine, 1% bovine serum albumine (BSA; WVR International, Amsterdam, The Netherlands, 1% glycine (Sigma-Aldrich) and 0.4% Triton X-100 (Merck, Darmstadt, Germany), the cryosections were stained immunohistochemically using the following primary antibodies: mouse anti-Cux1 (1:300, Abcam, ab242194, Cambridge, United Kingdom), rabbit anti-Tbr1 (1:1000, Abcam, ab31940), mouse anti-NeuN (1:500, Merck Millipore, MAB377, Bedford, MA, USA), rabbit anti-cleaved Caspase-3 (cl-Casp3, 1:500, Cell Signaling Technologies, ASP175, Danvers, MA, USA), rabbit anti-hydroxytryptamine (5-HT) (1:500, Sigma-Aldrich, SS545) and rabbit anti-tyrosine hydroxylase (TH) (1:500, Merck Millipore, AB152, Bedford, MA, USA), all diluted in BB. After overnight incubation, the slides were washed in PBS and incubated with the corresponding species-specific Alexa-conjugated secondary antibody (1:500, Thermo Fisher Scientific; A32732, AB32723) in blocking buffer for 30 min at room temperature. After washing in PBS, the sections were nuclear stained with DAPI diluted in PBS (1:1000, Thermo Fisher Scientific) for 15 min, washed extensively in PBS and embedded in 90% glycerol in PBS. For visualization, a Leica DMI6000B Automated high-content fluorescence microscope was used.
2.4. Quantification and analysis

All analyses were performed in a blind fashion on at least six sequential coronal sections of each mPFC subarea (infralimbic, prelimbic and cingulate), which were scanned for cell and fiber quantification. The counting area in the mPFC was obtained using a rectangle of 0.1 mm wide extending from the ventricular zone to the marginal zone, using Photoshop CS6 (Adobe, San Jose, California, USA). This rectangle was subdivided into 10 equal bins, in which bin 1 represents the deepest layer and bin 10 the most superficial layer. For counting of the cells, Photoshop CS6 tools were used and fiber lengths were measured using ImageJ software, including the NeuronJ plugin (National Institutes of Health, Bethesda, USA). Data were statistically analyzed by one-way ANOVA (α = 5%) followed by Tukey’s multiple comparisons test between groups using GraphPad Prism 6 (San Diego, California, USA). Data are expressed as means ± SEM.

3. Results

3.1. Perinatal exposure to EFV affects the cytoarchitecture of mPFC subareas

In a previous study, we found a clear effect of an EFV regimen during pregnancy and the lactating period on motor behavior and the cytoarchitecture of the motor cortex [17]. However, it is unclear whether this effect also occurs in other cortical structures that direct complex behavior. Therefore, we studied the effect of perinatal EFV exposure on prefrontal development, specifically the cytoarchitecture of various subdomains of the medial (m)PFC (i.e., the infralimbic, prelimbic and cingulate cortices; Fig. 1A) of adult (PND75) offspring. Compared to the vehicle-exposed group, we observed in rats perinatally exposed to EFV a significant reduction in the total number of DAPI-positive nuclei/cells in the adult infralimbic (p = 0.0065; Fig. 1B and C), prelimbic (p = 0.034; Fig. 1B and D), and cingulate cortex (p = 0.00085; Fig. 1B and E). This cellular reduction was distributed over the cortical width of all subdomains, but most apparent in the deeper layers (Fig. 1F, G, H). However, despite this significant cell loss, the cortical thickness between mPFC subdomains was not affected (data not shown).

To identify this reduced number of cells in the mPFC, we quantified the number of cells positive for the neuronal marker NeuN in animals perinatally exposed to EFV compared to the control group. We found a significant reduction in the total number of NeuN+ cells in various aspects of deep as well as superficial cortical layers of the infralimbic (bin1, p = 0.0071; bin2, p = 0.025; Fig. 1B and I), prelimbic (bin1, p = 0.0063; bin2, p = 0.012; bin3, p = 0.0060; bin4, p = 0.013; bin5, p = 0.016; bin6, p = 0.019; Fig. 1B and J) and cingulate cortex (bin1, p = 0.0022; bin2, p = 0.00091; bin3, p = 0.0018; bin4, p = 0.0016; bin5, p = 0.0011; bin6, p = 0.00016; bin7, p = 0.00014; bin8, p = 0.0060; bin9, p = 0.00044; Fig. 1B and I-K).

