Molecular Psychiatry (2016) 00, 1–7

www.nature.com/mp

ORIGINAL ARTICLE

Prevention of behavioral deficits in rats exposed to folate receptor antibodies: implication in autism

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Folate receptor alpha (FRα) autoantibodies have been associated with fetal abnormalities and cerebral folate deficiency-related developmental disorders. Over 70% of the children with autism spectrum disorders (ASD) are positive for these autoantibodies and high-dose folinic acid is beneficial in treating these children. Here we show that antibodies (Abs) to the rat FRα administered during gestation produce communication, learning and cognitive deficits in a rat model that can be prevented by folinic acid and dexamethasone. FRα Ab can trigger inflammation as well as block folate transport to the fetus and to the developing brain to produce the functional deficits. In humans, exposure to FRα autoantibodies during fetal development and infancy could contribute to brain dysfunction such as that seen in ASD and other developmental disorders. Identifying women positive for the autoantibody and treating them with high-dose folinic acid along with other interventions to lower the autoantibody titer are effective strategies that may be considered to reduce the risk of having a child with developmental deficits.

Molecular Psychiatry advance online publication, 20 September 2016; doi:10.1038/mp.2016.153

INTRODUCTION

Folate is an essential B-complex vitamin required for the transfer of carbon units in the intermediary metabolism of amino acids, purines, pyrimidines and in the production of S-adenosylmethionine for methylation reactions.[1] Folate is particularly essential during pregnancy as fetal development is a time of continuous DNA synthesis and cell division. Deficiency during pregnancy can produce fetal malformations and miscarriage.[2,3] Folate supplementation can prevent neural tube defect pregnancy[4] and decrease the risk of autism spectrum disorders (ASD).[5,6] Schmidt *et al.*[7] reported that the protection afforded by prenatal folic acid supplementation was particularly beneficial in mothers and children with the *MTHFR* 677 C>T variant genotype.[7]

Dietary folate insufficiency, gene defects or compromised transport can lead to metabolic folate deficiency. The identification of folate receptor alpha (FRα)-specific autoantibodies that can block folate transport provided a potential mechanism for folate deficiency in the fetus.[8] These FRα autoantibodies have been identified in a majority of women with a history of neural tube defect pregnancy,[8,9] as well as with subfertility and preterm birth.[10,11] Direct proof that FRα-specific antibodies (Abs) are teratogenic to the embryo came from observations in a pregnant rat model of exposure to FRα Ab that caused resorption of embryos at higher doses and neural tube and cranio-facial malformations at lower doses.[12] Folinic acid and dexamethasone prevented malformations, suggesting that blocking of folate transport to the embryo and Ab-mediated inflammation may have a role in the pathology.[12]

FRα autoantibodies have also been found in infants with cerebral folate deficiency (CFD), substantiating the important role of folate in brain development, whereby FRα-specific autoantibodies could block folate transport across the choroid plexus.[13–15] CFD is a developmental disorder with decreased level of 5-methyltetrahydrofolate (MTHF) in the cerebrospinal fluid.[16] Consequently, these patients suffer from severe neurological symptoms, including marked irritability, cerebellar ataxia, slow head growth, psychomotor retardation and pyramidal tract signs. One-third also suffer from dyskinesia (for example, choreoathetosis and ballismus) and seizures.[13,14] These autoantibodies have also been found in other developmental disorders, including low functioning autism,[15] Rett syndrome[17] and in ASD.[18,19] Owing to the high prevalence of ASD in children with CFD, and favorable response to folinic acid, reports have hypothesized that ASD may be a less severe manifestation of CFD.[18,20]

Considering the high prevalence of FRα autoantibodies in children with CFD (89%)[13] and ASD (~70%),[18] we sought to establish proof of hypothesis that FRα Abs can produce the pathology of behavioral and cognitive deficits. Preliminary studies indicated that exposure to FRα Abs during gestation and preweaning in a rat model produced severe learning and behavioral deficits.[21] Therefore, we determined behavioral deficits in pups born to dams exposed to FRα Ab during gestation and evaluated the effect of folinic acid, which would provide adequate folate, and dexamethasone, which would suppress inflammation, during gestational Ab exposure.

