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Prenatal corticosteroids modify glutamatergic and GABAergic synapse genomic fabric: Insights from a novel animal model of infantile spasms

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Summary

Prenatal exposure to corticosteroids has long-term postnatal somatic and neurodevelopmental consequences. Animal studies indicate that corticosteroid exposure-associated alterations in the nervous system include hypothalamic function. Infants with infantile spasms, a devastating epileptic syndrome of infancy with characteristic spastic seizures, chaotic irregular waves on interictal electroencephalogram (EEG; hypsarhythmia) and mental deterioration, have decreased concentrations of adrenocorticotrophic hormone (ACTH) and cortisol in cerebrospinal fluid strongly suggesting hypothalamic dysfunction. We have exploited this feature to develop a model of human infantile spasms by using repeated prenatal exposure to betamethasone and postnatal trigger of developmentally relevant spasms with N-methyl-D-aspartic acid (NMDA). The spasms triggered in prenatally primed rats are more severe compared to prenatally saline-injected ones and respond to ACTH, a treatment of choice for infantile spasms in humans. Using autoradiography and immunohistochemistry, we have identified a link between the spasms in our model and hypothalamus, especially the arcuate nucleus. Transcriptomic analysis of the arcuate nucleus after prenatal priming with betamethasone but before trigger of spasms indicates that prenatal betamethasone exposure down-regulates genes encoding several important proteins participating in glutamatergic and GABAergic transmission. Interestingly, there were significant sex-specific alterations after prenatal betamethasone in synapse-related gene expression but no such sex differences were found in prenatally saline-injected controls. A pair-wise relevance analysis revealed that, although the synapse gene expression in controls was independent of sex, these genes form topologically distinct gene fabrics in males and females and these fabrics are altered by betamethasone in a sex-specific manner. These findings may explain the sex differences in both normal behaviour and occurrence and severity of infantile spasms. Changes in transcript expression and their coordination may contribute to a molecular substrate of permanent neurodevelopmental changes (including infantile spasms) found after prenatal exposure to corticosteroids.

Prenatal corticosteroids as a factor in neurodevelopmental disorders

Every year, hundreds of thousands of women are treated with synthetic glucocorticoids during late pregnancy to improve the survival of neonates if there is a threat of premature delivery. Additionally, many women during their pregnancy are subjected to severe prenatal stress associated with elevated levels of endogenous corticosteroids, which are capable of overcoming enzymatic barrier of the placenta and affecting the foetus (1–6). Repeated increases in maternal corticosteroids (natural or synthetic) may have long-term postnatal deleterious effects for the offspring (3–12). These effects in exposed newborns are not only somatic (such as decreased birth weight or adrenal suppression), but include neurodevelopmental reprogramming with differential sex-specific outcomes depending on corticosteroid species (5, 13–17). For example, newborns of mothers receiving repeated antenatal courses of synthetic corticosteroids, a treatment which is life-saving for prematurely born babies as it accelerates their lung development, often demonstrate a transient hypertrophic cardiomyopathy (18). While this effect quickly diminishes, other effects of antenatal corticosteroid treatment are long-term, even permanent. Administration of multiple courses of prenatal betamethasone is associated with increased occurrence of neurodevelopmental disorders (15) and mortality, decreased foetal growth, birth weight and head circumference, as well as adrenal suppression at birth (11, 19). Similarly, the long-term stress during pregnancy (featuring prolonged elevated maternal corticosteroid levels) affects the child's development (late or poor walking, speech deficits) and behaviour (restlessness or fretfulness, poor interpersonal skill development) (20). Additional studies indicate that adverse effects of prenatally increased corticosteroid levels encompass impairments in distinct brain structures and neurotransmitter systems (i.e., glutamate and gamma-aminobutyric acid: GABA) that can lead to anxiety (21), impaired cognition (22), social behaviours (12) including autistic traits (23), and increased seizure susceptibility (24, 25). Children exposed prenatally to multiple corticosteroid courses display greater distractibility, attention deficits, hyperactivity and social behaviour problems compared to either untreated children or those exposed only to a single course (12, 22, 26). Similarly, *in utero* treatments with dexamethasone for congenital adrenal hyperplasia have negative effects on verbal working memory (26, 27). Alterations in these behavioural patterns may predispose to the development of depression, schizophrenia, autism and epilepsy (28).

Imprinting effects of prenatal corticosteroids on gene expression

Clinical and experimental findings indicate that prenatal exposure to excess corticosteroids may lead to long-term (perhaps permanent) reprogramming of the brain (15, 16, 29, 30), including control systems for anxiety (31), cognition, social behaviors and seizure susceptibility (32–34). The effects are brain region-specific, depend on timing of the impact, corticosteroid species, sex and genetic background (17).

