Abnormal instability, excess density, and aberrant morphology of dendritic spines in prenatally testosterone-exposed mice

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Abbreviations¹

¹ASD: autism spectrum syndrome; PCOS: polycystic ovary syndrome; YFP: yellow fluorescence protein

Abstract

Fetal brain development is programmed by the maternal intrauterine environment, and disturbance of the *in utero* environment leads to persisting deficits in brain functions of the offspring. Testosterone is an intrauterine environmental factor, and plays significant roles in fetal development. From human and animal model studies, it has been suggested that increased intrauterine testosterone concentration triggers subsequent autistic-like behavior of the offspring; however, the effects of maternal excess testosterone on synaptic development of the offspring remain unknown. In the present study, we employed prenatally testosterone-exposed mice, and by using *in vivo* two-photon imaging, we analyzed the dynamics, density, and morphology of the dendritic spine, an excitatory postsynaptic structure. We found that the offspring from testosterone-treated dams showed abnormal synaptic instability persisting into young adulthood, whereas dendritic spines in control mice became stabilized with normal synaptic maturation. In prenatally testosterone-exposed mice, the density of dendritic spines was excessively increased, and their morphology was abnormal. These results suggest that prenatally testosterone-exposed mice may have deficits in synaptic development, and furthermore that the observed pathological features of their dendritic spines may be the cause of the synaptic pathogenesis in prenatally testosterone-exposed mice.

Keywords

Dendritic spine; *in vivo* two-photon imaging; autism; polycystic ovary syndrome; testosterone; maternal environment

1. Introduction

The maternal intrauterine environment regulates fetal brain development, and its effects persist into adulthood and contribute to the behavioral phenotype; many lines of evidence suggest that there is a relationship between disturbances in the intrauterine environment and several neuropsychiatric disorders (Schuurmans and Kurrasch, 2013). Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by persistent impairment in social interaction, communication and restricted, repetitive, stereotyped patterns of behavior, often with resistance to change (Association). Recent studies have demonstrated an association between prenatal *in utero* exposure to testosterone and the development of abnormal cognitive function diagnosed as ASD (Baron-Cohen et al., 2005; Hines, 2011; Xu et al., 2013). Sex hormones play important roles in fetal brain development (Bonthuis et al., 2010; Keefe, 2002; Phoenix et al., 1959). Intrauterine levels of steroid hormones are directly affected by the physical condition of the mother. For example, psychological stress in women correlates with testosterone concentration (Chichinadze and Chichinadze, 2008; Lennartsson et al., 2012), and maternal stress during pregnancy is associated with increased risk of ASD in children (Keefe, 2002). Polycystic ovary syndrome (PCOS), characterized by ovarian dysfunction and polycystic ovarian morphology in women of childbearing age, is also associated with hyperandrogenism (Abbott et al., 2005; Azziz et al., 2005; Franks, 1995; Goodarzi et al., 2011), and it has been suggested that children from PCOS mothers may express more autistic traits (Palomba et al., 2012). An animal model study of maternal hyperandrogenism during pregnancy has demonstrated that the offspring exhibit autistic-like behavior from early childhood into adulthood (Xu et al., 2015). These lines of evidence suggest that prenatal exposure to testosterone causes behavioral deficits;

69 however, the synaptic pathogenesis of autistic-like behavior remains to be elucidated.

70 In the present study, we focused on the dynamics, density, and morphology of
71 dendritic spines, because these features of dendritic spines are strongly associated with
72 synaptic development (Grutzendler et al., 2002), plasticity (Trachtenberg et al., 2002),
73 and neuropsychiatric pathologies (Penzes et al., 2011). By using two-photon
74 laser-scanning microscopy, we investigated the synaptic pathologies of the frontal cortex
75 in prenatally testosterone-exposed mice *in vivo*. As rodent models of PCOS exposed to
76 androgens prenatally display abnormal reproductive and metabolic functions including
77 infertility, obesity, hyperinsulinism, an increased risk of type 2 diabetes, and
78 cardiovascular disease (Walters et al., 2012), it is important to image the synapses *in vivo*
79 to include any contributions from peripheral tissues. The present study is the first to
80 examine the synaptic basis of neurodevelopmental impairments in offspring exposed to a
81 prenatal hyperandrogenic environment.