MATERIALS AND METHODS

Rat model of FRα Ab exposure

Timed-pregnant postnatal day (PND) 50 Long Evans hooded rats (Charles River Laboratories, Wilmington, MA, USA) were anesthetized on gestational day (GD) 8 and a laparotomy was performed to record the number of implanted embryos. FRα Ab at a dose of 4 or 12 μg per embryo in 1 ml normal rat serum was administered by intraperitoneal (IP) injection, 1 h after the laparotomy. These doses were chosen because they allowed the implanted embryos to be carried to term and produce live pups. Treatment groups received 1 mg of folinic acid (GD7–GD12) IP and/or 0.5 mg

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Received 6 April 2016; revised 13 July 2016; accepted 18 July 2016

dexamethasone (GD7–GD10) intramuscularly. As the 12 μg per embryo pups showed more severe behavioral and cognitive deficits, this dose was chosen subsequently to evaluate response to folinic acid and dexamethasone treatments.

As shown in the flow chart (Supplementary Figure S1), pups born underwent behavioral tests between PND4 and PND70. As sham controls, we used rats that were injected with an equivalent amount of pooled rabbit immunoglobulin G (IgG) from preimmune serum (NRIgG). Following birth, the number of pups born to each litter in all groups were counted and compared with the number of implanted embryos noted during the laparotomy on GD8. Normal rats (simple controls) were also tested to determine baseline measures and behavior. All rats were fed a normal diet containing 2 mg folic acid per kg chow as recommended by the American Institute of Nutrition (1977) and had free access to food and water. The rats were maintained at 22 °C and on a 12 h light/dark cycle and the experimental protocols were approved by the Animal Care and Use Committee of the State University of New York, Downstate Medical Center, Brooklyn, NY, USA.

Testing for communication deficits

Isolated pups emit 40 kHz vocalizations, which normally elicits maternal search and retrieval of the pup, and thus has a crucial role in survival of the pup.[22] On PND4, pups were individually separated from the mother in a temperature-controlled room (25 °C) and ultrasonic vocalizations emitted by the pup over a 3-min period were recorded and analyzed (Supplementary Figure S2). Adult male ultrasonic vocalizations (on PND 50) upon interaction with a female were also analyzed to determine whether a deficit in communication persisted into adulthood.[22,23]

Sociability studies

Male rats underwent a sociability paradigm on PND40–PND45 where each rat's inclination to approach and engage in social interaction with a same sex rat was assessed[24] (Supplementary Figure S3).

Open field test

We used the open field test to assess locomotion as well as exploratory/anxiety-like behavior on PND40–PND45[25] (Supplementary Figure S4).

Place avoidance tasks

The place avoidance tasks require recognition and segregation of information obtained from both relevant and irrelevant stimuli and permits the assessment of spatial memory formation[26,27] (Supplementary Figure S5). The tests, consisting of passive, active and conflict place avoidance (PPA, APA, CPA), were conducted over 2 consecutive days between PND60–PND70.[21] Each place avoidance task consisted of 4–6 trials of 10 min each with 10 min rest periods. A number of parameters are recorded by an automated data acquisition system (Bio-Signal Group, Brooklyn, NY, USA).

Rabbit polyclonal antiserum to FRα

Recombinant rat FRα was produced and purified and polyclonal antiserum was generated in New Zealand white rabbits as previously described.[21] The IgG fraction was purified by affinity chromatography on a protein A column and used in the study. The antiserum contained two types of Abs: binding and blocking Abs to rat FRα. Binding Ab is defined as an Ab that binds to an epitope distant from the folate-binding site and, therefore, does not interfere with folate binding to FRα[28–30] (Supplementary Figure S6A). Blocking Ab is defined as an Ab that prevents the binding of folate when preincubated with apo FRα by virtue of the Ab directly or sterically interfering with folate binding[30] (Supplementary Figure S6B).

Immunohistochemistry

To examine inflammatory response induced by exposure to FRα Ab, GD14 rats were injected with FRα Ab or NRIgG (12 μg per embryo, for ~16 embryos). All animals received a second dose 16 h after the first dose. Six hours later, animals were killed and tissues were collected. A similar protocol was used to examine the effect of dexamethasone treatment in reducing the inflammation where animals were given 0.5 mg dexamethasone IP, 3 h prior to Ab injection. Tissues were fixed in Carnoy's solution, embedded in Paraplast (Fisher, Houston, TX, USA) and 5–7 μm sections were cut for immunohistochemistry. Injected FRα Ab was localized by probing sectioned tissue with peroxidase-conjugated goat anti-rabbit IgG (Pierce, Rockford, IL, USA) and then reacted with diaminobenzidine (DAB, Vector Laboratories, Burlingame, CA, USA) to develop the chromagen. Nuclear staining was carried out using hematoxylin (Vector Laboratories). For markers of inflammation, sections were incubated with mouse monoclonal anti-CD68 Ab (1:400 dilution, Abcam, Cambridge, MA, USA) or anti rat IL-1β Ab (5 μg ml^{-1}, R&D Systems, Minneapolis, MN, USA) overnight at 4 °C. Biotinylated anti-IgG was used as a secondary Ab, followed by incubation with ABC reagent and reaction with DAB to develop the chromagen.

