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Prenatal testosterone influence on reelin expression in association with autism

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Key words: testosterone; reelin; candidate gene; autism; spatial abilities

Abstract

Autism is the most genetically influenced neuropsychiatric disorder with unknown etiology. Reelin, one of the autism candidate genes plays a major role in neuronal migration during neurodevelopment and in regulation of synaptic plasticity in postnatal period. Autistic patients have decreased levels of reelin in plasma and frontal and cerebellar cortices. Testosterone pathway is suspicious in autism pathogenesis, since increased fetal testosterone correlates with strong autistic traits. Testosterone administration sharply decreases reelin expression in brains of male starlings. Our purpose was to reveal the possible relationship between testosterone pathway and reelin expression in context of autism pathogenesis using rat model. Pregnant rats were injected with testosterone. Reelin expression was measured in certain brain areas using real time PCR and immunohistochemistry methods. Reelin expression was decreased in hypothalamus (males) and cerebellum (females) in newborn pups of testosterone treated mothers. In adult offspring of testosterone treated mothers we have observed an opposite pattern. Immunohistochemical analysis has shown the presence of reelin positivity in parietal cortices of control group, whereas no rather than low reelin positivity was observed in parietal corti-

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ces of offspring of testosterone treated mothers.
We can conclude that testosterone may regulate reelin expression in the brain of mammals. However, further studies are needed to focus on this pathway in more details, since this pathway may play an important role in pathogenesis of autistic disorder.

Abbreviations:
RELN - reelin
fT - fetal testosterone
T group - offspring of testosterone treated mothers
CTRL group - offspring of oil treated mothers (control group)

INTRODUCTION

Autism is a neurodevelopmental disorder with unknown etiology. Incidence of autism spectrum is 0.1%. The most pronounced symptoms include social deficits, impaired speech and communication, obsessive and repetitive behaviors. Autistic disorder belongs to one of the most genetically influenced neuropsychiatric diseases (heritability 90%) (Folstein & Rosen-Sheidley 2002; Veenstra-VanderWeele & Cook 2004). Genome wide association and linkage studies on autism pointed out a number of candidate genes (Persico & Bourgeron 2006; Andres 2002; The Autism Genome Project Consortium 2007, Celec & Ostatnikova 2010). One of the candidate genes with known phenotypic autism association is RELN gene (7q22) encoding reelin. Reelin has a crucial role in regulation of neuronal migration and in regulation of synaptic plasticity (D´Arcangelo *et al.* 1995). Reduced reelin levels were found in blood and frontal and cerebellar cortices of autistic subjects (Fatemi *et al.* 2002, 2005). Polymorphic GGC repeats in 5´UTR of RELN were associated with autism (Persico *et al.* 2001, 2006).

One of the theories attempting to explain the autism etiology is extreme male brain theory. It suggests that autistic brain represents an extreme of the male brain pattern. Exposure to high levels of prenatal testosterone results in masculinized social behavior in the areas of spatial performance and cognitive abilities (Knickmeyer *et al.* 2005). The forementioned study showed a negative correlation between fetal testosterone (fT) levels and social relationships that are impaired in autism. Furthermore, children with high fT measured in amniotic fluid exhibit strong autistic traits, strong systemizing and deficits in empathy (Baron Cohen *et al.* 2005; Chapman *et al.* 2006; Knickmeyer *et al.* 2006; Auyeung *et al.* 2009; Krajmer *et al.* 2010). Other evidence of this theory is

that strong autistic traits are present in congenital adrenal hyperplasia patients (Knickmeyer *et al.* 2006).