Several molecular mechanisms responsible for brain reprogramming have been proposed based on observed changes in expression of genes encoding different receptors (35). Owing to transcriptomic networks (36, 37), by which expressions of individual genes are tied to each other, alteration of one key gene has ripple effects on many others (as observed in knockouts (e.g.: (37, 38)) and knockdowns (e.g. (36, 39)). Moreover, transcriptomic networks may even cross the cell boundaries (40, 41) mediated by intercellular signalling (42). Together, these findings indicate that intercellular communication integrates individual cells within multicellular structures so that transcriptomic events in one cell type modulate gene expression in others (43). Therefore, regulation of these receptors may change the organizational transcriptomic principles of the brain (44) with consequences on the dynamics and outcomes of multiple functional pathways. For instance, natural corticosteroids (corticosterone in the rat) can act either on mineralocorticoid (high affinity)

or glucocorticoid receptors (low affinity) (45, 46). In addition to the Hypothalamo-Pituitary-Adrenal (HPA) axis control structures, glucocorticoid and mineralocorticoid receptors are localized in limbic neurons, including hippocampal CA1 region, dentate gyrus granule cells, amygdala, neocortex and brain stem (47–50). Synthetic corticosteroids (betamethasone or dexamethasone) bind with high affinity specifically to glucocorticoid but not mineralocorticoid receptors (51). Activation of glucocorticoid receptors by corticosteroid binding is followed by receptor phosphorylation and dissociation from the complex of heat shock proteins (52, 53). The activated receptor-ligand complex translocates to cell nucleus and forms homodimers or monomers. The homodimers bind to hormone response elements in the DNA in synergy with a co-activator (54) resulting in activation of gene transcription (55). On the other hand, activated glucocorticoid receptor-ligand monomers interfere with transcription factors in a protein-protein interaction (53, 56) resulting in repression of gene transcription (55). All of these effects suggest strong epigenetic influence of the glucocorticoid action. Additionally, posttranscriptional and even posttranslational effects of corticosteroids have been described (57–59). Since mechanisms of action of corticosteroids are inherently associated with regulation of gene transcription, the gene expression can be reprogrammed by corticosteroids in a long-term manner.

Infantile spasms

Our research is focused on infantile spasms (West syndrome; epileptic spasms) (60, 61), a devastating neurodevelopmental epileptic syndrome of infancy. The term “infantile spasms” in the text below will be always referring to the human syndrome. The syndrome consists of a triad of spastic seizures, interictal diffuse EEG abnormalities (hypsarhythmia) and mental deterioration (60). Further characterization of infantile spasms involves ictal EEG electrodecrement (62–64). Every year in the US, there are 1500 new cases of infantile spasms, i.e., children who are newly diagnosed and require treatment. Infantile spasms have a strong developmental component. They occur between 3–12 months of age with incidence of approximately 1 per 3225 live births and with a slight predominance (60%) in males (65, 66). Worldwide, number of new cases of infantile spasms exceeds 45 000 children per year. Infantile spasms are associated with a significant mortality. A large study (67) demonstrated that about 30% of the patients with infantile spasms died during the follow-up period, many in the first three years of life. Among survivors, 45% were mentally retarded. While known aetiology and good response to the initially tried medication in infantile spasms predict better outcomes, many patients with infantile spasms continue to have epilepsy and cognitive disabilities long-term (68, 69).

Medical treatment options are different for infantile spasms than for any other epilepsy syndrome. There are two drugs with some evidence of efficacy: ACTH (62), and vigabatrin (63, 64), both approved by the United States Food and Drug Administration (FDA). ACTH, in addition to an enormous cost burden (70), has significant side effects, which are very serious in up to 43% of cases (71). Children treated with ACTH usually develop cushingoid obesity, irritability, arterial hypertension, electrolyte imbalance, gastric ulcer, growth retardation, cardiomyopathy and immunosuppression (72). In fact, in about 30% of patients who die by age of three, a common reason is the complications of ACTH therapy (73–76). Vigabatrin shows similar effectiveness as ACTH in short-term but it lags in effectiveness after one-year follow up (63, 64). In addition, vigabatrin has a significant risk for development of permanent concentric visual field defects (76). The major goals of infantile spasms therapy are to achieve a control of spasms as soon as possible using medications and treatment strategies with the least side effects. However despite the available treatment, up to 85% of patients become mentally impaired and 67% suffer from intractable epilepsy (65, 77–79). In these patients, usually new seizure types (including partial seizures with or without secondary generalization) emerge (67, 80–83) already during the childhood (84, 85).

There are two large etiology-based subgroups in infantile spasms: (1) Symptomatic infantile spasms, which are associated with detectable severe brain pathology and thus, a relatively poor prognosis, and (2) the cryptogenic/idiopathic group, which involves some type of suspected but yet unknown brain impairment but it has better prognosis if therapy is initiated early (61, 73).