Folate uptake and FRα Ab localization studies

To determine FRα Ab localization and its effect on folate uptake, GD14 rats were administered IP with 12 μg of FRα Ab or NRIgG per embryo and 5 μCi of ^{3}HPGA in 1 ml saline on GD15. The rats were killed on GD16 (48 h after the Ab dose). In another set of rats, a similar protocol was followed except the 5μCi of ^{3}HPGA was administered on GD16 and killed on GD17 (72 h after Ab dose). Tissue was homogenized in 0.1 M sodium phosphate buffer pH 7.4. Half of this homogenate was added to scintillation fluid to determine folic acid (^{3}HPGA) uptake. The other half of the tissue was treated with glycine/HCl pH 2.5 to detach Ab from receptor, supernatant fraction neutralized with 1 M dibasic sodium phosphate, followed by measuring immunoprecipitation of FRα labeled with ^{3}HPGA.

In order to examine the effect of dexamethasone treatment, GD14 rats were given 0.5 mg dexamethasone intramuscularly. Three hours later, they received FRα Ab or NRIgG (12 μg per embryo). All animals received a second dose of Ab 16 h later. Twenty hours after the first Ab or NRIgG injection, all rats were injected with 5 μCi of ^{3}HPGA. All animals were killed 4 h later and tissues were collected (placenta, embryo, yolk sac, uterus). Radioactivity in the tissues was then determined as above.

Statistical analysis

Statistical analysis of behavioral studies, which involved $\geqslant 3$ groups was carried out using one-way analysis of variance (ANOVA). If the one-way ANOVA showed statistical significance ($P < 0.05$), *post-hoc* analysis was carried out using Tukey's Honest Significant Difference. For analysis between two groups, Student's *t*-test was used to determine statistical significance (GraphPad Software, La Jolla, CA, USA). Values plotted are the mean and error bars represent s.e.m. Sample sizes (*n*) are indicated in the corresponding figure or figure legend. Based on our published data (2% of controls and 75% of Ab-exposed affected pups),[21] we calculated the necessary sample sizes. We needed pups from two dams in each group to yield a statistically significant result with 95% statistical power, accepting a 95% confidence interval in a two-tailed *post-hoc* test following a significant main effect ANOVA. This calculation was carried out using the Statistical Power Calculator from DSS Research, Fort Worth, TX, USA.

RESULTS

In the various experimental and treatment groups, no decrease in the number of live pups born was observed in any group. All pups were examined for any gross abnormalities, and none were noted. There was also no difference in the ratio of males to females in litters of each group. Pups were weighed on PND10 and PND25 and showed no significant weight differences in the various groups (18.6 ± 0.5 and 70.8 ± 2.0 g, respectively).

Early communication deficits

Following administration of the rat FRα Ab at 4 or 12 μg per embryo to pregnant dams on GD8, a dose-dependent decrease in isolation-induced vocalizations was observed on PND4 compared with sham controls ($P < 0.001$; Figure 1a). A significant improvement in the number of vocalizations was seen when folinic acid, dexamethasone or folinic acid and dexamethasone ($P < 0.001$) were administered along with the Ab. Additionally, these pups also presented with a significant delay to first call; corrected in all treatment groups (Figure 1b, $P < 0.001$) by folinic acid and dexamethasone.

Adult communication deficits

Communication deficits on PND4 among *in utero* Ab exposed rats persisted into adulthood as Ab exposed male rats significantly decreased vocalizations around a female, compared with controls. Folinic acid and dexamethasone normalized this behavior (Figure 1c, $P < 0.01$).

Sociability deficits

When adult male rats exposed *in utero* to Ab were tested in a sociability paradigm, they demonstrated significantly decreased social interaction as compared with controls ($P < 0.001$). This impaired behavior was prevented by folinic acid ($P < 0.01$), dexamethasone ($P < 0.02$) or folinic acid plus dexamethasone ($P < 0.01$; Figure 1d).

