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Are preconceptional stressful experiences crucial elements for the aetiology of ASD? Insights from an animal model

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Abstract

Autism spectrum disorders (ASD) are a group of neurodevelopmental disorders characterized by changes in social interactions, impaired language and communication, fear responses and presence of repetitive behaviours. Although the genetic bases of ASD are well documented, the recent increase in clinical cases of idiopathic ASD indicates that several environmental risk factors could play a role in ASD aetiology. Among these, maternal exposure to psychosocial stressors during pregnancy has been hypothesized to affect the risk for ASD in offspring. Here, we tested the hypothesis that preconceptional stressful experiences might also represent crucial elements in the aetiology of ASD. We previously showed that social isolation stress during adolescence results in a marked decrease in the brain and plasma concentrations of progesterone and in the quality of maternal care that these female rats later provide to their young. Here we report that male offspring of socially isolated parents showed decreased sociability, impairment in reversal learning, increased seizure susceptibility, reduced plasma oxytocin levels, and increased plasma and brain levels of BDNF, all features resembling an ASD-like phenotype. These alterations came with no change in spatial learning, aggression and anxiety. Altogether, the results suggest that preconceptional stressful experiences should be considered as crucial elements for the aetiology of ASD, and indicate that offspring of socially isolated parents may be a useful animal model to further study the neurobiological bases of ASD, avoiding the adaptations that may occur in other genetic or pharmacologic experimental models of these disorders.

Keywords: Parents' social isolation; social behaviour; reversal learning; seizures susceptibility; oxytocin; BDNF; rat; autism spectrum disorders

1. Introduction

Autism spectrum disorders (ASD) are a group of neurodevelopmental disorders characterized by changes in social interactions, impaired language and communication, fear responses and presence of repetitive behaviours. Although the genetic bases of ASD are well documented, the recent increase in clinical cases of idiopathic ASD indicates that congenital viral infections or dysregulation of the immune system, as well as several environmental risk factors, such as exposure to endocrine disrupting chemicals during development, could play a role in ASD aetiology (Chess et al., 1978; Beversdorf et al., 2005; Braunschweig et al., 2013; Kalkbrenner et al., 2014). Moreover, maternal exposure to psychosocial stressors across pregnancy has also been hypothesized to affect the risk for ASD in offspring (Ward, 1990; Kinney et al., 2008; Say et al., 2016).

Besides the gestational environment, which influences development of the fetal brain, other important factors to consider are preconceptional events. Newborns of mothers exposed to childhood maltreatment had a variation in brain structure (Moog et al., 2018) and were more likely to have ASD (Roberts et al., 2013); moreover, a relationship between ASD and both paternal and maternal psychopathologies has also been demonstrated in humans (Jokiranta et al., 2013). Likewise, pre-gestational stress in rats, induced by chronic unpredictable stress for 3 weeks, had enduring effects in the offspring, by significantly reducing sibling play behaviours and impacting plasticity in the hippocampus (Gemmel et al., 2017).

Our laboratory employs social isolation (ISO) of rats immediately after weaning as a model of chronic stress during adolescence that induces, in male adult animals, a plethora of biochemical and behavioural effects indicative of a state of anxiety and depression (Serra et al., 2000, 2003, 2005; Pisu et al., 2011a, 2011b). Additionally, social isolation in female rats results in a marked decrease in the brain and plasma concentrations of progesterone and allopregnanolone (Pisu et al., 2016), and a decrease in the quality of maternal care that these animals later provide to their young (Pisu et al., 2017).

We previously found that adult male offspring of ISO rats (ISO-O) shows increased basal cerebrocortical levels of allopregnanolone but no changes in emotional reactivity compared with the offspring of group-housed parents (GH-O). ISO-O rats also showed high circulating corticosterone levels, increased corticotrophin releasing hormone peptide and its type 1 receptor, indicative of an increased basal hypothalamic-pituitary-adrenal (HPA) axis activity, along with a reduced reactivity to stress (Pisu et al., 2013). The observation that these features are present in a mouse model of autism, the BTBT T+tf/J (Silverman et al., 2010; Frye and llaneza, 2010), prompted us to speculate that ISO-O may represent a useful animal model of ASD, in which preconceptional stressful