Hypothesis of this study is based on the findings of previous studies which revealed the connection between testosterone and reelin pathways in birds. Testosterone was described to enhance the quality and the frequency of birdsongs (Ball *et al.* 2003). Song behavior is controlled in brain by testosterone and its metabolite estradiol via their receptors that are expressed in many specialized forebrain song control nuclei. Furthermore, testosterone was found to influence the expression of reelin in the brain of male European starlings (Absil *et al.* 2003). Levels of testosterone change seasonally in the lifecycle of these birds. Reelin contributes to the incorporation of new neurons to the song control nucleus (HVC, high vocal center) of the songbird brain. Reelin expression in the songbird brain varies seasonally and could thus mediate seasonal incorporation of new neurons into the HVC. In addition, testosterone administration sharply decreased reelin expression in these birds (Absil *et al.* 2003). Based on these findings we suggest possible linkage between testosterone and reelin mediated neuronal development also in mammals including humans. The aim of this study was to reveal the influence of prenatal testosterone administration on reelin expression and spatial memory in rats. We propose that impairments in this mechanism could contribute to the manifestation of autistic phenotype.

MATERIALS AND METHODS

Animals

Animal experiment was approved by State veterinary and food control; experiment nr. Ro-3423/06-221/3. Wistar rats were used for the experiment (females, n=20; males, n=6, 290–320 g; Dobra Voda, Slovakia). Female rats were divided into two groups, testosterone and control group, 10 females in each. Both groups had the same climatic conditions-temperature 25 °C, the photoperiod was a 12-hr light: dark cycle. The experimental protocol was carried out under the guidelines of the Ethical Committee of the University of Comenius (Bratislava, Slovakia). Animals had free access to food (standard pellets mixture) and water ad libidum. After mating, rats of testosterone group were injected with 2.5 mg Testosteroni isobutyras i.m. within the days 11–15 of pregnancy. Control rats were injected with oil. One half of each litter was sacrificed at the postnatal day 10 (males, n=13–14, females, n=7–10). Blood and brain samples were collected. Second half of both genders of each litter were let to reach 2.5 months of age (males, n=7–15, females, n=6–10). Subsequently,

spatial memory was assessed using Morris Water Maze test. Afterwards, rats were sacrificed and blood and brain samples were collected.

Behavioral testing

Standard protocol of Morris water maze test was used for evaluating spatial memory (Brandeis *et al.* 1989). Briefly, during four learning days rats were gradually placed into the maze in all of the four quadrants. In case the rat did not manage to reach the platform within a minute, it was immediately allowed to rest on it for a whole minute. In probe trials the platform was removed, rats were placed for one minute in the same quadrant as they were placed for the first time during the learning trials. The time spent in the platform quadrant was measured. The time means of both groups were compared.

Measurement of testosterone levels in plasma

Plasma testosterone levels were assessed using ELISA method (DRG Diagnostics, Marburg, Germany) with intra- and inter-assay coefficients of variation <5%.

Analysis of gene expression

Total RNA was isolated from brain samples using TRIreagent. Concentration and purity of the isolates were assessed spectrophotometrically. Sybr Green one step RT PCR kit (Qiagen, Germany) was used for the real time PCR on Biorad IQ5 cycler. Melting curve analysis was performed to check the specificity of PCR. Transcript of target genes were determined relatively to the transcripts from the housekeeping gene (beta actin) using the delta Ct method.

Imunohistochemical analysis

Tissue samples of parietal cortex were fixed in 4% formaldehyde, embedded in paraffin and routinely processed for immunohistochemistry. The 5mm thick slices were deparaffinized, rehydrated in phosphate-buffered physiological salt solution (PBS) and incubated overnight with anti-reelin mouse antibody, clone 142 (Calbiochem, San Diego, CA, USA) diluted 1:10 00 in antibody diluent solution (Dako, Glostrup, Denmark). After 3 rinsing steps of 5 minutes each in PBS, sections were incubated for 30 minutes with the anti mouse antibody horse radish peroxidase conjugate (Envision, Dako, Glostrup, Denmark). After 3 rinsing steps of 5 minutes each in PBS, the peroxidase activity was visualized with diaminobenzidine (Dako, Glostrup, Denmark). Subsequently

the sections were counterstained with hematoxylin. The reelin positivity was analyzed semi-quantitatively in optical microscope (Leica DM 2000, Wetzlar, Germany) as follows: strong – more then 20% positive area; weak – less then 20% positive area; and negative.