Although the specific types of brain impairment in the cryptogenic/idiopathic groups of infantile spasms is unknown, because the infantile spasms occur early postnatally, the timing of the impact is suspected to fall into either prenatal or perinatal period. Additionally, infantile spasms are frequently associated with autism, another neurodevelopmental disorder (73, 86–88), which is also linked to early developmental impairments (15). For example, studies in Iceland found that about 35% of children with infantile spasms were also diagnosed with autism spectrum disorders (89, 90).

Hypotheses of infantile spasms origin

Infantile spasms represent a significant clinical problem with regards to treatment and long-term outcome. During the critical developmental window, in which infantile spasms occur, both spasms and their currently available treatments (with their attendant adverse effects) can contribute to long-term sequelae of cognitive impairments and autistic behaviours. The search for both novel effective treatments of infantile spasms and for mechanisms involved in the pathology of spasms and associated cognitive impairments requires the use of appropriate animal models (91).

Several interesting hypotheses have been presented in the past on the origin and pathogenesis of infantile spasms. Some of those hypotheses can be linked to current animal models of infantile spasms. Some hypotheses still lack a corresponding model. Yet, all these hypotheses bring interesting insights into complexity of the syndrome.

a. Serotonergic hypothesis

Based on FDG PET studies, Chugani (92) proposed that patients with infantile spasms have prominent alterations in the interaction between neocortex and subcortical areas (especially the brain stem). First, hypometabolic foci were found in the neocortex corresponding interictally to the areas with hypersarhythmia during interictal EEG recordings. Second, there were areas of hypermetabolism in the brain stem and in GABAergic basal ganglia. Thus Chugani proposed that infantile spasms are triggered focally in the neocortex and develop into generalized seizures by activation of the brain stem and basal ganglia. In the brain stem, he suggested that raphe nuclei may have a critical role in the propagation (generalization) of spasms because of their abundant projections throughout the brain. Raphe nuclei indeed connect to basal ganglia (striatum) as well as to the hypothalamus (arcuate nucleus) (93). The involvement of serotonergic raphe nuclei is supported by PET studies using α - $[^{11}\text{C}]$ methyl-L-tryptophan (AMT) showing significant cortical serotonergic hyperinnervation in patients with infantile spasms (92). Biochemical data of tissue obtained after surgery in patients with symptomatic infantile spasms associated with tuberous sclerosis suggest that such increase in AMT uptake may represent a shift in tryptophan metabolism from kynurenic to quinolinic acid, a compound with a strong convulsant properties mediated by activation of NMDA receptors (92). Pathological activation of NMDA receptors in infantile spasms has been also proposed previously (94).

The involvement of cortex-brainstem interaction in infantile spasms has also been entertained by others hypothesizing that increased excitability in motor and arousal systems of the brain stem is linked to inefficient descending cortical regulation (95).

b. Brain stress hypothesis (96)

Infantile spasms may occur in the context of an insulted and stressed developing brain (97). Many etiologies of infantile spasms lead to activation of stress responses including the release of CRF, which is a powerful convulsant in the immature brain (31, 98, 99). The convulsant effect is attributed to developmental abundance of CRF receptors in the brain (100, 101). Data from patients with infantile spasms also suggest involvement of the stress response: Several groups reported reduced ACTH levels in patients with infantile spasms (99, 102, 103) suggesting that reduced endogenous ACTH levels may be associated with increased CRF levels in those patients. Furthermore this hypothesis suggests that some of the effects of ACTH are directly mediated via melanocortin receptor system in the brain besides its hormonal activation of adrenal steroids (96). Direct central effects of ACTH may account ACTH efficacy even in patients with infantile spasms with ablated cortisol production (104, 105). Finally, ACTH is a very poor anti-seizure drug in models of seizures (106, 107).

c. The 11-deoxycorticosterone (DOC) hypothesis (107)

This hypothesis covers the anti-spasms effects of ACTH and anticipates that ACTH treatment leads to increased levels of DOC (also produced by adrenals) in addition to cortisol and considers activation of steroidogenesis in the adrenals as the major factor in the ACTH effects against infantile spasms. The attractive possibility here includes the conversion of DOC of the peripheral origin to a neurosteroid tetrahydro-DOC (THDOC) in the brain. Both DOC and THDOC have strong anticonvulsant properties (107, 108).

Search for animal model of infantile spasms

Original studies especially by Baram contributed significantly to the explanation of the involvement of hypothalamus-pituitary-adrenal (HPA) axis in seizure susceptibility in infants with possible relation to infantile spasms based on the findings of decreased ACTH and corticosteroid levels in the cerebrospinal fluid (CSF) and relevant effective treatments of infantile spasms (97, 98, 109, 110). However, until recently, there were no validated animal models of infantile spasms to further explore the underlying mechanisms of this epilepsy syndrome (91). Within the past five years several new models have emerged (111) (Table 1). While all these models yield interesting insights into different aspects of pathogenesis of infantile spasms, most of them lack an important validating step (112), which is the therapeutic response to ACTH.