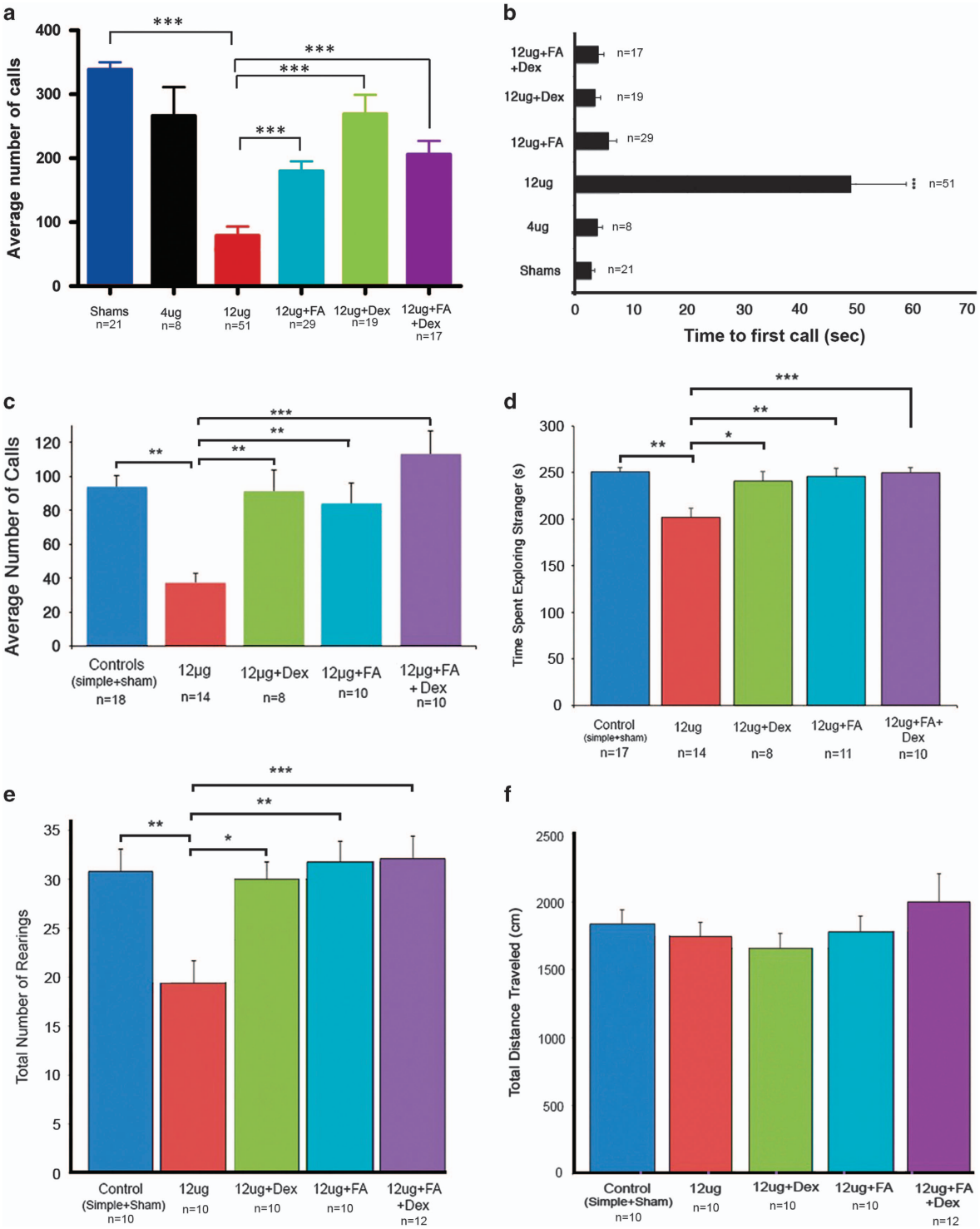

Figure 1. Vocalizations and social behavior in rats exposed to FRα antibodies *in utero*. (**a**) Isolation induced ultrasonic vocalization by pups when separated from the mother on postnatal day 4 and correction of this deficit by dexamethasone (Dex), folinic acid (FA) and combination of the two, (**b**) latency to first call, (**c**) male vocalizations in the proximity of a female, (**d**) male sociability (**e**) rearings and (**f**) movement in the open field test. FR, folate receptor. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