Using neocortex laminar/identity markers, we detected in EFV-exposed rats relative to control animals a significantly lower number of cells immunoreactive for the deep layer marker Trbl in all subareas of the mPFC (infralimbic, p = 0.0019; prelimbic, p = 0.017; cingulate cortex, p = 0.014; Fig. 2A-N). In addition, we found a significantly lower number of cells positive for the superficial layer marker Cux1 in all cortical layers of the infralimbic (p = 0.0083; Fig. 2B, I-N; Fig. 3A, B, E), prelimbic (p = 0.0013; Fig. 3C, F), and cingulate cortex (p = 0.0066; Fig. 3D, G). In a small population of cells in upper and deep layers, we identified double-positive Trbl+/Cux1+ cells [37] which showed in EFV-exposed versus vehicle-exposed rats a significant decrease in the deeper aspects of laminar distribution of mPFC prelimbic (bin2, p = 0.033; Fig. 2M) and cingulate cortex (bin1, p = 0.029; bin2, p = 0.030; bin3, p = 0.019; bin4, p = 0.030; bin5, p = 0.035; Fig. 2N). However, in the infralimbic cortex, a significant reduction of these double-labeled cells was found, not only in deeper but also in superficial layers (bin2, p = 0.029; bin5, p = 0.048; bin6, p = 0.0085; bin7, p = 0.00081; Fig. 2B, I, L). Together, the data suggest that perinatal EFV exposure alters the cytoarchitecture of mPFC subareas by reducing the number of neurons, along with an altered expression of deep and superficial cortical layer/identity markers.

3.2. Perinatal EFV exposure results in neuron death in mPFC subareas

Programmed cell death is a natural and critical process during brain development [38]. Various apoptotic signaling pathways are mediated by activation of, among others, serotonergic signaling [38–41]. In the prelimbic and cingulate cortex, we found a significant difference in the total number of mature apoptotic neurons between the EFV and control groups (p = 0.027; Fig. 4A-D). We observed a significant increase in cells positive for the apoptotic marker cleaved Caspase-3 in various aspects of the prelimbic (bin4, p = 0.040; bin5, p = 0.049; Fig. 4A, C, F) and cingulate cortex (bin1, p = 0.011; bin2, p = 0.048; bin4, p = 0.015; bin6, p = 0.023; bin8, p = 0.0039; bin9, p = 0.039; Fig. 4D, G) of adult animals prenatally exposed to EFV when compared to control animals. No differences were identified in the infralimbic cortex (p = 0.16; Fig. 4B, E). Interestingly, not all cleaved Caspase-3+ cells co-expressed NeuN (Fig. 4A). We conclude that perinatal EFV exposure results in cellular apoptosis of mature neurons in mPFC subareas of adult animals.

3.3. Perinatal EFV exposure causes disturbances in the serotonergic and dopaminergic innervation of mPFC subareas

Disturbances in serotonergic and dopaminergic innervation of the cortex during critical periods of brain development are associated with various brain disorders [42–43]. Both neurotransmitters are able to control important neurodevelopmental processes such as proliferation, migration, differentiation, programmed cell death and synaptic organization during the embryonic period, adolescence, and up to adulthood [41,44]. Therefore, we quantified the lengths of 5-HT+ and TH+ fibers in the infralimbic, prelimbic and cingulate cortices of adult animals perinatally exposed to EFV and compared this to fiber lengths in control animals. We found a significant increase in 5-HT+ fiber length in the more deeper layers of the infralimbic (bin2, p = 0.033; bin3, p = 0.021; bin4, p = 0.017; Fig. 5A, B, E), prelimbic (bin1, p = 0.016; bin2, p = 0.027; bin3, p = 0.037; Fig. 5C and F) and cingulate cortex (bin2, p = 0.031; bin3, p = 0.022; Fig. 5D and G).