Our quest for an animal model of infantile spasms began more than 20 years ago, when we found that systemic administration of N-methyl-D-aspartic acid (NMDA) in rats during development induced very peculiar spastic seizures (flexion spasms, emprosthotonus) (113), which were semiologically similar to human infantile spasms (114) and were associated with attenuation of EEG during the spasms (similar to ictal electrodecrement in humans with infantile spasms). Importantly, the occurrence of spasms was restricted to early development, we recorded the spasms only in 7–18 days old rat pups (Figure 1), the age relevant to human “infancy” (115, 116) (Table 2). Older animals developed semiologically different type of seizures following NMDA administration. This restricted developmental occurrence of flexion spasms in rat pups corresponds to human infantile spasms, which can be diagnosed/observed only during infancy but later on the seizures change their character and phenotype. To our disappointment, detailed pharmacological analysis of these spasms did not show sensitivity to corticosteroids (117), though we have seen suppression of spasms by pyridoxine (B6 vitamin), which is frequently used for treatment of infantile spasms in Japan (118–120). Interestingly, early developmental experience of NMDA-induced spasms was associated with long-term cognitive impairments and increased susceptibility to

provoked seizures in adulthood (121) reflecting mental impairments in children with infantile spasms as well as their susceptibility to develop other seizure types with continuing development (77).

Although, the original model of NMDA-induced spasms in developing rats carries certain features of human infantile spasms, it does not respond to corticosteroids (or ACTH), which represents a serious shortcoming. Therefore, we have been searching for additional features of infantile spasms that would help to adjust the model accordingly. Clinical studies have determined that the patients with severe infantile spasms have decreased ACTH and corticosteroid levels in the CSF (Table 3) (99, 102, 103, 122–125). Not only does this finding indicate that the very specific sensitivity of at least some patients with infantile spasms to ACTH or corticosteroids may be due to insufficient endogenous levels of these hormones, it also strongly suggests an impairment of the HPA axis function in these patients (97, 99). As mentioned above, infantile spasms develop very early after birth, indicating impairments arising from either prenatal or perinatal impacts. Therefore, we searched for models in rodents, which include impacts during prenatal (also perinatal) period resulting in similar decreases of the CSF hormones as found in infants with infantile spasms. Experimental studies investigating effects of prenatal administration of synthetic corticosteroids in guinea pigs suggested that administration of synthetic corticosteroids during second half of pregnancy should decrease, at least temporarily, the levels of ACTH and corticosterone in the guinea pig fetuses (126).

Based on this information, we have combined repeated prenatal exposure to betamethasone on gestational day 15 (G15) followed by a trigger of spasms using NMDA on postnatal day 15 (P15) (Figure 2). As a result of this approach, in 2007, we reported a novel model of cryptogenic infantile spasms in infant rats (32, 127–129) and also validated many features of the model similar to human condition (25). This new model represents a clinically relevant animal model of infantile spasms because: (1) Phenotype is developmentally specific and semiologically similar to human infantile spasms, including clustering of spasms. The phenotype of spasms persists only up to 21 days of age in rats (correlating with human infancy and early childhood). (2) EEG features correspond well to human infantile spasms, with interictal high amplitude asynchronous waves similar to hypsarhythmia and ictal EEG suppression similar to electrodecrements. See Figure 3 for comparison. (3) The spasms (in repeated bouts) are associated with cognitive impairments. (4) The spasms in this model respond to treatment with ACTH (in a prospective randomized trial), long-term treatment with corticosteroids (methylprednisolone) as well as to vigabatrin (25).

Importantly, to better reflect human situation we have demonstrated that the ACTH treatment is effective also in animals already experiencing the spasms prior to the treatment initiation using a prospective randomized trial (Figure 4) (25). We have modified the model in such a way that prenatally betamethasone exposed rats were subjected to a trigger of spasms using NMDA on P12. After the bout of spasms dissipated, the pups were randomly divided into two groups (assuring an identical latency to onset and number of spasms in each group; Figure 4A, B): one that received treatment with ACTH (2 doses of 0.3 mg/kg on P12 after the spasms, and 3 doses on both P13 and P14) and a second group receiving the vehicle instead. On P13, in between the ACTH/vehicle treatments, the rats were subjected to an additional bout of spasms. Finally, another bout of spasms was triggered on P15, at least 12 hours after the last ACTH treatment. First, we found that treatment with ACTH ensured 100% survival in the treated group compared to 56% survival in the vehicle group (Figure 4C). Further, on P15, ACTH treatment significantly decreased number of spasms per subject (Figure 4E), an effect consistent with ACTH effects in infantile spasms in humans. However, there was no effect of chronic treatment with ACTH on the latency to onset of spasms measured from the trigger (Figure 4D).

The serotonergic hypothesis (92) significantly connects to our model: Using 2-DG autoradiography, we demonstrated activation of raphe nuclei and its connected structure during the flexion spasms (32). Raphe nuclei in the rat are significantly and reciprocally linked to the hypothalamic arcuate nucleus (93), which also showed activation during the flexion spasms (32). A side metabolite of kynurenic acid pathway - quinolinic acid - activates NMDA receptors, which is relevant to our NMDA-trigger of spasms (32). The brain stress hypothesis (97) also relates to our model. Prenatal betamethasone exposure is consistent with impaired HPA regulation, specifically with elevated CRF expression in the HPA control centers and decreased glucocorticoid receptor expression (130). In addition, to confirm that the priming effect of prenatal corticosteroids exposure is a general feature and does not apply only to the synthetic steroids, we expanded the model and showed that severe prenatal stress (administered as two 45 min periods of restraint stress on G15) has a similar outcome in terms of acceleration of NMDA-triggered spasms, increasing their number and sensitivity to ACTH treatment (33).

Infantile spasms involve impaired control of hypothalamic functions

Studies in humans point to involvement of the hypothalamus in infantile spasms (125, 131, 132). Efficacy of ACTH against the infantile spasms as well as findings of decreased ACTH and cortisol levels in the CSF of patients with infantile spasms indicate impairments in hypothalamic control of the HPA axis (99, 102, 103, 122–125). A recently found decrease in insulin-like growth factor (IGF) in patients with infantile spasms (122) suggests involvement of ventromedial hypothalamus, an area with high concentration of IGF receptors (133). Growth hormone deficiency or ACTH deficiency have been documented in case reports of children with infantile spasms (125). In addition, hypothalamic hamartomas are sometimes associated with infantile spasms (131, 132, 134–136). Finally, a recent study indicated that severe stress during pregnancy may contribute to increased risk of developing infantile spasms in offspring (137). While this retrospective study is based on self-reporting by mothers regarding the occurrence and level of prenatal stress and it is retrospective, importantly, there was an attempt to quantify the stress perceived by pregnant women and stratify the level of stress versus occurrence of spasms in the offspring (Table 4). This approach indicated that effect of prenatal stress on increased risk to develop infantile spasms was highly correlated with stress severity. Finally, this study used an interesting control group of age-matched children with epilepsy other than infantile spasms. Analysis of prenatal stress level indicated that those children suffering from other types of epilepsy than infantile spasms did not experience any significant prenatal stress (Figure 5). Thus, the stress factor seems to be correlated specifically with infantile spasms and is less involved in pathophysiology of other epilepsy syndromes.

Additional support for involvement of hypothalamus in infantile spasms comes from the efficacy of ketogenic diet in affected children (138, 139). The ketogenic diet is based on high fat, low carbohydrate intake and it is often used in attempt to control intractable seizures especially in pediatric epilepsies (140). First order CNS neurons sensing adiposity signals (e.g., leptin or ghrelin) from the periphery are located in the hypothalamic arcuate nucleus (141–143), strongly suggesting significant participation of the arcuate nucleus and its relay hypothalamic nuclei in the control of seizures including the infantile spasms.

Hypothalamus is involved in our model of infantile spasms

We studied the brain structures involved in spasms in this model. Initial studies included [¹⁴C]2-deoxyglucose (2DG) autoradiography (32). 2DG is a glucose analogue, which undergoes identical uptake process as glucose. However, as a metabolic substrate following the glycolysis path, it cannot pass the phosphorylation step and becomes trapped within the

cell (144, 145). Thus, the brain regions with high glucose uptake (high metabolic activity) display increased radioactivity compared to structures with less glucose uptake. We found an early (25 min after the onset of spasms) activation of the hypothalamic arcuate nucleus, later (at 45 minutes) followed by supraoptic and medial tuberal nuclei (32). We confirmed these findings by immunohistochemical assessment of the c-fos product. C-fos is an early gene, which marks areas of excitation in the brain (146, 147). Already 30 min after the onset of spasms, we found significantly increased number of c-fos immunopositive cells within hypothalamus, specifically in the arcuate, supraoptic and medial tuberal nuclei as well as dorsal part of the dorsomedial hypothalamic nucleus (Figure 6). Additional early activation was seen in the brainstem raphe nuclei, which have significant and bilateral connections with the arcuate nucleus, in the medial amygdala (again bilaterally connected with the arcuate nucleus) and medial septum.

Thus both clinical investigations in patients with infantile spasms and our experimental model indicate that hypothalamus is involved during this syndrome and among hypothalamic structures the arcuate nucleus may play a central role in this syndrome.

Transcriptomic analysis of the rat model of infantile spasms

Our studies have identified that the prenatal raise in corticosteroids is essential for increased susceptibility to develop spasms in the NMDA-triggered model as well as for the effects of ACTH to occur. There are reports demonstrating that prenatal exposure to corticosteroids, both natural (stress-derived) or synthetic, may have long-lasting effects on many molecules within CNS such as glucocorticoid receptors (130, 148, 149) or subunits for the NMDA receptors (150, 151). Moreover, effects of prenatal stress or prenatal exposure to synthetic corticosteroids may be sex-specific (126, 152, 153). Since we have linked the hypothalamic nuclei and especially the arcuate nucleus to the expression of spasms in the model, in order to determine the molecular substrate of the increased susceptibility to spasms we used our standard protocol (154) to profile the arcuate nucleus transcriptome in P14 rats (without any spasms), that have been prenatally exposed to either betamethasone or vehicle. Although we have quantified the expression level, control and coordination of a total of 18,094 distinct genes, the study focused on the 121 genes involved in the glutamatergic synapse and the 92 genes involved in GABAergic function at the synapse. The two subsets have a significant overlap of 41 genes including *Adcy1/2/3/4/5/6/7/8/9*, *Cacna1a/c/d*, *Gnai1/2/3*, *Gnb1/2/3/4/5*, *Gng2/3/4/5/10/11/12/13*. Alteration of expression and networking of these genes, selected by KEGG (Kyoto Encyclopedia for Genes and Genomes, <http://www.genome.jp>) software, may account for postnatal neurological impairment owing to the brain circuitry remodelling. As there are many sex-specific differences in the outcome of prenatal exposure to corticosteroids, we evaluated and compared male and female rats separately. Detailed methods and the raw and processed data have been deposited and are publicly accessible at <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE44858>.

As illustrated in Figures 7A–D, some of the genes of interest were significantly down-regulated but none was up-regulated in betamethasone exposed animals with respect to the corresponding controls. According to our standard protocol, a gene was considered as *significantly regulated* if the absolute fold-change was >1.5 and the p-value of the heteroscedastic *t*-test with a Bonferonni type correction of the means equality was < 0.05 . Interestingly, while no glutamatergic or GABAergic synapse gene was found as differentially expressed between control males and females, after prenatal betamethasone, the two sexes responded by altered regulation of common genes and additionally with sex-specific genes. Thus, the glutamatergic synapse genes *Adcy2* and *Grin2c* and the GABAergic synapse genes *Adcy2* and *Nef* were altered by betamethasone in both sexes. However, *Grik4*, *Ppp3cb* and *Prkcg* in the glutamatergic, and *Hap1* and *Prkcg* in the

GABAergic synapse were altered only in males. In contrast, *Gng13*, *Slc1a1*, *Gnas* and *Gnao1* in the glutamatergic, and *Gng13* and *Slc1a1* in the GABAergic synapses were altered only in females.

In addition, we have used Pair-Wise Relevant Analysis, which is more sensitive than analysis of transcript expression, to determine the changes in genomic fabrics of the glutamatergic and GABAergic synapse. The genomic fabric is defined as the most inter-coordinately and stably expressed gene web whose encoding proteins form the two major transmitter systems (154). As illustrated in Figure 8, (41), the very perceptive Pair-Wise Relevant Analysis revealed major alterations in the glutamatergic and GABAergic synapse genomic fabric topologies in betamethasone exposed animals as well as the presence of substantial sex differences in controls and sex-specific alterations in betamethasone-exposed animals. In addition to the expression levels, this analysis also considers contribution of expression controls and coordination of the paired genes to the inter-coordination and stability of the genomic fabric.

Together, the transcriptomic findings indicate that:

1. Prenatal exposure to betamethasone down-regulated several glutamatergic and GABAergic synapse genes, but it did not up-regulate any of these genes.
2. Although no gene included in glutamatergic or GABAergic synapse gene fabric was differentially expressed between the two sexes in control animals prenatally-exposed to saline, males and females exposed to betamethasone exhibited altered expression in both common and sex-specific genes.
3. If only expression levels of genes in synaptic fabrics were considered, there was no difference between the two sexes in prenatally saline-exposed (control) animals. However, if the sensitive topological analysis was applied, male and female synapse genomic fabrics were significantly different in controls and presented sex-specific alterations in betamethasone-exposed animals as well.