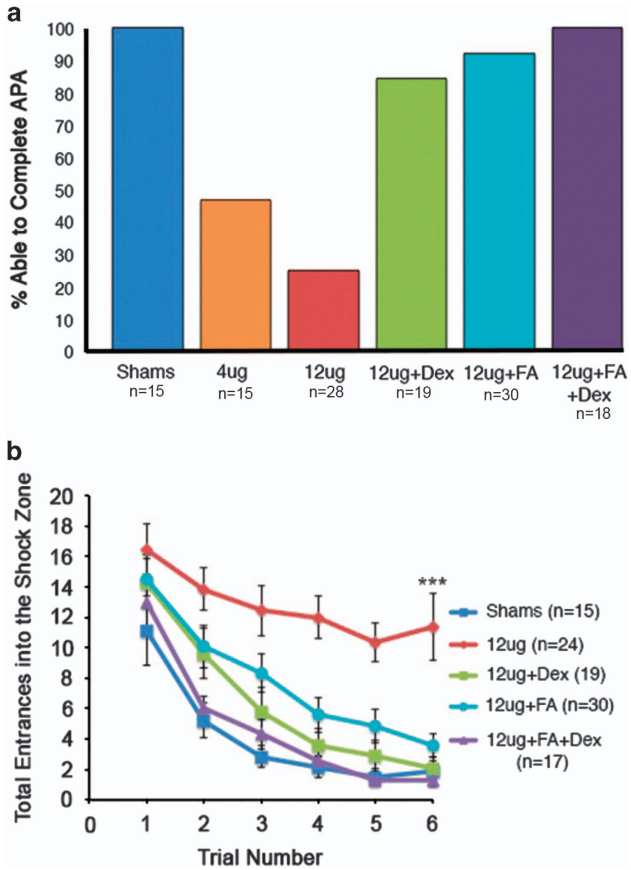

Figure 2. Learning deficits in rats exposed to FRα antibody *in utero*. (**a**) Active place avoidance test results and (**b**) learning curves for various treatment groups as indicated by decreased entrances into the shock zone in subsequent trials. ***$P < 0.001$ compared with all the groups. APA, place avoidance; Dex, dexamethasone, FA, folinic acid.

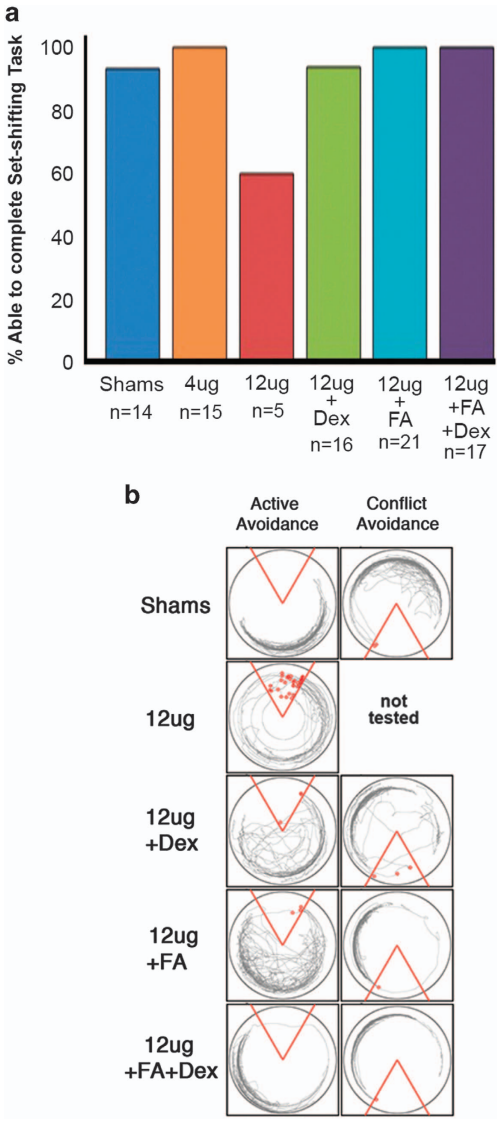

Figure 3. Set-shifting deficits in rats exposed to FRα antibody *in utero*. (**a**) Conflict place avoidance test results for various treatment groups. Of the animals exposed to antibody during gestation, only 25% could pass the active place avoidance task and 40% of these could not complete the conflict avoidance task. This is indicative of impairment in completing set-shifting tasks as 93% of sham animals that passed active place avoidance could pass conflict avoidance. This impairment was corrected by dexamethasone (Dex), folinic acid (FA) and folinic acid plus dexamethasone treatment. (**b**) Tracing the movement of the rat in the fourth trial of the active and conflict place avoidance tasks.