2. Material and methods

2.1. *Experimental animals*

Mice expressing yellow fluorescence protein (YFP) predominantly in layer 5 pyramidal neurons (*Thy1-YFP* H-line) were purchased from the Jackson Laboratory and backcrossed with C57BL/6J mice. Mice were housed four per cage under controlled temperature ($25 \pm 1^\circ\text{C}$) and lighting (12-h light/dark cycle), and were provided with and water ad libitum (Feng et al., 2000). On days 16–18 of pregnancy, dams were subcutaneously with 0.1 mg/day testosterone propionate (Sigma-Aldrich, St Louis, MO, USA) or 50 μL sesame oil vehicle alone as a control. Only male offspring heterozygous for YFP were used for all analyses. Animal procedures were in strict accordance with the guidelines of the National Institute of Neuroscience, National Center of Neurology and Psychiatry (Japan).

2.2. *Surgical procedure for in vivo imaging*

We used the thinned-skull cranial window technique (Yang et al., 2010) because it is less invasive than the open-skull method (Xu et al., 2007). Male pups expressing YFP were deeply anesthetized with ketamine (0.1 mg/g body weight, intraperitoneally) and xylazine (0.015 mg/g body weight, intraperitoneally). Body temperature was maintained at 37°C with a heating pad during the surgery and imaging. Eyes were lubricated with eye ointment. After the scalp incision, the frontal association cortex (2.8 mm anterior to the bregma and 1.0 mm lateral from the midline) was identified with stereotactic coordinates. A small metal plate with a round hole was glued onto the skull with cyanoacrylate glue and acrylic resin dental cement (UNIFAST; GC, Japan), and mice were fixed to a custom-made skull immobilization stage via the metal plate. The skull

above the imaging area, located in the center of the hole in the metal plate, was thinned to a thickness of ~20 μm with a high-speed microdrill (UG23A/UC210C; Urawa, Japan) and microsurgical blade (USM-6400; Sable Industries, Vista, CA, USA). The hole in the metal plate was filled with artificial cerebrospinal fluid during surgery and imaging.

2.3. *In vivo transcranial two-photon imaging*

Thy1-YFP pups from sesame oil- or testosterone-treated dams were imaged under anesthesia using two-photon laser-scanning microscopy (FV1000-MPE; Olympus, Japan) with a water-immersion objective lens (25 $\times$, NA 1.05) at 8 $\times$ digital zoom, yielding high-magnification images suitable for quantification of dendritic spines. A Ti-sapphire laser (MaiTai HP DeepSee-OL; Spectra-Physics, Mountain View, CA, USA) was tuned to 950 nm. To minimize phototoxicity, laser intensity was kept between 10 and 30 mW at the focus. Image-stacks (512 $\times$ 512 pixels; 0.124 μm /pixels; 0.75 μm z-step) were taken within ~70 μm below the pial surface, where layer 1 dendrites of layer 5 pyramidal neurons were present. Images of two identical dendrites were taken at intervals of 24 h. Mice were recovered from anesthesia after the first imaging session and returned to their home cages until the next session.

2.4. *Image analysis*

The turnover rate, density, head width, and neck length of postsynaptic dendritic protrusions were analyzed with Neurolucida (MicroBrightField, Williston, VT, USA) from three-dimensional two-photon or confocal z-stacks. Morphometric analysis of dendritic protrusions was in accordance with a previous report (Grutzendler et al., 2002).

Filopodia were identified as long and thin structures (ratio of head width to neck width $< 1.2:1$; ratio of protrusion length to neck width $> 3:1$). The other protrusions were classified as spines. These protrusions were considered the same between first and second images based on their spatial relationship to adjacent landmarks (e.g., axonal and dendritic orientations) and their relative position to immediately adjacent protrusions. Protrusions between two successive frames were considered different if they were located $> 0.7 \mu\text{m}$ from their expected positions based on the first image. The formation and elimination rates for the protrusions were defined as the fraction of protrusions that appeared and disappeared, respectively, between two successive frames, relative to the total protrusion number. Protrusion turnover rate was defined as the sum of the protrusions formed and eliminated divided by twice the total number of protrusions. Data were collected from 10–12 dendrites and 601–1350 protrusions in four mice.

2.5. Statistical analysis

To determine statistical significance, we used the Student's t test, one-way ANOVA followed by Tukey's multiple-comparison test, two-way ANOVA followed by Bonferroni's multiple-comparison test, or a Kolmogorov–Smirnov test. All statistical analyzes were performed with GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA). All data are presented as the mean $\pm$ SEM.

3. Results

3.1. *Prenatal testosterone exposure induces increased instability of dendritic in juvenile and young adult mice*

We performed *in vivo* two-photon imaging in L1 dendrites of the frontal cortex of prenatally testosterone-exposed mice and control mice whose dams were treated with sesame oil, and analyzed the dynamics of dendritic protrusions during a 24-h period (Figure 1a, e). Dendritic protrusions were classified into two groups: spines and filopodia, and formation or elimination rates were analyzed in each group. Filopodia become less abundant with synaptic development, and are less stable than the spines (Grutzendler et al., 2002). In 4-week-old prenatally testosterone-exposed mice, the ratio of spines to total protrusions was significantly lower than that in age-matched control mice (Figure 1b; control: $82 \pm 2\%$; testosterone: $73 \pm 2\%$; $p = 0.0090$; unpaired t test), whereas the ratio of filopodia was significantly higher (Figure 1b; control: $18 \pm 2\%$; testosterone: $27 \pm 2\%$; $p = 0.0090$; unpaired t test). In the spine dynamics of 4-week-old prenatally testosterone-exposed mice, both formation and elimination rates were significantly higher than those in control mice (Figure 1c; formation rate: control: $5 \pm 1\%$; testosterone: $17 \pm 5\%$; $p = 0.0457$; elimination rate: control: $3 \pm 1\%$; testosterone: $21 \pm 2\%$; $p < 0.0001$; unpaired t test). No significant differences were observed in the filopodium dynamics of 4-week-old testosterone-exposed mice (Figure 1d; formation rate: control: $50 \pm 10\%$; testosterone: $70 \pm 10\%$; $p = 0.2981$; elimination rate: control: $48 \pm 7\%$; testosterone: $70 \pm 9\%$; $p = 0.0596$; unpaired t test). In 8-week-old testosterone-exposed mice, no differences were observed in both the ratio of spines (Figure 1f; control: $91 \pm 2\%$; testosterone: $86 \pm 2\%$; $p = 0.0715$; unpaired t test) and filopodia (Figure 1f; control: $9 \pm 2\%$; testosterone: $14 \pm 2\%$; $p = 0.0715$; unpaired t test)

to total protrusions between 8-week-old testosterone-exposed mice and age-matched control mice. Both formation and elimination rates in 8-week-old testosterone-exposed mice were higher than those in control mice (Figure 1g; formation rate: control: $3 \pm 1\%$; testosterone: $14 \pm 4\%$; $p = 0.0195$; elimination rate: control: $4 \pm 1\%$; testosterone: $13 \pm 4\%$; $p = 0.0230$; unpaired t test). There were no significant differences between 8-week-old control and testosterone-exposed mice in filopodium formation and elimination rate (Figure 1h; formation rate: control: $60 \pm 20\%$; testosterone: $90 \pm 30\%$; $p = 0.4760$; elimination rate: control: $40 \pm 10\%$; testosterone: $70 \pm 20\%$; $p = 0.0670$; unpaired t test).

To evaluate protrusion stabilization with neuronal circuitry development and its disruption in prenatally testosterone-exposed mice, we analyzed the 24-h turnover rate of spines and filopodia at 4 and 8 weeks of age during synaptic development. In prenatally testosterone-exposed mice, spine turnover rate was significantly higher than that in control mice throughout synaptic maturation (Figure 2a; 4 weeks: $p = 0.0013$; 8 weeks: $p = 0.0090$; two-way ANOVA followed by Bonferroni's test). There were no significant differences in filopodium turnover rate between testosterone-exposed and control mice at 4 and 8 weeks of age (Figure 2b; 4 weeks: $p = 0.6028$; 8 weeks: $p = 0.2144$; two-way ANOVA followed by Bonferroni's test). This may be because of the high turnover rate of filopodia over 24 h, even in a control group. These results indicate that normal development of dendritic protrusions is disrupted in testosterone-exposed mice.

3.2. Dendritic spines and filopodia are increased in prenatally testosterone-exposed mice

Next, we analyzed the density of dendritic spines and filopodia in control and prenatally testosterone-exposed mice. In 4-week-old testosterone-exposed mice, the density of filopodia was significantly higher than that in age-matched control mice, whereas spine density was not different between the groups (Figure 3a; protrusions: control: 0.51 ± 0.02 protrusions/ μm ; testosterone: 0.62 ± 0.02 protrusions/ μm ; $p = 0.0011$; spines: control: 0.42 ± 0.02 spines/ μm ; testosterone: 0.45 ± 0.02 spines/ μm ; $p = 0.2114$; filopodia: control: 0.09 ± 0.01 filopodia/ μm ; testosterone: 0.17 ± 0.01 filopodia/ μm ; $p = 0.0010$; unpaired t test). However, 8-week-old testosterone-exposed mice exhibited a higher density of both dendritic spines and filopodia than age-matched control mice (Figure 3b; protrusions: control: 0.39 ± 0.01 protrusions/ μm ; testosterone: 0.54 ± 0.03 protrusions/ μm ; $p < 0.0001$; spines: control: 0.35 ± 0.01 spines/ μm ; testosterone: 0.46 ± 0.03 spines/ μm ; $p = 0.0016$; filopodia: control: 0.034 ± 0.007 filopodia/ μm ; testosterone: 0.07 ± 0.01 filopodia/ μm ; $p = 0.0038$; unpaired t test). These results suggest that prenatal testosterone exposure causes an abnormal increase of synapses.

3.3. *Prenatally testosterone-exposed mice show abnormal morphology of dendritic protrusions*

Prenatal testosterone exposure caused a left shift of frequency distribution of dendritic protrusion width both at 4 (Figure 4a; $p < 0.0001$; Kolmogorov–Smirnov test) and 8 weeks of age (Figure 4b; $p < 0.0001$; Kolmogorov–Smirnov test). These results show that the dendritic protrusion width of prenatally testosterone-exposed mice shrank throughout synaptic development. Frequency distribution of dendritic protrusion length in testosterone-exposed mice was shifted to the right at 4 weeks of age (Figure 4c; $p < 0.0001$; Kolmogorov–Smirnov test), whereas it was shifted to the left at 8 weeks of age

222 (Figure 4d; $p = 0.0002$; Kolmogorov–Smirnov test). These results indicate that the
223 dendritic protrusion length of testosterone-exposed mice is longer than that of control
224 mice at the immature stage of synapse development and it becomes shorter at a mature
225 stage. This is because of increased density of not only filopodia but also spines in
226 8-week-old testosterone-exposed mice.

4. Discussion

ASD is a neurobehavioral syndrome with a heterogeneous phenotype, and its overall prevalence is about 1/100 (Fernell and Gillberg, 2010). Men are more likely to be affected than women, with a 4:1 ratio (Abrahams and Geschwind, 2008; Freitag, 2007). Many lines of evidence, including human pathological, genetic, and animal model studies, suggest that disturbance in synaptic homeostasis may be a biological process associated with ASD (Delorme et al., 2013; Toro et al., 2010). The pathogenesis of ASD is most likely polygenic and potentially epistatic; however, perinatal environmental factors may also interact with genetic factors to increase risk (Gardener et al., 2011; Hallmayer et al., 2011). In the present study, we found that prenatally testosterone-exposed mice showed abnormal synaptic instability in the frontal cortex at the developmental stage of synapse formation, and the synaptic instability persisted into young adulthood. Both dendritic spines and filopodia were increased excessively, and their morphology was abnormal during this period in prenatally testosterone-exposed mice. These results suggest that synaptic instability, excess density, and abnormal morphology of dendritic spines and filopodia are the synaptic basis of neurodevelopmental deficits in prenatally testosterone-exposed mice, which exhibit autistic-like behavior. This study provides novel insights into the pathology of neurodevelopmental disorders.

We found synaptic instability in prenatally testosterone-exposed mice. Other studies using genetic animal models of neurodevelopmental disorders associated with autistic syndrome have also demonstrated abnormal dynamics of the synapse. Genetic mouse models of ASD, human 15q11-13 duplication and neuroligin-3 R451C mutation, exhibit increased instability of PSD-95-positive dendritic spines (Isshiki et al., 2014). A

mouse model of fragile X syndrome, *FMR1* knock-out mice, shows synaptic instability and insensitivity to sensory experience (Cruz-Martin et al., 2010; Pan et al., 2010). *MECP2* duplication syndrome model mice, expressing the human *MECP2* gene at twice the normal levels, also show abnormal instability of the synapse (Jiang et al., 2013). These reports and our present study suggest that neurodevelopmental disorders associated with autistic syndrome may share a common synaptic pathology.