Conclusions

In our rat model of infantile spasms, prenatal priming using the exposure to synthetic corticosteroids is associated with enhanced susceptibility to develop NMDA-triggered spasms during the early postnatal period and renders the spasms responsive to ACTH treatment. Autoradiographical and immunohistochemical analyses support involvement of hypothalamus, especially the arcuate nucleus, in the model of infantile spasms. Transcriptomic analysis of the effects of the prenatal priming condition (exposure to betamethasone) indicates downregulation of transcripts encoding proteins participating in glutamatergic and GABAergic transmission in the immature arcuate nucleus. Furthermore, after prenatal betamethasone, there is a significant sex difference in gene interactions of both major transmitter systems. Transcriptomic findings, if substantiated by further proteomic analysis may provide molecular substrates for long-term reprogramming of neurotransmission in the arcuate nucleus, which may be associated with increased expression of neurodevelopmental disorders including infantile spasms.

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Figure 1. Age-specific occurrence of flexion spasms induced by systemic (intraperitoneal) administration of N-methyl-D-aspartate in developing rats

x-axis: logarithmic scale of i.p. doses of NMDA

y-axis: incidence of spasms in NMDA-injected groups in %. n.t. – not tested.

From top to bottom: 7-, 12-, 18-, and 25-day-old-rats (P7 through P25). Please note high incidence of flexion spasms in P7 and P12 groups, a decreased incidence in P18 rats, and complete disappearance of flexion spasms in P25 rats. It should be emphasized however, that tonic-clonic seizures have occurred in all age groups if the dose of NMDA was sufficiently high. Number of subjects per group = 8, except for the 200 mg/kg or 300 mg/kg doses of NMDA.

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Figure 2. Scheme of the model of cryptogenic infantile spasms in rats

Prenatally, the rats are primed by two injections of betamethasone to pregnant mother (Sprague-Dawley; Taconic Farms; 0.4 mg/kg of maternal weight each) administered at 8:30 and 18:30 on gestational day (G) 15 (25, 32). Alternatively, a restraint stress of 45 min (two episodes at the corresponding times) is also effective (33). The rats deliver almost exclusively on G23=postnatal day 0 (P0). On P15, spasms are triggered with injection of NMDA (15 mg/kg i.p.) in the primed offspring. The spasms reliably occur between P7-P21 (113). For the prospective ACTH trial we additionally triggered spasms on P12 and P13 while the ACTH treatment spanned P12-P14 with onset after the first bout of spasms on P12 diminished (25).

Figure 3. Comparison of EEG recordings in a patient with infantile spasms and the rat model of infantile spasms

(A) EEG in a patient with cryptogenic infantile spasms: Ictal EEG (during spasms) in a patient who developed spasms at age 6 months shows symmetrical paroxysmal high amplitude waves followed by voltage attenuation (electrodecrement). Calibration 100 μ V, time mark 1 s. Reprinted from (162) under license from Elsevier (#315429133357).

(B) EEG in the rat model of cryptogenic infantile spasms: Ictal EEG during a cluster of three spasms is shown in a prenatally betamethasone-exposed P13 rat after the spasms were triggered with 12 mg/kg, NMDA i.p. LF, RO – monopolar recordings from the left frontal and right occipital cortex (vs. reference electrode in the nasal bone). RF-LO – bipolar recording between right frontal and left occipital neocortex. Arrowheads mark onsets of spasms; arrows indicate the end of the spasms. Each of the three spasms was associated with a significant decrease in the EEG amplitude, an electrodecrement. Between the spasms, large-amplitude irregular waves were observed. Calibration 200 μ V, time mark 1 s.

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Figure 4. ACTH suppresses spasms and decreased lethality

(A) Latency to onset of spasms in the first bout of spasms triggered on P12 by 7.5 mg/kg of NMDA, i.p. "Saline" and "ACTH" represent the P12 data (mean $\pm$ SEM) of rats randomized to the saline (n=11) or ACTH (n=11) treatments, respectively. Randomized treatment started immediately after the spasms of the first bout disappeared and continued through evening of P14. Second bout of spasms was induced between the treatments on P13. There was no triggering of spasms on P14. (B) Number of spasms in the first bout of spasms triggered on P12 by 7.5 mg/kg of NMDA, i.p.; details as above. (C) Survival rate of rats through P15 (prior to entering the P15 trigger of spasms). Saline and ACTH indicate the data of rats receiving saline or ACTH, respectively. ACTH-treated rats had 100% survival, a significant increase compared to 56% in the saline group (Fisher's exact test, * $p = 0.035$). (D) Latency to onset of spasms in the bout triggered on P15 by 15 mg/kg of NMDA was not affected by chronic ACTH treatment. (E) Chronic ACTH treatment significantly decreased number of spasms in the P15 bout compared to saline controls (Student's t-test; * $p = 0.003$). Reprinted from (25) under license from John Wiley and Sons (#3154300140526).

Figure 5. Comparison of prenatal stress of infants among the three groups

IS patients (infantile spasm; case group); non-IS epilepsy patients (positive control group); and healthy infants (negative control group). Stress level is indicated by the mean of the weighted score on the Pregnant Woman Life Event Scale. There was a statistically significant difference in the prenatal stress level among mothers of infants in the three groups ($P < 0.05$).

