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REVIEW

Regeneration of neurotransmission transcriptome in a model of epileptic encephalopathy after antiinflammatory treatment

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Abstract

Inflammation is an established etiopathogenesis factor of infantile spasms (IS), a therapy-resistant epileptic syndrome of infancy. We investigated the IS-associated transcriptomic alterations of neurotransmission in rat hypothalamic arcuate nucleus, how they are corrected by antiinflamatory treatments and whether there are sex differences. IS was triggered by repeated intraperitoneal administration of N-methyl-D-aspartic acid following anti-inflammatory treatment (adreno-cortico-tropic-hormone (ACTH) or PMX53) or normal saline vehicle to prenatally exposed to betamethasone young rats. We found that treatments with both ACTH and PMX53 resulted in substantial recovery of the genomic fabrics of all types of synaptic transmission altered by IS. While ACTH represents the first line of treatment for IS, the even higher efficiency of PMX53 (an antagonist of the complement C5a receptor) in restoring the normal transcriptome was not expected. In addition to the childhood epilepsy, the recovery of the neurotransmission genomic fabrics by PMX53 also gives hope for the autism spectrum disorders that share a high comorbidity with IS. Our results revealed significant sex dichotomy in both IS-associated transcriptomic alterations (males more affected) and in the efficiency of PMX53 anti-inflammatory treatment (better for males). Our data further suggest that anti-inflammatory treatments correcting alterations in the inflammatory transcriptome may become successful therapies for refractory epilepsies.

Key Words: *adreno-cortico-tropic-hormone; autism; cholinergic transmission; hypothalamus; infantile spasms; microarrays; PMX53; sex differences*

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Infantile spasms (IS; West syndrome; also epileptic spasms during infancy first described by Dr. West in 1841) is a severe epileptic encephalopathy of infancy. Etiology of IS is quite complex and still not fully understood. Infantile spasms are characterized by a triad of brief spastic seizures, large amplitude asynchronous waves in the electroencephalography (EEG) recordings (hypsharrhythmia) and mental deterioration underlying the seriousness of this condition. Despite many years of investigation, the complete therapeutic solution for infantile spasms is far from available. Infantile spasms have also significant (up to 30%) comorbidity with autism spectrum disorders (ASD). Because of this close link, an effective treatment of IS covering common etiopathogenesis with ASD (such as neuroinflammation Ohja et al, 2018) may contribute a treatment option for ASD as well. Neuroinflammation has been established as one of the contributors to the pathophysiology of epilepsy (Aronica et al., 2017). In both experimental animals and human patients, transcriptomic analysis of different seizure types and epilepsies indicates significant changes in profiles of inflammatory transcriptome as well as the transcriptome pertinent for synaptic transmission (Laurén et al., 2010; Das et al., 2012). Tuberous sclerosis (a condition resulting from *TSC1* or *TSC2* gene mutations and characterized by occurrence of neuroectodermal tubers) in humans is frequently associated with infantile spasms that progress to other types of epilepsy. Patients with tuberous sclerosis have increases expression of cell adhesion and inflammatory genes in the tubers plus wide disarray of gene expression including genes involved in synaptic transmission in the perituberal tissue (Boer et al., 2010). Hence, we were interested in rodent models of seizures and epilepsy, how transcriptomic profiles would change as a function of seizure type, sex, and developmental stage of the animal and whether treatments affecting inflammation can stabilize transcriptomic profiles of genes belonging to synaptic transmission.

In two previous papers (Iacobaş et al., 2013, 2018a) we analyzed the alterations of the gene expression profiles in the hypothalamic arcuate nucleus of our rat model of IS (Velíšek et al., 2007). These papers are part of our larger effort to understand the impact and commonalities of different forms of epilepsy on the transcriptomes in various brain areas. In addition to the arcuate nucleus in the IS model, we have also profiled the CA1 (Friedman et al., 2013) and dentate gyrus (Velíšková et al., 2015, 2017; Iacobaş et al., 2018b) regions of the hippocampus involved in other models of seizures and epilepsy. We have studied also the protective role of estrogen against the seizure-induced neuronal injury

in the hippocampus (Velíšková et al., 2015, 2017; Iacobaş et al., 2018b). Data from the paraventricular nucleus and the prefrontal cortex in the animal model of IS following ACTH and PMX53 treatments are yet to be published.

We have had particular interest in how the spasms in our model can affect the transcriptomic networks responsible for the glutamatergic (denoted by GLU), GABAergic (GABA), dopaminergic (DA), cholinergic (ACH) and serotonergic (5HT) neurotransmission. Genes pertaining to each type of neurotransmission were selected using Kyoto Encyclopedia of Genes and Genomes developed by Kanehisa Laboratories (http://www.genome.jp). While the impairment of neurotransmission can significantly contribute to the expression of spasms, the neurotransmission recovery may become a measure (marker) of treatment efficacy. This is particularly important as current treatments of infantile spasms (adrenocorticotropic hormone-ACTH, corticosteroids and vigabatrin) are only partially effective (less than 55% efficacy after one year), leaving many infants and their families without hope for improvement in future.

In our two-hit IS rat model (Velíšek et al., 2007; Yum et al., 2012), the animals were prenatally primed with two doses of betamethasone delivered intraperitoneally to pregnant dams (control animals received saline). The spasms in infant, betamethasone-primed, rats were postnatally triggered by repeated intraperitoneal administration of N-methyl-D-aspartic acid (NMDA). After the first bout of spasms on postnatal day (P) 12, the rats were randomized into treatment groups or received vehicle (saline). Treatment delivery and spasm trigger continued through P14 and P15, respectively. On P15, rats were anesthetized and brain regions of interest were collected. Hypothalamic arcuate nucleus was selected as the main region of interest because our previous study indicated its prominent activation during experimental spasms (Velíšek et al., 2007). Arcuate nuclei of both sexes were profiled using Agilent two-color 4x44k gene expression microarrays and our optimized protocol (Iacobaş et al., 2018a), with 4 biological replicas of each condition (20 males and 20 females randomly distributed in 5 sex homogeneous groups). Raw data and full description of the experimental protocol are available as GSE81061 and GSE84585 at http://www.ncbi.nlm.nih.gov/geo.

Since no neurotransmission gene was differently expressed between males and females in control rats (SNS; saline (S)-primed, no (N) induced spasms, postnatally injected with saline (S)) we asked what transcriptomic feature might explain the known behavioral sex differences. The response came from our genomic fabric paradigm (Iacobaş, 2016) that does not limit the characterization of the transcriptome to the expression levels of individual genes but considers also their expression control and coordination with expression of each other gene. By definition, the genomic fabric of a functional pathway is the transcriptome associated to the most interconnected and stably expressed gene network responsible for that biological function. Thus, the pair-wise relevance analysis (that takes into account the contributions off all gene-pairs to the expression level, control and coordination to the neurotransmission transcriptomic landscape) has shown substantial differences between the two sexes (Figure 8 in Iacobaş et al., 2013). Years of working with animal models taught us that prenatal priming with betamethasone is not only essential for ACTH efficacy against experimental spasms but it also increases the propensity of rats to develop spasms after the NMDA trigger (Velíšek et al., 2007). This pathophysiological finding can be explained by the major alterations of the pair-wise relevant topology of the genomic fabrics of the synaptic pathways in response to the betamethasone priming.

Data in our randomized study of induced spasms clearly showed that male pups displayed more spasms at the end of the experiment compared to females. Indeed, the microarray analyses indicated that the transcriptomic effects of the induced spasms on the neurotransmission are sex specific, on average 3× larger in males than in females (Iacobaş et al., 2018a). In addition to the percentage of the regulated genes, we have evaluated the transcriptomic alterations using the novel Weighted Pathway Regulation analysis that takes into account all genes regardless of whether they were considered as significantly regulated (according to arbitrarily introduced cut-offs). Further analysis determined that in males, specifically GABA pathway transcriptome was significantly impaired, while in females, the DA pathway was affected the most by the spasms.

In the set of experiments with treatments, the betamethasone-primed (B) rats after the first trigger of spasms (Y) were randomized into treatment groups treated either with full molecule of rat ACTH (BYA group), PMX53 (BYP group) or Saline (BYS group). ACTH (adrenocorticotropic hormone) is the FDA-approved first line of treatment for epileptic spasms in human infancy (Knupp et al., 2016). PMX53, an antagonist of the C5a receptor, was suggested as an alternative because of the positive results with this drug in other models of epilepsy (Benson et al., 2015). Rationale for using PMX53 was that neuroinflammation, including that regulated *via* innate immune complement activation through C5ar1, is significantly involved in both epilepsy and ASD (Aronica et al., 2017; Ohja et al., 2018). Inhibition of C5ar1 decreases production of tumor necrosis factor alpha (TNFα), one of the key pro-inflammatory molecules responsible for microglial activation leading to neuronal damage and hyperexcitability. Thus, by attenuation of neuroinflammation, PMX53 might improve excitatory/inhibitory balance, which is impaired in epilepsy (Benson et al., 2015) and also in ASD. Since both infantile spasms and ASD have male predominance (male:female ratio is approximately 1.5:1 in infantile spasms and 3–4:1 in ASD), we evaluated males and females separately and then compared the results.

After treatment (ACTH or PMX53) we noticed a significant suppression of spasms in males, but not as much in females with respect to the corresponding betamethasone-primed pups with spasms, “treated” with saline only (the BYS group). PMX53 had at least equal if not better effects as ACTH on the suppression of spasms in males. It should be noted here that we evaluated only those animals entering the transcriptomic analysis (4 from each group) hence the cohort size was small and could have contributed

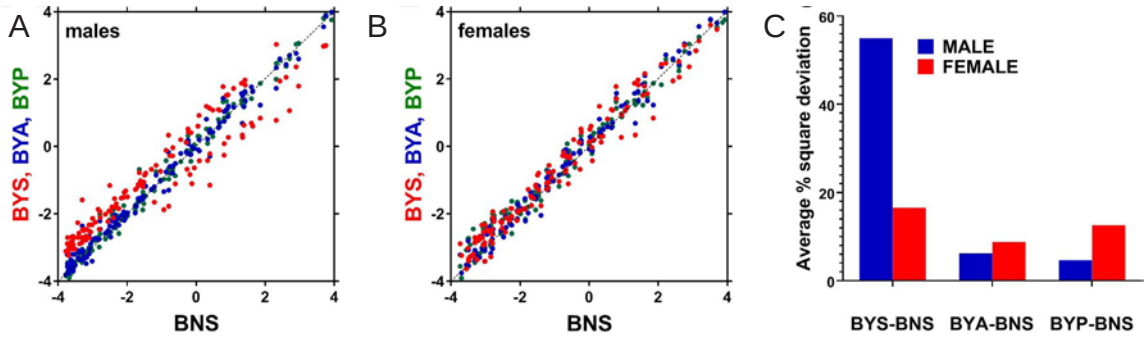

Figure 1 Experimental spasms alter the cholinergic transmission genes but both adreno-cortico-tropic-hormone (ACTH) and PMX53 treatments partially restore their normal expression.

In the set of experiments with treatments, the betamethasone-primed (B) rats after the first trigger of spasms (Y) were randomized into treatment groups treated either with full molecule of rat ACTH (BYA group), PMX53 (BYP group) or Saline (BYS group). (A, B) (log_2) expression levels of cholinergic genes in BYS, BYA and BYP plotted against (log_2) expression levels in non-spasm values (BNS). In these diagrams, the representative points of the unaltered genes (same expression as in BNP) are located on the diagonal. Note that expression levels in BYA (blue dots) and BYP (green) are significantly closer to those in BNS than expression levels in BYS (red) in both males (A) and females (B). (C) Average % square deviation of the expression of cholinergic genes from the BNS in male and female rats. Note that males were more affected by the spasms (larger average square deviation) but also benefit significantly more from the treatments (larger reduction of the average % square deviation).

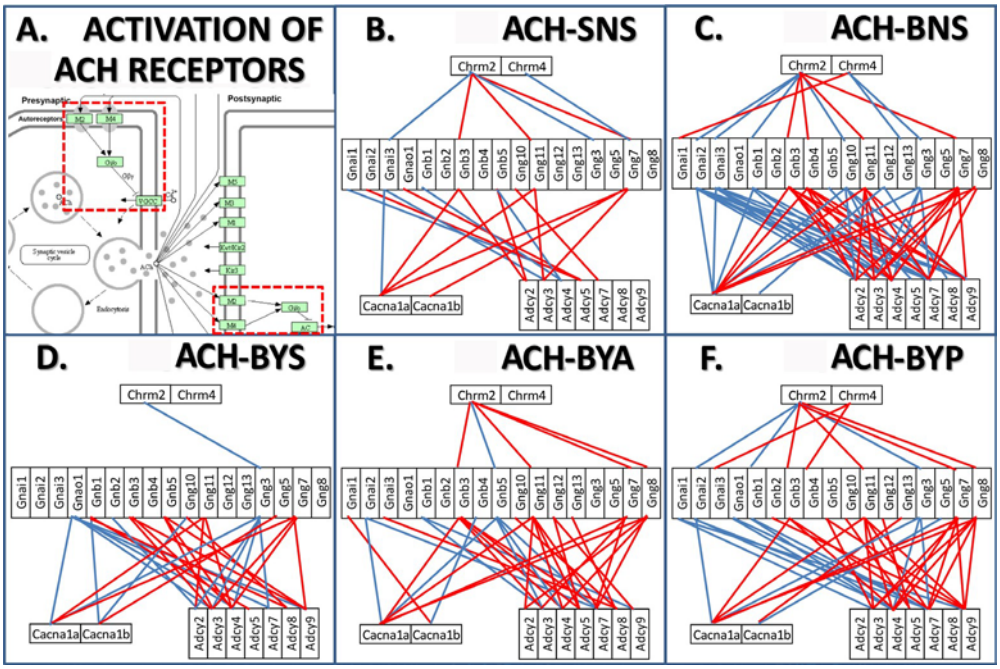

Figure 2 Remodelling of the cholinergic transmission gene networks.

In the set of experiments with treatments, the betamethasone-primed (B) rats after the first trigger of spasms (Y) were randomized into treatment groups treated either with full molecule of rat ACTH (BYA group), PMX53 (BYP group) or Saline (BYS group). (A) Part of the pathway regulated by the activation of the cholinergic receptors of the presynaptic and postsynaptic neurons. M2/4: Muscarinic metabotropic acetylcholine receptors (*Chrm 2/4*); Gi/o: G-proteins (*Gnai1/2/3, Gnao1, Gnb1/2/3/4/5, Gng10/11/12/13/3/5/7/8*); VCCC: voltage-dependent calcium channels (*Cacna1a/b*); AC: adenylate cyclases (*Adcy2/3/4/5/7/8/9*). (B–E) Gene expression correlation in the SNS (the absolute control: not betamethasone-primed no spasms pups treated with saline), BNS, BYS, BYA and BYP conditions.

to the lack of statistical significance for ACTH and PMX53 effects in females. The microarray experiment confirmed that both treatments (ACTH or PMX53) contributed to significant restoration of the synaptic pathway transcriptomes and the relative effect was larger in males. In the 2018 treatment paper (Iacobaş et al., 2018a), the effect was evaluated both by the reduction of percentage of regulated genes, and by the recently introduced pathway restoration efficiency (Iacobaş et al., 2018c). However, the same conclusion can be drawn by using several other quantifiers. For instance, as illustrated in **Figure 1A** and **B** by plotting the (log_2) expression values in BYS, BYA and BYP conditions against the corresponding values in BNS condition there were more alterations but also more regenerations following the treatments in males than in females. In **Figure 1C** we used the average square deviation from the non-spasm value to evaluate alteration in animals with spasms treated with ACTH (BYA), PMX53 (BYP) or just saline (BYS).

We have also obtained interesting results in the coordination analysis (Iacobaş and Iacobaş, 2010) of the synapse genes in each of the five groups of rats (BYS, BYA, BYP, BNS, SNS). In this analysis, two genes were considered as synergistically expressed if the abundances of their transcripts increase or decrease simultaneously within biological replicas (the four animals of the group). The genes are antagonistically expressed if their levels manifest opposite tendencies and independently expressed if the variation of one gene has no effect on the other's level. Thus, we found

that the transcriptomic networks by which the expressions of genes synchronize to ensure the "transcriptomic stoichiometry" (Iacobaş et al., 2007) of the synaptic functional pathways can be impaired by spasms and partially restored by the anti-spasm treatments.

Figure 2 represents the transcriptomic network, by which activated cholinergic receptors control the synaptic transmission in the arcuate nucleus of the male rat in each of the five conditions. In **Figure 2**, a red/blue line indicates that the connected genes are (P-value < 0.05) significantly synergistically/antagonistically expressed in that condition. Missing connecting lines indicate not enough statistical evidence for expression coordination. Of note is the substantial remodelling of the gene networks by prenatal exposure to betamethasone (compare BNS with SNS). Further, the seizure induction decoupled the cholinergic receptors from the G-proteins in (BYS) and that the anti-spasms treatments (BYA and BYP) restored most of this coupling. The results were similar for all neurotransmission types in both male and female rats. This profound remodelling of the neurotransmission genomic fabrics clearly shows how sensitive the synapse-mediated brain circuitries are to the neurological diseases and treatments addressing these diseases.

Conclusions

The brain circuitry is continuously rebuilding (regenerating) itself, responding with remodeling of the neurotransmission transcriptome to the induced spasms and recovering in response to treatment of spasms. We determined that attenuation of neuroinflammation with PMX53 may become an effective treatment approach for infantile spasms, which may additionally improve comorbidity of infantile spasms with the ASD. Using novel transcriptome analytical tools developed on the genomic fabric paradigm (Iacobaş, 2016), we determined that treatment efficacy (in both ACTH and PMX53) is associated with significant transcriptomic regeneration of synaptic pathways and can inform about possible efficacy of treatments. We also determined sex specificity of transcriptome changes and recovery which adds another dimension to the developmental profile of changes after seizures (Azevedo et al., 2018). Our novel tools improved quantification measures of both alterations and recovery of the synaptic neurotransmission transcriptome in the model of infantile spasms.

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