Open field testing for anxiety

Rats exposed to Ab *in utero* showed increased anxiety in the open field test. These animals had significantly decreased numbers of rearings compared with controls (Figure 1e, $P < 0.02$). They did not present with any significant decrease in total distance traveled, indicating no motor abnormalities (Figure 1f).

Learning, memory and set-shifting deficits

The rats were further tested for learning and memory in a series of tests of increasing complexity that evaluated their ability to learn and remember a task over an extended period and then be able to switch to a new task. When animals exposed to FRα Ab *in utero* were tested in a hierarchy of place avoidance tasks between PND60 and PND70, all animals could learn the PPA task by successfully avoiding a stationary shock sector using olfactory and visual cues. However, when they continued on to the APA task, 50% of the 4 μg, and 75% of 12 μg Ab-exposed animals failed to learn the task (Figure 2a). Protection from these deficits was provided by administration of folinic acid, dexamethasone and folinic acid plus dexamethasone along with the FRα Ab (Figure 2a). A majority of the animals in all treatment groups could successfully acquire the APA task, with the learning curve for animals treated with folinic acid plus dexamethasone being almost identical to control animals (Figure 2b).

Not all of the 12 μg Ab/embryo-exposed rats that passed the active avoidance task were able to pass the CPA task, (Figure 3a). Further, administration of folinic acid and dexamethasone rescued these rats from the CPA learning deficits (Figure 3a). Representative tracings illustrating the movement of rats in the APA and CPA tasks are shown in Figure 3b, demonstrating the inability of the Ab-exposed animals to learn the tasks as indicated by repeated entry into the shock sector and the ability of the animals in the treatment groups to learn the tasks. No significant difference between the treatment groups of folinic acid, dexamethasone or folinic acid plus dexamethasone following Ab exposure was noted in any of the behavioral and cognitive studies, suggesting no detrimental effect of the steroid use.