Statistical analysis

The hormonal levels and relative expression of RELN were compared between rats of testosterone treated mothers (T group) and control group (CTRL group) using unpaired T-test (two-tailed). The same test was used to assess the differences between T and control group in the scoring of time spent in the platform quadrant in the Morris water maze test.

RESULTS

We have found a decrease of plasma testosterone levels in 10 days old pups of both genders (males, p=0.003; females, p<0.001) of testosterone treated mothers (Fig. 1A). Reelin mRNA expression was significantly decreased in hypothalamus of male pups (p=0.002) and in cerebellum of female pups (p=0.025) of testosterone treated mothers (Fig. 2). In 2.5 months old grown-ups we have found an increase of plasma testosterone levels in male (p<0.001) and female (p=0.032) offspring of testosterone treated mothers (T group) in comparison to control group (CTRL group) (Fig. 1B). Surprisingly, reelin mRNA expression was significantly increased in cerebellum (p=0.031), frontal cortices (left, p=0.039; right, p=0.016), parietal cortex (p=0.009) and hippocampus (p=0.011) of males and in cerebellum (p=0.024), frontal left cortex (p=0.017)

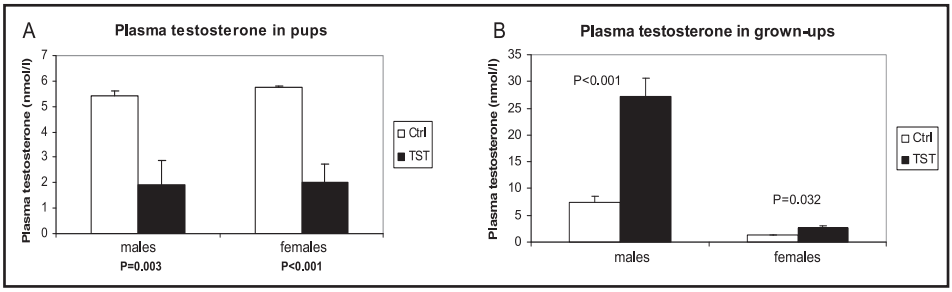

Fig. 1. Plasma testosterone levels in pups (A) and adult offspring (B) of testosterone treated mothers (T group) and control group (CTRL group). In pups, we have measured decreased plasma testosterone in T group of both genders, no intersexual differences were observed. In adult offspring plasma testosterone levels were higher in T group in comparison to CTRL group in both genders, lower testosterone levels were observed in females.

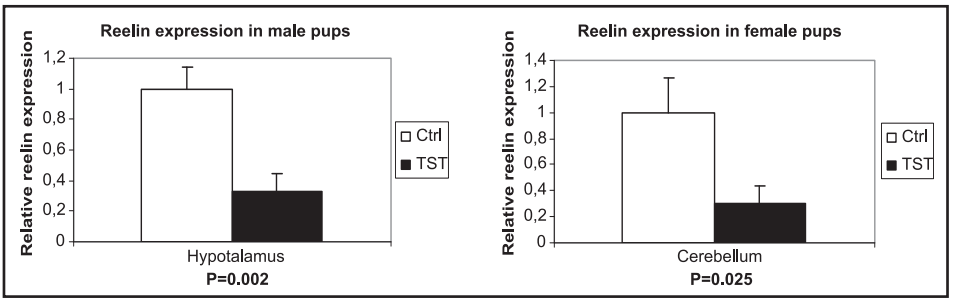

Fig. 2. Relative reelin expression in pups of testosterone treated mothers (T group) and control group (CTRL group). Reelin mRNA expression was decreased in hypothalamus (males) and cerebellum (females) of T group in comparison to C group.

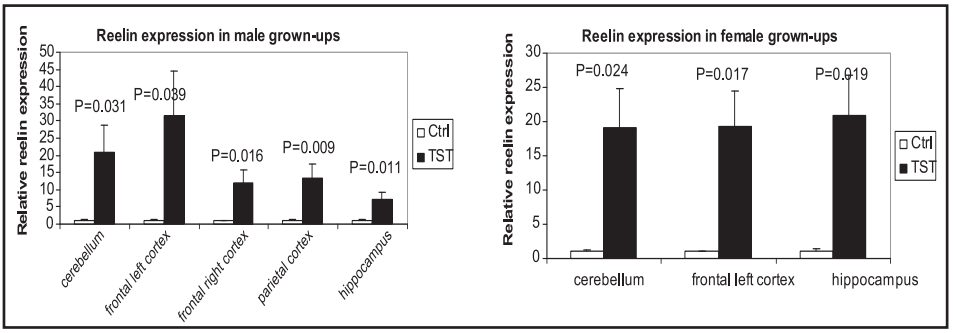

Fig. 3. Relative reelin expression in adult offspring of testosterone treated mothers (T group) and control group (CTRL group). Reelin mRNA expression was increased in cerebellum, frontal, parietal cortices and hippocampus (males) and in cerebellum, frontal left cortex and hippocampus (females) of T group in comparison to C group.

and in hippocampus (p=0.019) of females of T group in comparison to CTRL group (Fig. 3).

We have found no significant differences in Morris water maze results in both genders (males, p= 0.918; females, p=0.151).

Immunohistochemical analysis showed the strong positivity of extracellular matrix in parietal cortex of control animals, whereas the only weak or no positivity was found in the offspring of testosterone treated animals (Fig. 4).

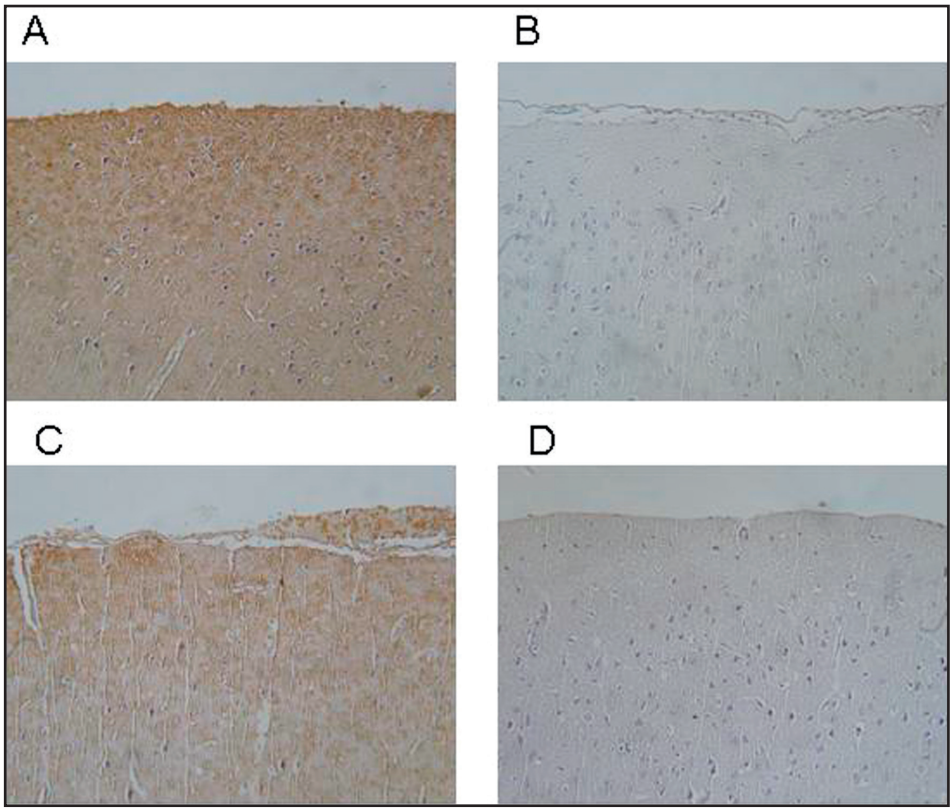

Figure 4. Immunohistochemical analysis showed strong reelin positivity of extracellular matrix in parietal cortex of male (A) and female (C) control animals. Only weak or no positivity was found in parietal cortex of adult male (B) and female (D) offspring of testosterone treated mothers. Anti-reelin mouse antibody, anti mouse-peroxidase complex, diaminobenzidine, magnification 200x.

DISCUSSION

The aim of our study was to reveal the connection between testosterone and reelin pathways in mammals in context of autism pathogenesis. Testosterone administration sharply decreases reelin expression in songbird brain (Absil *et al.* 2003). Furthermore, testosterone pathway is suspicious in autism pathogenesis, since high fetal testosterone levels correlate with autistic traits (Baron Cohen *et al.* 2005; Chapman *et al.* 2006; Knickmeyer *et al.* 2006; Auyeung *et al.* 2009). In the present study, testosterone administration during pregnancy in rats seems to have an effect on pup development. We have observed decreased plasma testosterone levels in pups of testosterone treated mothers (Fig. 1A). This phenomenon could be a result of negative feedback of increased testoster-

one levels on hypothalamus-pituitary axis. Male pups of testosterone treated mothers have decreased reelin mRNA expression in hypothalamus and female pups of the same group have decreased reelin mRNA expression in cerebellum in comparison to control pups (Fig. 2). These results confirm the hypothesis that testosterone administration in pregnancy negatively regulates the expression of reelin in newborn rats.

We have observed no significant differences in Morris water maze test between the adult offspring of testosterone treated mothers (T group) and offspring of control mothers (CTRL group) with high interindividual variability in the measured parameter. This variability might have been caused by hormonal differences especially in females during the estrous cycle.

In the adult offspring plasma testosterone levels were significantly higher in T group in comparison to CTRL group (Fig. 1B). Interestingly, the reelin mRNA expression was increased in cerebellum, frontal and parietal cortex and in hippocampus of males of T group in comparison to CTRL group. Reelin mRNA expression was also increased in cerebellum, frontal left cortex and in hippocampus of females of T group in comparison to CTRL group. The limitation of hormonal measurements interpretation in adult rats is given by the fact that strong interindividual and intraindividual variability in plasma testosterone levels was observed in adult rats of both genders during repeated plasma collections within 15 days (unpublished data). Our results propose that reelin expression is differentially regulated in relation to the ontogenetic phase, the explanation of these findings is however difficult.

In addition, the level of mRNA expression does not necessarily have to correspond with the protein levels. Immunohistochemical analysis has shown strong reelin positivity of extracellular matrix in parietal cortex of CTRL group of both genders, whereas weak or no positivity was observed in parietal cortex of T group. Contrary, increased reelin mRNA expression was found in parietal cortices of T males. One possible explanation of this discrepancy could be the presence of altered translational or posttranslational regulation of reelin expression in T rats. On the other hand, relatively small number of offspring in each group lowers the accuracy of the statistical results of reelin mRNA expression. In this experiment we aimed to use the highest dosage of testosterone as possible to demonstrate the effect of the treatment. During repetitive treatment of pregnant rats with testosterone we have observed high prenatal mortality. However, single testosterone treatment was also followed by some level of postnatal morbidity and mortality, being the main cause of relatively small sample size.

In summary, we can conclude that testosterone may regulate reelin expression in the brain of mammals. However, further studies are needed to focus on this pathway in more details. This pathway may play an important role in pathogenesis of autistic disorder.

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