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Figure 6. Counts of c-fos-immunoreactive (IR) cells after N-methyl-D-aspartic acid (NMDA) spasms in prenatally betamethasone exposed rats

C-fos IR cells were counted in matching brain sections at 30 (n = 4), 60 (n = 3), and 120 minutes (n = 4) after the occurrence of NMDA spasms. Only significantly different findings of 92 compared structures are shown. The c-fos IR was concentrated in three major brain areas: limbic structures (without the involvement of the dorsal hippocampus), hypothalamus, and the brainstem. Asterisk indicates a significant difference (Analysis of Variance with post hoc Fisher's Protected Least Square Difference test with $p < 0.05$ corrected for multiple comparisons) versus combined control group (interval-matched control rats injected with saline instead of NMDA were not significantly different from each other and, therefore, were pooled; combined n = 10).

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A. Glutamatergic synapse of male exposed to betametasone

B. Glutamatergic synapse of female exposed to betamethasone

C. GABAergic synapse of male exposed to betamethasone

D. GABAergic synapse of female exposed to betamethasone

Figure 7. Regulation of genes involved in glutamatergic (A, B) and GABAergic (C, D) function at the synapse in male and female P14 rats prenatally exposed to betamethasone with respect to corresponding saline-exposed controls

The synapse pathways were assembled by KEGG, each block representing a group of genes. Orange background indicates that no gene in that block was significantly altered, while green/red background indicates significantly down-/up-regulated genes, respectively. For instance, block Gi/o is composed of the genes: Gnai1, Gnai2, Gnai3, Gnao1, Gnb1, Gnb2, Gnb3, Gnb4, Gnb5, Gng2, Gng3, Gng4, Gng5, Gng7, Gng8, Gng10, Gng11, Gng12, Gng13, Gngt1, and Gngt2. No gene of this block was altered in male and only Gng13 was down-regulated in females. Each of four subgroups analyzed (males, females; prenatal betamethasone, saline) consisted of 4 independent samples

Figure 8. The Pair-Wise Relevant topologies of the glutamatergic and GABAergic synapse genomic fabrics of male and female P14 rats exposed prenatally to betamethasone or saline
 The height of each peak is the square root of the product of expression levels, controls (163) and square of the Pearson correlation coefficient between the expression levels of the paired genes in the four biological replicas. Note both the alteration induced by the prenatal betamethasone and the sex differences in both control and betamethasone-exposed animals.

TABLE 1

Overview of the current models of infantile spasms

Model	ACTH response	reference
prenatal corticosteroid priming with postnatal NMDA trigger	yes	(25, 32)
the tetanus toxin model	requires 2 weeks	(155)
multiple hit model	no	(156)
<i>Ts65Dn</i> mouse model (infantile spasms in Down syndrome)	yes, ACTH ₁₋₂₄	(157)
<i>Arx</i> ^{(GCG)10+7} triplet repeat expansion mouse model	no	(158)
<i>Arx</i> ^{-N} / <i>Dlx5/6</i> ^{CI} knock-out mouse model	no	(159)
prenatal stress priming with postnatal NMDA trigger	yes	(33)

TABLE 2

Comparative development of human and rat brain

milestone	human	rat
brain development	at birth	full term newborn
	during infant period	1 month – 1 year
	toddler	1–3 years
	childhood	3–9 years
puberty onset	9–10 years	P32–P36
end of puberty	14–17 years	around P55
life expectancy	74–79 years	2 years

This table has been compiled and approximated based on the studies comparing 2-deoxyglucose uptake, expression of transporters such as KCC2/ NKCC1, neurotransmitters (GABA), NMDA receptors, development of synaptogenesis, myelination, cell maturation, connectivity, brain growth and other factors evaluated and reviewed in (115, 116, 160, 161).

TABLE 3

CSF hormones in infantile spasms: Summary

Hormone	Controls (n)	IS (n)	P
Cortisol (ng/ml)	2.81 ± 0.46 (12)	1.49 ± 0.24 (16)	.0094*
ACTH (pg/ml)	45.6 ± 4.0 (15)	29.9 ± 2.4 (19)	.0029*

Values are means ± standard error.

* Two-tailed; Mann-Whitney U-test.

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Table 4

Risk for infantile spasms at four different levels of maternal prenatal stress

Prenatal Stress	Odds Ratio (95% Confidence Interval)			
	Level 1	Level 2	Level 3	Level 4
Level 1	–	7.00 (1.76–27.89)*	22.50 (5.29–95.76)*	34.50 (7.75–153.59)*
Level 2	–	–	3.21 (1.10–9.44)*	4.93 (1.58–15.38)*
Level 3	–	–	–	1.53 (0.45–5.18) †
Level 4	–	–	–	–

* $P < 0.05$ † $P > 0.05$

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