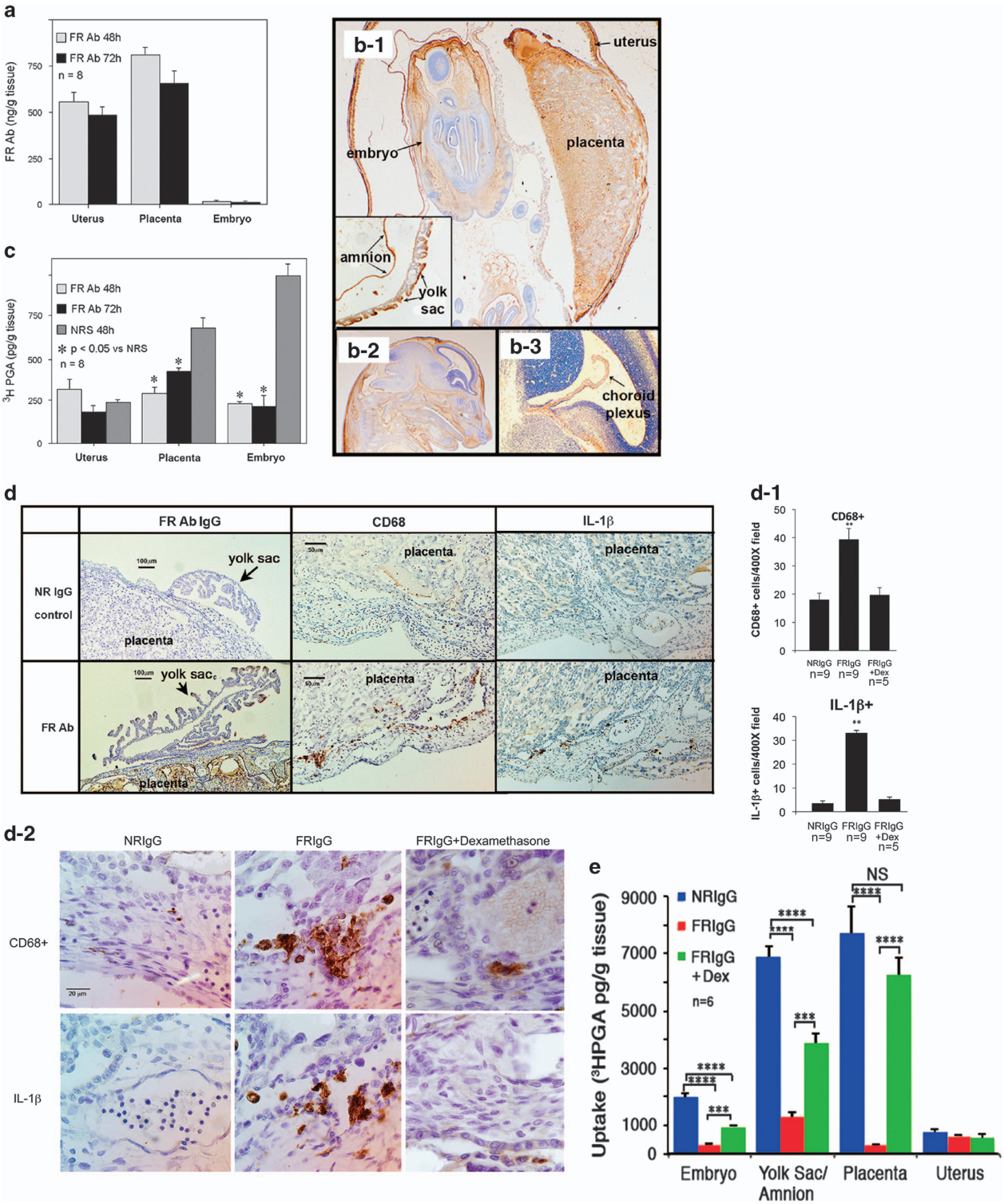

Figure 4. Antibody (Ab) localization, effect on ^{3}HPGA uptake and inflammation produced in gestational day 15 pregnant rats. (**a**) Antibody accumulation, (**b**) immunohistochemical localization, (**c**) blocking of ^{3}HPGA uptake by antibody, (**d**) increase in inflammatory markers and (**e**) correction of this by dexamethasone (Dex), (**f**) correction of ^{3}HPGA uptake by dexamethasone. IL, interleukin; NRIgG, immunoglobulin G from preimmune serum; NS, not significant. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

Folate uptake, localization of Ab and inflammation

During fetal development, maternal folate is the only source of folate and this has to traverse the placental barrier in order to enter fetal circulation.[31] One potential mechanism by which the Abs can affect brain development is by blocking folate transport to the fetus. Ab localization was seen mostly in the placenta and in the uterus and much less in the embryo (Figure 4a). Immunohistochemical analysis showed extensive accumulation in the placenta, the uterine wall and the yolk sac (Figure 4b-1) Within the embryo, Ab was mostly localized to epithelial cells in the head

region (Figure 4b-2) and to the epithelial cells of the choroid plexus (Figure 4b-3). During this period, folate transport to the embryo was significantly compromised as indicated by a 56% and 41% decrease in ^{3}HPGA accumulation in the placenta at 48 and 72 h, respectively, and ~76% decrease in the embryo (Figure 4c). Ab localization within the placenta and yolk sac appeared to affect folate transfer from the mother to the fetus and folate uptake in the fetus. Localization of IgG was not seen in the NRIgG-injected dam.

Rodent studies of maternal inflammation during pregnancy that increase risk of neurodevelopmental deficits in the offspring have shown increased $CD68^{+}$ cells and interleukin (IL)-1β in the placenta.[32,33] This prompted us to examine whether the localization of this Ab in the placenta induced a similar inflammatory response. Consistent with the presence of Ab on the placenta, increased expression of $CD68^{+}$ cells and IL-1β was seen in similar areas (Figure 4d). Dexamethasone treatment significantly decreased the expression of both these markers for inflammation (Figures 4(d-1 and d-2)). Dexamethasone treatment also significantly increased folate transport to the placenta, yolk sac and amnion and the embryo (Figure 4e). Rats exposed to Ab *in utero* were killed after PND 60 and examined for the presence of any structural changes in the brain using coronal sections stained with hematoxylin–eosin and for markers of inflammation. No discernable histological changes were observed compared with sham or simple controls.

DISCUSSION

In this study, we provide evidence that exposure to FRα-specific Ab during gestation in a rat results in the birth of pups with severe behavioral and learning deficits. Many of these deficits mirror core deficits of ASD, including deficits in communication, sociability and difficulty with set-shifting tasks. Lack of bonding, social interaction and verbal communication are some of the early indicators of autism development although these may not fully manifest until later in life. In the rodent model, ultrasonic vocalizations are one of the earliest forms of communication between the pups and their mother.[22] Thus evaluating the rat pups on PND4 is ideal. As observed in this study, lack of communication has been reported in many rodent models of autism-like behavior.[34–36] Additionally, we have shown the presence of variable symptoms of ASD, including increased anxiety in the open field test and learning deficits in the place avoidance tasks. We have shown that both of these core deficits and variable symptoms produced by Ab exposure can be prevented, or at the least attenuated, using gestational treatment with folinic acid and dexamethasone.