From studies of post-mortem ASD human brains, it has been demonstrated that ASD patients show an increase in spine density in the frontal, temporal, and parietal lobes (Hutsler and Zhang, 2010). The brains from fragile X patients also show elevated spine density (Irwin et al., 2001). These lines of evidence are consistent with our present study that demonstrated excess density of spines in prenatally testosterone-exposed mice. It should be noted that the above genetic models of neurodevelopmental disorders associated with autistic syndrome have not demonstrated increased spine density (Cruz-Martin et al., 2010; Isshiki et al., 2014; Jiang et al., 2013; Pan et al., 2010). Prenatally testosterone-exposed mice reproduced both features of ASD synaptic pathology: synaptic instability and excess spines.

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References

- Abbott, D.H., Barnett, D.K., Bruns, C.M., Dumesic, D.A., 2005. Androgen excess fetal programming of female reproduction: a developmental aetiology for polycystic ovary syndrome? *Hum Reprod Update* 11, 357-374.
- Abrahams, B.S., Geschwind, D.H., 2008. Advances in autism genetics: on the threshold of a new neurobiology. *Nat Rev Genet* 9, 341-355.
- Association, A.P., 2013. *Diagnostic and Statistical Manual of Mental Disorders*, 5th ed. American Psychiatric Association.
- Azziz, R., Marin, C., Hoq, L., Badamgarav, E., Song, P., 2005. Health care-related economic burden of the polycystic ovary syndrome during the reproductive life span. *J Clin Endocrinol Metab* 90, 4650-4658.
- Baron-Cohen, S., Knickmeyer, R.C., Belmonte, M.K., 2005. Sex differences in the brain: implications for explaining autism. *Science* 310, 819-823.
- Bonthuis, P.J., Cox, K.H., Searcy, B.T., Kumar, P., Tobet, S., Rissman, E.F., 2010. Of mice and rats: key species variations in the sexual differentiation of brain and behavior. *Front Neuroendocrinol* 31, 341-358.
- Chichinadze, K., Chichinadze, N., 2008. Stress-induced increase of testosterone: contributions of social status and sympathetic reactivity. *Physiol Behav* 94, 595-603.
- Cruz-Martin, A., Crespo, M., Portera-Cailliau, C., 2010. Delayed stabilization of dendritic spines in fragile X mice. *J Neurosci* 30, 7793-7803.
- Delorme, R., Ey, E., Toro, R., Leboyer, M., Gillberg, C., Bourgeron, T., 2013. Progress toward treatments for synaptic defects in autism. *Nat Med* 19, 685-694.
- Feng, G., Mellor, R.H., Bernstein, M., Keller-Peck, C., Nguyen, Q.T., Wallace, M., Nerbonne, J.M., Lichtman, J.W., Sanes, J.R., 2000. Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. *Neuron* 28, 41-51.
- Fernell, E., Gillberg, C., 2010. Autism spectrum disorder diagnoses in Stockholm preschoolers. *Res Dev Disabil* 31, 680-685.
- Franks, S., 1995. Polycystic ovary syndrome. *N Engl J Med* 333, 853-861.
- Freitag, C.M., 2007. The genetics of autistic disorders and its clinical relevance: a review of the literature. *Mol Psychiatry* 12, 2-22.
- Gardener, H., Spiegelman, D., Buka, S.L., 2011. Perinatal and neonatal risk factors for autism: a comprehensive meta-analysis. *Pediatrics* 128, 344-355.

308 Goodarzi, M.O., Dumesic, D.A., Chazenbalk, G., Azziz, R., 2011. Polycystic ovary
 309 syndrome: etiology, pathogenesis and diagnosis. *Nat Rev Endocrinol* 7, 219-231.
 310 Grutzendler, J., Kasthuri, N., Gan, W.B., 2002. Long-term dendritic spine stability in the
 311 adult cortex. *Nature* 420, 812-816.
 312 Hallmayer, J., Cleveland, S., Torres, A., Phillips, J., Cohen, B., Torigoe, T., Miller, J.,
 313 Fedele, A., Collins, J., Smith, K., Lotspeich, L., Croen, L.A., Ozonoff, S., Lajonchere,
 314 C., Grether, J.K., Risch, N., 2011. Genetic heritability and shared environmental factors
 315 among twin pairs with autism. *Arch Gen Psychiatry* 68, 1095-1102.
 316 Hines, M., 2011. Prenatal endocrine influences on sexual orientation and on sexually
 317 differentiated childhood behavior. *Front Neuroendocrinol* 32, 170-182.
 318 Hutsler, J.J., Zhang, H., 2010. Increased dendritic spine densities on cortical projection
 319 neurons in autism spectrum disorders. *Brain Res* 1309, 83-94.
 320 Irwin, S.A., Patel, B., Idupulapati, M., Harris, J.B., Crisostomo, R.A., Larsen, B.P.,
 321 Kooy, F., Willems, P.J., Cras, P., Kozlowski, P.B., Swain, R.A., Weiler, I.J., Greenough,
 322 W.T., 2001. Abnormal dendritic spine characteristics in the temporal and visual cortices
 323 of patients with fragile-X syndrome: a quantitative examination. *Am J Med Genet* 98,
 324 161-167.
 325 Isshiki, M., Tanaka, S., Kuriu, T., Tabuchi, K., Takumi, T., Okabe, S., 2014. Enhanced
 326 synapse remodelling as a common phenotype in mouse models of autism. *Nat Commun*
 327 5, 4742.
 328 Jiang, M., Ash, R.T., Baker, S.A., Suter, B., Ferguson, A., Park, J., Rudy, J., Torsky, S.P.,
 329 Chao, H.T., Zoghbi, H.Y., Smirnakis, S.M., 2013. Dendritic arborization and spine
 330 dynamics are abnormal in the mouse model of MECP2 duplication syndrome. *J*
 331 *Neurosci* 33, 19518-19533.
 332 Keefe, D.L., 2002. Sex hormones and neural mechanisms. *Arch Sex Behav* 31, 401-403.
 333 Lennartsson, A.K., Kushnir, M.M., Bergquist, J., Billig, H., Jonsdottir, I.H., 2012. Sex
 334 steroid levels temporarily increase in response to acute psychosocial stress in healthy
 335 men and women. *Int J Psychophysiol* 84, 246-253.
 336 Palomba, S., Marotta, R., Di Cello, A., Russo, T., Falbo, A., Orio, F., Tolino, A., Zullo,
 337 F., Esposito, R., La Sala, G.B., 2012. Pervasive developmental disorders in children of
 338 hyperandrogenic women with polycystic ovary syndrome: a longitudinal case-control
 339 study. *Clin Endocrinol (Oxf)* 77, 898-904.
 340 Pan, F., Aldridge, G.M., Greenough, W.T., Gan, W.B., 2010. Dendritic spine instability

and insensitivity to modulation by sensory experience in a mouse model of fragile X syndrome. *Proc Natl Acad Sci U S A* 107, 17768-17773.

Penzes, P., Cahill, M.E., Jones, K.A., VanLeeuwen, J.E., Woolfrey, K.M., 2011. Dendritic spine pathology in neuropsychiatric disorders. *Nat Neurosci* 14, 285-293.

Phoenix, C.H., Goy, R.W., Gerall, A.A., Young, W.C., 1959. Organizing action of prenatally administered testosterone propionate on the tissues mediating mating behavior in the female guinea pig. *Endocrinology* 65, 369-382.

Schuurmans, C., Kurrasch, D.M., 2013. Neurodevelopmental consequences of maternal distress: what do we really know? *Clin Genet* 83, 108-117.

Toro, R., Konyukh, M., Delorme, R., Leblond, C., Chaste, P., Fauchereau, F., Coleman, M., Leboyer, M., Gillberg, C., Bourgeron, T., 2010. Key role for gene dosage and synaptic homeostasis in autism spectrum disorders. *Trends Genet* 26, 363-372.

Trachtenberg, J.T., Chen, B.E., Knott, G.W., Feng, G., Sanes, J.R., Welker, E., Svoboda, K., 2002. Long-term in vivo imaging of experience-dependent synaptic plasticity in adult cortex. *Nature* 420, 788-794.

Walters, K.A., Allan, C.M., Handelsman, D.J., 2012. Rodent models for human polycystic ovary syndrome. *Biol Reprod* 86, 149, 141-112.

Xu, H.T., Pan, F., Yang, G., Gan, W.B., 2007. Choice of cranial window type for in vivo imaging affects dendritic spine turnover in the cortex. *Nat Neurosci* 10, 549-551.

Xu, X.J., Shou, X.J., Li, J., Jia, M.X., Zhang, J.S., Guo, Y., Wei, Q.Y., Zhang, X.T., Han, S.P., Zhang, R., Han, J.S., 2013. Mothers of autistic children: lower plasma levels of oxytocin and Arg-vasopressin and a higher level of testosterone. *PLoS One* 8, e74849.

Xu, X.J., Zhang, H.F., Shou, X.J., Li, J., Jing, W.L., Zhou, Y., Qian, Y., Han, S.P., Zhang, R., Han, J.S., 2015. Prenatal hyperandrogenic environment induced autistic-like behavior in rat offspring. *Physiol Behav* 138, 13-20.

Yang, G., Pan, F., Parkhurst, C.N., Grutzendler, J., Gan, W.B., 2010. Thinned-skull cranial window technique for long-term imaging of the cortex in live mice. *Nat Protoc* 5, 201-208.

Legends

Figure 1: Prenatal exposure to excess testosterone causes instability of dendritic spines in the frontal cortex. (a, e) *In vivo* time-lapse two-photon imaging of L1 dendrites of L5 pyramidal neurons in 4- and 8-week-old control (control: $n = 10$ dendrites from four mice and $n = 12$ dendrites from four mice) and prenatally testosterone propionate-exposed (testosterone: $n = 11$ dendrites from four mice and $n = 12$ dendrites from four mice) mice. Repeated images of the same dendrites over 24 h in each group show increased formation (filled arrowheads) and elimination (open arrowheads) of dendritic spines in prenatally testosterone-exposed mice. Images are maximum projections (3–7 optical sections, 0.75 μm apart). (b, f) Percentages of spines and filopodia as a proportion of total dendritic protrusions in control and prenatally testosterone-exposed mice at 4 (b) and 8 (f) weeks of age. The percentage of spines was lower and that of filopodia was greater in 4-week-old testosterone-exposed mice than in control mice, whereas neither of them were altered in 8-week-old testosterone-exposed mice. (c, g) Percentages of spines formed or eliminated as a proportion of total spines. Testosterone-exposed mice showed higher formation and elimination rates of spines at 4 (c) and 8 (g) weeks of age. (d, h) Percentages of filopodia formed or eliminated as a proportion of total filopodia. No differences were observed both in formation and elimination rates of filopodia between control and testosterone-exposed mice at 4 (d) and 8 (h) weeks of age. Data are presented as the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Student's t test. Scale bar, 5 μm .

Figure 2: Instability of dendritic spines in prenatally testosterone-exposed mice becomes persistent during synaptic maturation. (a) Compiled turnover rates of spines

(ratio of spines formed and eliminated to twice the total number of spines) of 4- and 8-week-old control and prenatally testosterone-exposed mice. Prenatally testosterone-exposed mice displayed higher spine turnover rates than control mice throughout synaptic development. **(b)** Compiled turnover rates of filopodia at 4 and 8 weeks of age. No differences were observed between control and testosterone-exposed mice. Data are presented as the mean $\pm$ SEM. ** $p < 0.01$, two-way ANOVA followed by Bonferroni's test.

Figure 3: Dendritic spines and filopodia are increased in prenatally testosterone-exposed mice. Density of dendritic spines and filopodia in 4- **(a)** and 8- **(b)** week-old control and testosterone-exposed mice. Only filopodium density was increased in 4-week-old testosterone-exposed mice, whereas both spine and filopodium density were increased in 8-week-old testosterone-exposed mice. Data are presented as the mean $\pm$ SEM. ** $p < 0.01$, *** $p < 0.001$, Student's t test.

Figure 4: Prenatally testosterone-exposed mice show abnormal morphology of dendritic protrusions. Morphology of dendritic protrusions including spines and filopodia. **(a, b)** Cumulative frequency distribution of protrusion width in control and prenatally testosterone-exposed mice at 4 **(a)** and 8 **(b)** weeks of age. At both ages, dendritic protrusions were shrunk in testosterone-exposed mice. **(c, d)** Cumulative frequency distribution of protrusion length in control and prenatally testosterone-exposed mice at 4 **(c)** and 8 **(d)** weeks of age. Testosterone-exposed mice exhibited longer dendritic protrusions at 4 weeks of age and shorter protrusions at 8 weeks of age. *** $p < 0.001$, Kolmogorov–Smirnov test.

Figure 1.

Figure 2

a

b

Figure 3

a

b

Figure 4

a

4-week-old

b

8-week-old

c

4-week-old

d

8-week-old