We furthermore observed a significant increase in TH+ fiber length in various aspects of cortical layers of the infralimbic (bin3, p = 0.045; Fig. 6A, E), prelimbic (bin1, p = 0.039; Fig. 6A, F) and cingulate cortex (bin1, p = 0.010; bin2, p = 0.011; bin5, p = 0.0054; bin6, p = 0.018; Fig. 6A, G). Together, the data suggest that animals perinatally exposed to EFV show changes in serotonergic and catecholaminergic innervation of the subdomains of the mPFC and that these changes persist into adulthood.

4. Discussion

We have explored the question whether exposure of rats to EFV during the embryonic period and the first postnatal week results in adverse effects on mPFC development. Our results demonstrate that perinatal EFV exposure causes a significant decrease in the total number of cells, including mature neurons, in all subareas of the mPFC without affecting cortical thickness. Furthermore, EFV exposure results in disturbances in the cytoarchitecture as evidenced by a significantly reduced number of cells expressing Cux1 and/or Trbl1, markers of superficial and deep cortical layers, respectively. This may be due to the increased activation of apoptotic pathways as demonstrated by the increase of cleaved Caspase-3+ cells in all mPFC subdomains or an altered or delayed expression of the markers. In addition, we observed a significant increase in the serotonergic and catecholaminergic
innervation of the infralimbic, prelimbic and cingulate cortex of EFV-exposed rats. Together, the data suggest that perinatal EFV exposure causes prefrontal alterations at the cellular level in all subdomains of the mPFC and that these changes persist into adulthood.

The development of the PFC undergoes a number of stages. Following proliferation, cells progress to stages of neural migration, maturation, synapse formation, cell death, and network maturation [45–49]. All of these events are mediated by local neurotrophic factors and other external afferents, including serotonergic and catecholaminergic ones [21]. It has been demonstrated that 5-HT, via various receptors present on different neural progenitor cells, glial cells and Cajal-Retzius cells, can regulate specific events during cortical development, including neuronal maturation, excitability and programmed cell death [20,23,24,50]. The effects of EFV on the immature brain may be broad, since this drug binds as an agonist or antagonist to a number of receptors and transporters of the 5-HT signaling pathway as well as of other neurotransmitter systems [18,19]. This interaction of EFV with different serotonergic receptors and the transporter may underlie the disturbances in cytoarchitecture, increased programmed neuronal death and increased serotonergic and dopaminergic innervation of the mPFC found by us. There is furthermore an interrelationship between the developing serotonergic and catecholaminergic systems which could have enhanced the effects observed during the various steps of development [65,66]. Recently, we found that rats exposed perinatally to EFV showed an increase in serotonergic innervation of the motor cortex [17]. A similar effect was found in 5-HTT knockout (5-HTT−/−) rats [21,33]. In addition, a disorganised cortical cytoarchitecture was found in young 5-HTT−/− rats, as demonstrated by variances in layer/specific Satb2, Cux1 and Tbr1 expression in mPFC subdomains [21,33]. Yet, answering the questions how EFV interferes with the normal course of development and how this influences other developing systems necessitates further longitudinal studies.

Interference of EFV with the 5-HT signaling pathway may explain the increase we found in serotonergic axonal length in the mPFC. Thus, the serotonergic system may well have been the major target for the effects of EFV we observed. Previous studies have demonstrated that the 5-HTT−/− rats display a delay in motor development [51], comparable to the effects of perinatal EFV exposure in rats [17]. The knockout rats furthermore display increased affective behavior [52], lower levels of social interaction [53], and a decrease in memory for learning and memory tasks [54]. The mPFC plays a critical role in these behaviors [55], and EFV exposure may have affected its connectivity with other brain regions involved in these functions.
Fig. 2. Perinatal EFV exposure affects specific subsets of neurons in mPFC subareas. (A) Immunostaining of the infralimbic subarea of the mPFC of EFV-exposed (n = 5) and control (n = 5) rats showing the deep layer marker Tbr1 (red). Scale bar: 50 µm. (B) Co-immunostaining of the infralimbic subarea of the mPFC of EFV-exposed (n = 5) and control (n = 5) rats showing Tbr1 (red) and Cux1 (green). Significant decrease in the number of Tbr1+ cells in the infralimbic (C), prelimbic (D) and cingulate cortex (E) of the mPFC of EFV-exposed rats compared to that in the control rats. Significant decreases in the number of Tbr1+ cells in several aspects of the infralimbic (F) prefrontal (G) and cingulate cortex (H) of the mPFC of the EFV-exposed rats compared to that in the control rats. Significant decrease in the total number of Tbr1+/Cux1+ cells in the infralimbic (I) and cingulate cortex of EFV-exposed versus control rats and not in the prelimbic cortex (J). Differences in the number of Tbr1+/Cux1+ cells in several aspects of superficial and deep cortical layers of the infralimbic (L), prelimbic (M) and cingulate cortex (N) of EFV-exposed rats compared to that in control rats. Data show mean number of cells ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001, analyzed using one-way ANOVA (α = 5%). EFV: efavirenz; Ctrl: vehicle. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. Perinatal EFV exposure affects the expression of superficial layer marker Cux1 in mPFC subareas. (A) Immunostaining of the infralimbic cortical subarea of the mPFC of EFV-exposed (n = 5) and control (n = 5) rats for the superficial layer marker Cux1. Scale bar: 50 µm. (B-D) Significant decrease in the number of Cux1+ cells in the total area of the infralimbic, prelimbic and cingulate cortices of EFV-exposed rats compared to that in the control rats. (E-G) Significant decrease in the number of Cux1+ cells in both cortical superficial as well as deep layers of mPFC subdomains of the EFV-exposed rats compared to that in the control rats. Data show mean number of cells ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001, analyzed using one-way ANOVA (α = 5%). EFV: efavirenz; Ctrl: vehicle.
neutral information, but an increase in memory for valenced (rewarding, aversive) information [54,55]. Whether perinatal EFV exposure causes a similar behavioral profile remains to be investigated.

A line of investigation to be targeted in future research involves examining the effect of perinatal EFV exposure on other brain-specific biomolecules such as enzymes in the cholesterol pathway. Cholesterol is unable to cross the blood-brain-barrier and is produced locally [67]. It has recently been shown that EFV elevates the in vitro as well as the in vivo activity of the brain-specific cholesterol metabolism enzyme cytochrome P450 46A1 (CYP46A1), thereby increasing the levels of the cholesterol metabolite 24(S)-hydroxycholesterol (24-HC) [68-71]. During development 24-HC is a potent modulator of NMDA receptors which are expressed on radial glial cells [72,73]. Of note, most neurotransmitter systems, including serotonin, also increase CYP46A1 activity, and during development EFV can bind directly to CYP46A1 as well as to components of the serotonin pathway.

A limitation of this study is that we used a daily concentration of EFV (100 mg/kg) that produced plasma levels within the therapeutic range in humans (1.0–4.0 mg/l) [56]. However, a dose in the therapeutically similar range led to neurotoxic effects in rats [57,58], because the neurotoxic metabolite accumulates in brain tissue [59]. Further, our animals were exposed to EFV during the perinatal period and first postnatal week, and the analyses were performed in adult life, allowing compensatory changes to take place during this developmental period, such as reduction of cell death or induction of proliferation.

A number of studies have reported on the neurological effects of ART during pregnancy [73,74]. As many of these treatment regimens differ between low-income countries or even between regions, studying the effects of the various individual compounds on the offspring’s brain development is difficult. EFV is the preferred NNRTI in ART regimens in many low-income countries in Africa [3]. EFV is somewhat longer on the market than for example the second-generation NNRTI Rilpivirine, however similar neurological side effects have already been reported [1]. EFV and Rilpivirine differ in their ability to cross the blood-brain-barrier though [2], which may be important in view of their presence during brain development. Considering the results of the DOLPHIN-study, it is likely that dolutegravir will become the first line therapy although neurological effects have been reported there as well [76,77]. Clearly, more studies are necessary on the safety of NNRTIs during pregnancy and their effect on brain development.

The high efficacy of EFV in inhibiting cross-infection between mother and embryo together with its low price still makes EFV the first drug of choice for the treatment of HIV in pregnant women [1-4]. However, this may come at a cost. In vitro, EFV can alter the physiological function of neuronal and glial cell lines [60-63] as well as the proliferative state of neural stem cells [64]. In rats, perinatal exposure to EFV caused a developmental delay and a clear loss of neurons in the limbic and cingulate cortices of their offspring [57,58]. Hence, the mPFC is responsible for cognitive functions, language, comprehension, decision making, planning, memory and attention, these findings may have implications for the social and cognitive functioning of adult offspring of expectant women exposed to EFV. This is of great importance when considering EFV as the drug of choice for treating pregnant, HIV-infected women.

CRediT authorship contribution statement

L.P. Garcia: Methodology, Formal analysis, Visualization, Investigation, Funding acquisition, Writing - original draft. L. Van de Wijer: Conceptualization, Methodology, Validation, Writing - review & editing. S.I. Hanswijk: Project administration, Methodology. J. Rando: Project administration, Methodology. J.S. Witteveen: Validation, Visualization, Writing - review & editing. A. Middelman: Project administration, Methodology. R. ter Heine: Resources.

Fig. 4. Perinatal EFV exposure results in cell death in mPFC subareas. (A) Immunostaining for NeuN (green) and cleaved Caspase-3 (clCasp3, red), counterstained with DAPI (blue) in the prelimbic subarea of the mPFC of EFV-exposed (n = 5) and control (n = 5) rats. Scale bar: 50 µm. (B-D) Quantification of the total number of clCasp3+/NeuN+ neurons in the mPFC subdomains of EFV-exposed rats compared to that in the control rats. (E-G) Significant increase in clCasp3+ cells per area in aspects of superficial and deep layers of the prelimbic and cingulate cortices, but not in the infralimbic cortex of EFV-exposed versus control rats. Data show mean number of cells ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001, analyzed using one-way ANOVA (α = 5%). Ctrl: vehicle; EFV: efavirenz. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Fig. 5. Perinatal EFV exposure causes disturbances in serotonergic innervation of mPFC subareas. (A) Immunostaining showing 5-HT⁺ fibers (white) in deep and superficial layers of the infralimbic cortex in EFV-exposed (n = 5) and control (n = 5) rats. Scale bar: 50 µm. (B-D) Quantification of serotonergic fiber length in the total swatch area in mPFC subareas of EFV-exposed rats compared to that in the control rats. (E-G) A significant increase in 5-HT⁺ fiber length in deep layers of the infralimbic, prefrontal and cingulate cortex area of EFV-exposed rats compared to that in the control rats. Data show mean length ± SEM, *p < 0.05 analyzed using one-way ANOVA (α = 5%). EFV: efavirenz; Ctrl: vehicle; 5-HT: serotonin.

Fig. 6. Perinatal EFV exposure causes disturbances in dopaminergic innervation of mPFC subareas. (A-G) Immunostaining showing TH⁺ fibers in infralimbic, prefrontal and cingulate cortex in EFV-exposed (n = 5) and control (n = 5) rats. Scale bar: 50 µm. (B-D) Quantification of the total fiber length in mPFC subareas of the EFV rats compared to that in the control rats. (E-G) A significant increase in TH⁺ fiber length in some aspects of deep layers of the infralimbic, prefrontal and cingulate cortex of EFV-exposed versus control rats was found. Data show mean length ± SEM, *p < 0.05 analyzed using one-way ANOVA (α = 5%). EFV: efavirenz; Ctrl: vehicle; TH: tyrosine hydroxylase.
Methodology, Writing - review & editing. Q. de Mast: Writing - review & editing. G.J.M. Martens: Supervision, Resources, Validation, Writing - review & editing. A.J.A.M. van der Ven: Conceptualization, Writing - review & editing. A.F.A. Schellekens: Conceptualization, Writing - review & editing. J.R. Homberg: Conceptualization, Methodology, Supervision, Fundraising acquisition, Validation, Writing - review & editing. S.M. Kolk: Conceptualization, Methodology, Supervision, Resources, Funding acquisition, Data curation, Writing - review & editing.

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Contributors

The study was designed and directed by J.R.H and S.M.K. Immunohistochemical experiments and analyses were performed by L.P.G. The drug treatment and animal care were performed by L. van de W., S.H., A.F.M., R. ter H. and J.R.H. All authors reviewed by J.S.W, S.H. and L.P.G. The manuscript was written by L.P.G. and S.M.K., extensively reviewed by L. van de W., Q.de M., A.J.A.M. van der V., G.J.M.M., A.F.A.S. and J.R.H. All authors refined and approved the final version of it.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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