Further, intellectual disability has been found to be highly comorbid with ASD.[37] We have previously shown that exposure to Ab during gestation and the preweaning period in rats leads to learning deficits.[21] We have confirmed this effect of gestational exposure in the current study and, more importantly, shown that these learning deficits can be prevented by treatment with folinic acid and dexamethasone.

Overall, it appears that FRα Abs exert their effect on the fetus by decreasing transplacental transport of folate, which results in fetal folate deficiency. This explains why protection is afforded by folinic acid treatment where folinic acid, a reduced form of folate, is taken up by the reduced folate carrier, rather than by FRα, which is hindered by the Abs from transporting folate.

Epidemiological studies suggest a clear association between maternal exposure to infection and inflammation and increased risk of ASD in the offspring.[38] Maternal cytokine expression, including expression in the placenta, can affect the developing embryo or fetus, resulting in neurological and behavioral abnormalities.[39] These studies suggest that FRα Ab exposure in an animal model leads to a type II autoimmune reaction,[40] where there are increased Fc-receptor-bearing phagocytes, as demonstrated by the increased presence of $CD68^{+}$ macrophages. These macrophages appear to secrete increased amounts of IL-1β cytokine in the presence of FRα Ab, leading to increased inflammatory response. When the inflammatory response is suppressed with dexamethasone, there is more folate uptake along with rescue of behavioral deficits. Hence, the benefit afforded by dexamethasone treatment also appears to be the result of increased folate uptake, where the dexamethasone suppresses inflammatory reaction around FRα. The inflammatory response owing to autologous Abs in the FRα autoimmune disorder in humans may vary from that owing to the heterologous Abs we have used in our rat model. Regardless, the Abs appear to cause immune-mediated damage and block folate transport.

These mechanisms of impaired folate transport can potentially explain pregnancy-related disorders, including neural tube defects in the presence of FRα autoimmune disorder. Daily folate intake and folate supplementation during pregnancy may minimize the effects of the autoantibody but may not be sufficient in those with higher Ab titer. Therefore, identifying women positive for the autoantibody and treating them with high-dose folinic acid along with other interventions to lower the Ab titer are effective strategies for a favorable outcome. A case that utilized such a strategy showed positive results in preventing pregnancy-related complications owing to the presence of FRα autoimmune disorder.[41] Our rat study suggests that steroids and other immunosuppressant drugs could decrease placental inflammation induced by the FRα autoantibody. Consequently, further investigation of similar treatment options to prevent pregnancy-related complications owing to FRα autoimmune disorder is warranted.

It should be noted that the methods for the detection of FRα-specific autoantibodies in the serum of patients provide a measure of autoantibody titer in serum.[30] The FRα protein is a glycosylphosphatidylinositol-linked peripheral membrane protein and Abs directed against this protein would seek the target antigen on the membrane.[42] Therefore, Ab in circulation is likely to represent excess free Ab. As in many autoimmune disorders, fluctuations in Ab titer is a common occurrence,[43,44] and therefore testing patients multiple times during 2–6 months may be necessary to rule out the autoimmune disorder. The prevalence of autoantibodies in $>70\%$ of children with autism[18] and improvements in the core symptoms of autism with folinic acid treatment[15,18] provides compelling evidence linking the autoimmune disorder with autism. Parental FRα autoantibodies with either mother or father positive have also been linked to autism in children.[19] Particularly, mothers of children with ASD were found to have a significantly higher prevalence of FRα autoantibodies compared with controls (26% vs 3.3%, $P < 0.01$),[19] and therefore identifying and treating these mothers could potentially reduce the risk of autism in the offspring.

In conclusion, the findings of this study suggest severe behavioral and cognitive changes mirroring ASD symptoms following gestational Ab exposure in a rat model and protection afforded by folinic acid and dexamethasone treatment. This has major implications in the treatment of FRα autoimmune disorder in women during pregnancy and reducing the risk of autism in the offspring.

CONFLICT OF INTEREST

Two of the authors (JMS and EVQ) are inventors on a US patent for the detection of FRalpha autoantibodies issued to the Research Foundation of the State University of New York, USA. The other author declares no conflict of interest.

ACKNOWLEDGMENTS

Funding for this work was provided by Autism Speaks grant no. 8202.

AUTHOR CONTRIBUTIONS

AD, JMS and EVQ designed the studies. AD and JMS conducted all experiments. AD, JMS and EVQ participated in the writing of the manuscript.

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp)