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# Proteomic analysis on infantile spasm and prenatal stress

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**Summary** Infantile spasms (IS) are an age-dependent epileptic encephalopathy with severe cognitive dysfunction. Prenatal stress (PS) has been reported to increase the risk for IS through clinical and animal studies. We aim to investigate the mechanism of brain damage caused by IS and the effect of PS. Animals were divided into 4 groups: PS-spasm model, PS-saline control, NS-spasm model, and saline control. N-methyl-D-aspartate (NMDA) was used to induce spasm and swimming in cold water was used to induce PS. A proteomics-based approach was used to compare the NS-spasm model vs. saline control, and PS-spasm model vs. NS-spasm model. Gel image analysis was followed by mass spectrometric protein identification and bioinformatics analysis. We observed an increased spasm frequency ($t = 8.65$, $P < 0.001$), and a shorter latency period ($t = 3.96$, $P < 0.001$) in the PS-spasm model vs. the NS-spasm model. In the NS-spasm model vs. saline control, the main differentially expressed proteins were CFL1, PKM2, PRPS2, DLAT, CKB, DPYSL3, and SNAP25. In the PS-spasm model vs. NS-spasm model, MDH1 and YWHAZ were differentially expressed. YWHAZ was directly connected with CFL1 in protein networks. YWHAZ and CFL1 were further validated by Western blot analysis.

The biological function of differentially expressed proteins indicates the pathogenesis of IS maybe relevant to energy metabolism, brain development, and neural remodeling. PS aggravated seizures in the NMDA-induced spasm model, YWHAZ, and CFL1 may be involved.


## Introduction

Infantile spasms (IS) are an age-dependent epileptic encephalopathy with the characteristics of repeatedly spasm seizures, hypsarrhythmia electroencephalography, and severe cognitive dysfunction. Approximately 70—90% patients are associated with mental retardation (Primec

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et al., 2006). It is associated with more than 200 etiological factors, including brain insults, metabolic disorders, malformations, genetic errors, and dysmorphogenetic syndromes (Pellock et al., 2010), whereas, complex causes result in the similar clinic feature. There are several hypotheses that attempt to explain this disorder that involve corticotrophin-releasing hormone (CRH), N-methyl-D-aspartate (NMDA), and serotonin (5-HT)—kynurenine (Rho, 2004). Thus far, the pathogenesis of IS remains unknown. In the present study, we analyzed an animal model of IS by using proteome, and examined changes in protein expression during the pathogenic progress.

In addition, we focus on prenatal stress (PS) in IS. Because this is a case-control study, we found that the degree of PS is higher among mothers of IS patients than among controls; within a specific range, the risk of IS increases with the severity of PS (Shang et al., 2010). In an NMDA animal model, PS could enhance offspring susceptibility to spasms (Wang et al., 2012a; Yum et al., 2012). Previous reports reveal the influence of PS on infants covers multiple systems and aspects. PS offspring show delayed motor development, greater emotional and anxious reactions in unfamiliar environments, and impaired cognitive functions as an adult (Rho, 2004). PS exposure can alter hormone levels and neurotransmitter receptor expression in developing rats (Mulder et al., 2002). When the body regulates stress, the hypothalamus—pituitary—adrenal cortex (HPA) axis and the sympathetic nervous system—adrenal medulla system are activated. In the rat, PS can increase glucocorticoid levels in the fetus, delay HPA activation, and cause low rat glucocorticoid levels after birth (Baquedano et al., 2011). CRH plays a major role in coordinating the HPA axis and the behavioral response to stress (Bale and Vale, 2004). PS also can increase the concentration of NMDA receptors in the cerebral cortex and basal ganglia in infants (Nair and Bonneau, 2006). PS is related to the functional impairment of 5-HT1A receptors in the hippocampus (Savitz et al., 2009), and alters 5-HT2A receptor expression in mouse frontal cortex (Holloway et al., 2013). Based on these previous findings, we found that PS is likely involved in the 3 of the hypothesis of IS pathogenesis. Therefore, we hypothesized that multiple causes converge into a final common pathway to produce a specific epilepsy phenotype, and PS plays an important role in the onset of IS. Here, we examine changes in protein expression in an animal model exposed to PS, and tried to find PS effect on pathogenic progress for IS.

NMDA induced spasms in young developing rats can show hyperflexion seizures similar to those observed in IS (Kábová et al., 1999; Velísek et al., 2007). Electroencephalography (EEG) in this model shows generalized amplitude reduction in ictal periods and large-amplitude, nonsynchronous waves in interictal periods. Furthermore, the adult rats show cognitive deficits in with spatial learning and memory impairment (Stafstrom and Sasaki-Adams, 2003). Because of the characteristic spasms, EEG features, and cognitive dysfunction that is similar to IS. Using C-fos immunohistochemistry and 2-deoxyglucose imaging, we identified limbic areas (except the dorsal hippocampus), hypothalamus, and brainstem regions that participate in the development of flexion spasms. We used the NMDA-induced spasm model in this study. Swimming in cold water is commonly used to PS animal model (Yum et al., 2012; Neeley et al., 2011). PS can delay HPA activation in the rat fetus. We previously studied the NMDA-induced spasm model and adrenal dysfunction by adrenalectomy (ADX), and found the latency of seizures decreased, the severity of seizures increased significantly, and CRH mRNA was overexpressed in ADX rats (Wang et al., 2012b). Therefore, proteomes may provide additional information on neuropathological changes.

## Materials and methods

### Animals

All studies were approved by the ethics committee of the General Hospital of the Chinese People's Liberation Army (Approval ID no. 2008B079). Three-month-old Wistar rats (31 females, 10 males, obtained from the Experimental Animal Center, Capital Medical University) were used in the study. The animals were maintained on a 12 h light/dark cycle with food and water available ad libitum. Female Wistar rats were mated with males and the time of appearance of a vaginal plug was considered gestational day 1. The pregnant rats were randomly divided into PS and non-stressed (NS) groups ($n = 10$). PS rats were exposed to stressful conditions daily, from day 1 to parturition, by immersing the rats in cold water (4 °C) for 5 min. The water was contained in a Plexiglas cylinder (diameter: 20 cm, height: 50 cm). After the immersion, rats were removed from the water and allowed to dry for 10 min in a heated container before they were returned to their home cages. Their offspring were assigned to one of the four following groups ($n = 15$): PS-spasm model, PS-saline control, NS-spasm model, and saline control. On postnatal 13 day, NMDA (7 mg/kg Sigma, St. Louis, MO, USA) was injected intraperitoneally (i.p.) in the pups of the PS-spasm model and NS-spasm model; 2 mL saline as control i.p. inject to the pups of PS-saline control and saline control. All of the pups were observed continuously for 3 h after the i.p. injection. Spasm onset was determined by hyperflexion that included flexion of the head and trunk, forelimbs, hindlimbs, and hips. Spasm latency was the duration between drug injection and spasm onset; spasm frequency was the total number of spasms that occurred during the 3 h observation period. The extent of spasm was quantified by the spasm latency and spasm frequency.

### Sample preparation

After rapid decapitation, the brains were isolated and stored at −70 °C. We selected five samples randomly from each group, froze them in liquid nitrogen, and then ground the sample into a powder. The powder was then added to a lysis buffer (8 M Urea, 4% CHAPS, 0.5% Pharmalyte pH 3—10, 65 mM DTT) and protease inhibitor cocktail. Following a 1 h dissolution, the solution was sonicated (4 s per cycle, 200 W, 12 s intervals), added to DNAse I and RNAse A, incubated at 4 °C, and the centrifuged at 40,000 × $g$ for 1 h. The supernatant was removed and the protein concentration was measured using the Bradford method (Bio-Rad Protein Assay, Bio-Rad) with Coomassie Brilliant Blue (CBB) staining against a standard protein bovine serum albumin (BSA).