experiences of their parents may have played a crucial role for the aetiology of these disorders. Thus, the general aim of this paper is to confirm that the endophenotype of ISO-O rats is coherent with a model of ASD. Patients with ASD show a high degree of variability not only in the core symptom domains (social communication and restricted, repetitive behaviour, DMS-V) but also in the presence/severity of additional behaviours such as anxiety, increased sensitivity to convulsions, aggression, altered sensory processing (Argyropoulos et al., 2013). To reproduce core symptoms, we selected two behavioural patterns corresponding to those frequently observed in ASD for examination under laboratory conditions: (i) social interaction in the social transmission of flavour preference, and (ii) cognitive flexibility in a reversal learning task in the Morris water maze test. As associated comorbid traits we studied (i) seizures susceptibility by assessing sensitivity to isoniazid; (ii) anxiety-like behaviour by evaluating the exploratory response when a novel object was introduced in their home cage, and (iii) aggression in the resident-intruder test. At the completion of the study we measured brain and plasma levels of brain derived neurotrophic factor (BDNF), and plasma levels of oxytocin that, in rodents, are correlated with impairment in cognitive flexibility and social interaction (de Souza et al., 2013; Armeanu et al., 2017; Brondino et al., 2018). Likewise, abnormal serum concentrations of BDNF and blunted plasma oxytocin levels are prognostic biomarkers of ASD in children (Heinrichs et al., 2009; Leung and Zakzanis, 2014; Roberts et al., 2016). We hypothesized that exposure to chronic stress during life and in particular during critical periods such as adolescence may be a risk factor for ASD in the next generation.

2. Materials and Methods

2.1 Animals

Experiments were performed in the male offspring of Sprague-Dawley male and female rats from our colony, generated from breeders obtained from Charles River (Calco, Italy). All animals were maintained under an artificial 12-h-light, 12-h-dark cycle at a constant temperature of 23 ± 2°C and 65% humidity. Food and water were freely available at all time. Adequate measures were taken to minimize pain or discomfort of animals whose care and handling throughout the experimental procedures were in accordance with the European Parliament and the Council Directive of 22 September 2010 (2010/63/EU) and were approved by the Italian Ministry of Health (no. 1062/2016-PR) according to the Italian Legislative Decree no. 26 of 4 March 2014.

2.2 Juvenile social isolation in parents

Male and female Sprague-Dawley rats at 21 days of age, immediately after weaning, were housed for 30 days either in groups of five per cage (59 by 38 by 20 cm; group-housed, GH) or individually in smaller cages (42 by 26 by 15 cm; socially isolated, ISO). For the breeding procedure, 51 days old males and females were paired for 5 days; ISO females were bred with ISO males, and GH females were bred with GH males, in order to match the same protocol previously described by Pisu et al. (2013). The day in which sperm was detected in the vaginal smear was designated as gestational day 0 (G0). Upon gestational status identification, female rats were either singly housed or group-housed (depending on which experimental group they belonged to, ISO or GH) until gestational day 20, when every rat was singly housed for parturition and subsequent nursing. Male offspring of GH and ISO parents (GH-O and ISO-O, respectively) were weaned between 25 and 30 days after birth and then housed in groups of five per cage until 2 months of age, when experiments were performed.

2.3 Social transmission of flavour preference

2.3.1 Animals. Rats were assessed in the social transmission of flavour preferences (STFP), a social learning test that is well established in rats (see for review Galef, 2012). The test was performed as previously described (Berretti et al., 2014). 60 experimental rats (n=30 GH-O and n=30 ISO-O) were tested as observers. An additional 40 adult male rats (naïve rats, group-housed, that had not been subjects in other experiments) served as demonstrators.

2.3.2 Test Fluids. Diluted (25%) whole milk solutions flavoured with either 0.03% anise (Erboristeria Magentina, Turin, Italy) or 0.015% mint (Erba Vita Group S.p.A., Italy) essential oils were used. We performed pilot tests to evaluate the preference for the two flavoured solutions. Both mint and anise solutions were roughly equipalatable; out of 40 naive male rats that were offered a choice between mint and anise for 24 hr, only 30 drank an average of 6 g solution/rat (preference for mint or anise was around 50%); in this way we obtained 15 demonstrators per flavour. Ten demonstrator rats were discarded from the test because of the little amount of solution drank. New fresh solutions were prepared the day of the experiment for the observer's choice test.

2.3.3 Procedure. Both observer rats and their demonstrators were exposed to water and food deprivation schedules the day before the experiment. Demonstrators and observers were deprived from food and water 20 and 15 hr, respectively, before the experiment session. At least 12 h before testing, demonstrators' fur was marked with black magic marker, then they were moved into the experimental room and left undisturbed. The observers were left with only a medium-sized food pellet so that the food deprivation was not total and they did not get too hungry on the day of the experiment, so that they did not throw randomly on the milk bottles. On the test day observer rats

were housed in single clean cages (42 x 26 x 15 cm) and left undisturbed for at least 30 min to acclimate. Demonstrators were removed from their home cages, individually placed in an empty cage (42 x 26 x 15 cm) with a bottle containing the flavoured solution (randomly distributed such that half received each diet), and allowed to drink for 1 h. At the end of the 1-hr feeding period, bottles were removed from the cage and weighted (demonstrators that had not drunk at least 1g of the flavoured solution were discarded from the test). Demonstrators were then transferred to their respective observer's cage and let to freely interact for 30 min (taste reactivity test). Clear Plexiglas lids were used to allow videotaping from above with an 8-mm Sony Handycam (in nightshot) for subsequent behavioural analysis of the interactions. One trained observer, unaware of the animals' experimental group, scored the videotaped social interactions for the first 10 minutes. The interactions were analyzed using the ethogram by Grant and Mackintosh (1963) (see also Clipperton Allen et al. 2010, for behaviour descriptions) using Behavior Tracker (a shareware software available for download at ww.behaviortracker.com).

At the end of the 30-min interaction period, observer rats were placed in a new clean cage and were allowed to drink from two bottles containing mint and anise flavoured milk solutions (bottles were weighted before being presented to the rats) for 24 hours. The weight of the bottles was recorded at 1, 2, 4 and 24 hours to calculate the amount of fluid intake for each solution. For each observer an anise preference score was calculated (amount of the anise flavoured solution drunk/total fluid intake [anise+mint]) to assess the presence of social learning. The bottles' position was counterbalanced to avoid any position effect. Demonstrators were placed back into their home cage and immediately returned to the colony.

2.4 Morris water maze test: learning and memory and behavioural flexibility

The Morris water maze consisted of a circular pool (150 cm in diameter, 60 cm in depth) whose interior was painted black. It was located in the centre of a room dedicated to measurement of this behavioural paradigm. The room was illuminated (120 lx) by four light bulbs aimed at the ceiling to avoid light reflections in the water. The water temperature was maintained at 25 ± 2°C with the use of a submersible digital water-heating system. The pool was divided into four virtual quadrants, and a removable circular escape platform (10 cm in diameter, 32 cm in height) was introduced into one of the quadrants (target quadrant) at a depth of 2 cm below the water surface. The initial assessment of spatial learning occurred on 5 consecutive days followed by a probe trial on day 6; for evaluation of behavioural flexibility an additional 4 days of training (reversal learning) occurred, starting from day 7.

Each rat (n = 20 animals/experimental group) was subjected to four training trials on each of five consecutive days (by first placement into the pool in the quadrant next to the target quadrant). Once the animal had climbed onto the platform, it was allowed to remain there for 15 s before the next trial; if it had not found the escape platform at the end of 120 s, it was gently guided to the platform and allowed to rest there for 15 s. Cumulative search error [i.e., the corrected cumulative distance from the platform that represents deviation from an optimal search, that is, from a direct path to the goal (Gallagher et al., 1993)] was recorded on training trials. On day 6, 24 h after the last training trial, each rat was subjected to a probe trial, in which the escape platform was removed from the pool and the animal was released from the quadrant opposite to the original platform location and allowed to freely swim for 60 s. Behavioural data from the training and probe tests were acquired and analyzed using an automated tracking system (Ethovision XT 5.0, Noldus, Wageningen, The Netherlands). Using this software, the precise rat location (in x, y coordinates) was recorded throughout the probe test (capture rate: 10 frames/s). From this spatial distribution, the proximity measure from target (i.e. the average distance from the target location) or opposite (average distance from a comparable location in the opposite quadrant of the maze, that is, 180° from the target) was calculated automatically (Gallagher et al., 1993).
Behavioural flexibility was assessed, in ISO-O and GH-O animals, during a reversal learning. Twenty-four hours after the probe trial, rats were subjected to a new 4-day training session, starting from day 7; the platform was placed in the opposite side of the initial target quadrant, and rats relearned the new platform location. Performance in this reversal learning session was indexed by cumulative search error.

2.5 Resident-intruder test

ISO-O and GH-O were assessed for the display of offensive aggressive behaviours against an unfamiliar conspecific intruder, according to Clipperton Allen et al. (2010) and to Berretti et al., (2014). 2 weeks before the experiment took place rats used as intruders were castrated and the light-dark cycle was inverted for all animals, in order to test social interactions between residents and intruders during the dark phase. 15 ISO-O and 15 GH-O rats were used as residents; they were randomly selected from 4 different cages for each experimental group. Resident rats were singly housed for 7 days prior to testing; no cage change took place during this time to allow the residents to establish a territory in their home cages. To assist in identification, the intruder rats were painted with black magic marker at least 12 h prior to testing. At this time, all residents and intruders were moved into the testing room and left undisturbed for at least 12 h. On the test day, the castrated male intruder (same size as the resident) was introduced in the resident's home cage. Rats were left

undisturbed to freely interact for 10 min while being videotaped. Clear Plexiglas lids were used to allow videotaping from above with an 8 mm Sony Handycam (in nightshot) for subsequent behavioural analysis of the interactions. One trained observer, unaware of the animals' treatment, scored the videotaped social interactions.

The interactions were analyzed for behaviours based on the ethogram by Grant and Mackintosh (1963) using Behavior Tracker (a shareware software available for download at www.behaviortracker.com). Behavioural analysis focused on the resident rat; the behaviour of the intruder was collected only in relation to the resident's behaviour (i.e., agonistic behaviours delivered and received, social investigation). Agonistic behaviours delivered include: follow the intruder, attacks delivered and dominant behaviours (pinning of the intruder, aggressive grooming, crawling over or on top). Agonistic behaviours received include: avoidance of the intruder, attacks received, submissive behaviours (crawl under, supine posture, prolonged crouch and any other behaviour in which the intruder is dominant). The dominance score was calculated for each pair of rats by subtracting the amount of total agonistic behaviour received from the amount of total agonistic behaviour delivered. A negative score indicates that the resident was the submissive animal in the pair, while a positive score signifies that the resident was the dominant animal. Social investigation includes oronasal investigation, body investigation, anogenital investigation, stretched approaches, and attend to/approach intruder. Total social behaviours refer to the sum of the following behaviours: follow intruder, dominant behaviours, attacks delivered, aggressive postures, reciprocal attacks, avoid intruder, submissive behaviours, attacks received, defensive upright posturing, inactive together, oronasal investigation, body investigation, anogenital investigation, stretched approaches, and attend to/approach intruder. Total activity includes all behaviours involving activity, both social and non-social; excluded from this group are inactive alone, inactive together, and self-grooming. Non social behaviours refer to horizontal exploration, vertical exploration, dig, stereotypies, inactive alone, and self-grooming.

2.6 Novel object exploration test

To assess novelty-induced behavioural inhibition, a novel object exploration test was conducted, consisting in the evaluation of the exploratory response of rats when a novel object was introduced in their home cage. 60 days old ISO-O and GH-O rats were used for the novel object exploration test (n=15 animals per group). The day of the test, rats were moved to the test room, the food was removed from the home cage (leaving only four pellets in each cage) and they were left undisturbed for 1 hour. Afterward, the novel object (graphite pencil Staedtler Noris, HB no.2) was perpendicularly introduced in their home cage through the grid cover, until it made contact with the

cage bedding. To facilitate observation, each cage was pulled from the rack about 20 cm. Latency to the first exploration (the time elapsed until the first exploration of the novel object) and the total time spent exploring the pencil for each individual rat were recorded in a 3-min test. The observer, blind to the experimental group, was standing at 50 cm from the cage front (Río-Alamos et al., 2015).

2.7 Isoniazid-induced seizures

ISO-O and GH-O (n= 18 animals per group) were injected with isoniazid (isonicotinic acid hydrazide, Sigma-Aldrich), and observed by a blind experimenter for at least 150 min for the appearance of convulsions. Isoniazid (350 mg/Kg/2 ml) was dissolved in physiological saline and administered subcutaneously. Data are presented as the mean ± SEM of values from 18 animals per group and represent the latency (min) to appearance of seizures, the number of animals showing seizures, the latency to death (min), and the number of animals that died from seizures.

2.8 BDNF, oxytocin and testosterone assays

ISO-O and GH-O rats (n=15 animals per group) were sacrificed by decapitation; the brain was rapidly removed for dissection of the hippocampus and prefrontal cortex on frosted glass kept cold on crushed ice. The dissected tissue samples were immediately frozen in dry ice and stored at -80°C until immunoblot analysis for brain BDNF content. Blood was collected from the trunk into heparinized tubes and centrifuged at 900 × g for 10 min at 4°C. The resulting plasma supernatant was frozen at -80°C until use for BDNF, oxytocin and testosterone assays.

Immunoblot assay for brain BDNF: frozen tissue was homogenized in a solution (~800 μl per 100 mg of tissue (wet weight) containing 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, 1% Triton X-100, 1% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, aprotinin (1 mg/ml), 1 mM benzamidine and bacitracin (200 μg/ml). Protein concentrations in the extracts were determined using a DC Protein assay kit (Bio-Rad, Milan, Italy). The extract (40 μg of protein in 15 μl) was incubated for 10 min at 70°C and then fractionated by SDS-polyacrylamide gel electrophoresis (NuPAGE Novex 4-12% Bis-Tris Midi Gel, Life Technologies, Monza, Italy). The separated proteins were transferred to a polyvinylidenedifluoride membrane (Immobilon-P; Millipore, Milan, Italy) with the use of a Criterion Blotter (Bio-Rad); the membrane was then incubated for 60 min at 25°C with 5% non fat dried milk in Tris-buffered saline containing 0.01% Tween-20, and then overnight at 4°C with rabbit polyclonal antibodies to BDNF (1:200 dilution, Santa Cruz Biotechnology); immune complexes were detected with horseradish peroxidase–conjugated secondary antibodies and chemiluminescence reagents (GE Healthcare Biosciences). Optical density of the bands was

determined with the use of an imaging system (Geliance 600; Perkin Elmer, Monza, Italy), and associated image acquisition (GeneSnap, Perkin Elmer) and analysis (GeneTools, Perkin Elmer) software. Values for BDNF were normalized by the corresponding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amount.

Plasma BDNF assay: an enzyme-linked immunosorbent assay (ELISA) kit was used to quantify plasma levels of BDNF. ELISA was performed according to the manufacturer's instruction (Sigma-Aldrich, Milan, Italy) using a 96-well plate that was pre-coated with a primary antibody against rat BDNF. Each sample was run in duplicate. Data are presented as the mean ± SEM of 15 animals per group and are expressed as pg/ml of plasma BDNF.

Oxytocin assay: an enzyme immunoassay (EIA) kit was used to quantify plasma levels of oxytocin. EIA was performed according to the manufacturer's instruction (Oxytocin EIA kit, Phoenix Europe GmbH, Karlsruhe, Germany) using a 96-well plate that was pre-coated with a secondary antibody (while nonspecific binding sites are blocked) that can bind to the Fc fragment of the primary antibody (peptide antibody) whose Fab fragment will be competitively bound by both biotinylated peptide and peptide standard or targeted peptide in samples. The kit also provided a five-point standard curve. Each sample was run in duplicate. Data are presented as the mean ± SEM of 15 animals per group and are expressed as ng/ml of plasma oxytocin.

Testosterone assay: testosterone was extracted from plasma three times with 3ml of ethyl acetate, and the combined organic phases were dried under vacuum. The recovery (80 to 90%) through the extraction procedure was monitored by addition of a trace amount (7000 to 8000 cpm, 78 Ci/ mmol) of [3H] testosterone (Perkin Elmer Italia, Monza, Italy) to the plasma samples. Testosterone levels were quantified by radioimmunoassay as previously described (Santoru et al., 2014) with a specific antiserum to testosterone (MP Biomedicals, Solon, OH, USA).

2.9 Statistical analysis

Quantitative data are presented as means ± SEM. Unpaired Student's t-test was used to analyze behavioural data in STFP, seizures onset, resident-intruder and novel object exploration tests, as well as BDNF and oxytocin levels; the $\chi 2$ test was utilized to conclude if observed frequency differed from expected frequency in the seizures experiment; repeated measures ANOVA was used to analyse preference ratio in the STFP test and cumulative search error in the Morris water maze test considering parents' housing condition and time effects; two-way ANOVA considering the parents' housing condition (GH-O vs. ISO-O) and zone specificity (target vs. opposite) effects was used to analyze the average proximity in the probe trial of the Morris water maze test.

ANOVAs were followed by Bonferroni post hoc test. A value of $p<0.05$ was considered statistically significant.

3. Results

3.1 Offspring of socially isolated rats showed impaired social learning in the STFP test.

STFP was evaluated in ISO-O and GH-O rats. As shown in figure 1, there was a significant increase in anise preference in the first 4 hours interval in the GH-O group ($p<0.01$; Fig. 1A), an index of social learning. By contrast, ISO-O did not shown any difference in the preference ratio (Fig. 1B), suggesting impaired social learning. No differences were detected in total fluid intake between the two groups (Fig. 1C). The ANOVA for the anise preference ratio revealed a main effect of demonstrator diet [$F(1,56)=27.93$, $p=0.0001$], no significant effect of parents' housing condition [$F(1,56)=0.66$, $p=0.79$] or time [$F(3,168)=0.87$, $p=0.46$] and a significant interaction between factors (parents' housing*demonstrator diet) [$F(3,168)=9.09$, $p=0.004$] (Fig. 1A-1B).

ISO-O and GH-O rats were also tested for social behaviour in the STFP paradigm, during the first 10 min of the 30 min free social interaction (taste-reactivity test). As shown in table 1, ISO-O rats showed a significant decrease in the frequency and duration of the following behaviours: follow demonstrator (frequency, [$t(58)=4.112$, $p=0.0001$]; duration [$t(58)=3.395$, $p=0.0013$]); dominant behaviour (frequency, [$t(58)=4.189$, $p=0.0001$]; duration, [$t(58)=3.553$, $p=0.0008$]); attacks delivered (frequency, [$t(58)=4.264$, $p=0.0001$]); avoidance (frequency, [$t(58)=2.453$, $p=0.0173$]); submissive behaviour (frequency, [$t(58)=2.966$, $p=0.0044$]; duration, [$t(58)=2.255$, $p=0.0281$]); attacks received (frequency, [$t(58)=2.912$, $p=0.0052$]); defensive upright posture (frequency, [$t(58)=4.746$ $p=0.0001$]; duration, [$t(58)=3.206$, $p=0.0022$]). By contrast, no changes in social investigation were observed (Table 1).

3.2 Learning and memory performance and behavioural flexibility in offspring of socially isolated rats – Morris water maze test

The impairment in social learning, observed in ISO-O rats in the STFP test suggests a loss of learning and memory capability. We further investigated these processes in the Morris water maze test. As shown in figure 2A, during the first five days of training (days 1 - 5) both GH-O and ISO-O rats showed a significant reduction in the cumulative search error, suggesting a similar learning ability. Repeated measures ANOVA revealed a significant effect of time [$F(4,632)=28.76$, $p=0.000001$)], no effect of parents' housing condition [$F(1,158)=1.67$, $p=0.198$] or interaction

between factors [F(4,632)=1.82, p=0.123], suggesting no differences in learning performance between the two groups. To evaluate spatial memory, the average proximity was calculated during the probe trial (day 6); figure 2B shows a decrease in the average proximity to the target vs. the opposite zone in both groups of animals, suggesting no impairment in memory performance. Two-way ANOVA revealed a significant effect of zone specificity [F(1,76)=120,21, p=0.00001]; no effect of parents' housing condition [F(1,76)=0.01, p=0.971] and a significant interaction between factors [F(1,76)=5.42, p=0.037].

To evaluate the behavioural flexibility, rats were subjected to a reversal learning from day 7 to day 10; in this test the platform was moved to a quadrant opposite to the original one (south quadrant). Across the days of training (days 7 – 10), the cumulative search error was significantly decreased in both GH-O and ISO-O rats (Fig. 2C); however, it was overall significantly higher in ISO-O compared to GH-O animals, suggesting that ISO-O rats have impaired behavioural flexibility. ANOVA revealed a significant effect of parents' housing condition [F(1,158)=15.538, p=0.0010)], a significant effect of time [F(3,474)=23.327, p=0.0001], and no interaction between factors [F(3,474)=1.188, p=0.313].

3.3 Offspring of socially isolated rats did not show altered aggressive behaviour in the resident-intruder test

ISO-O and GH-O rats were tested for aggressive behaviour in the resident-intruder paradigm. As shown in table 2, the frequency of agonistic behaviours delivered (follow the intruder, dominant behaviours and attacks) and dominance score, (total agonistic behaviour delivered minus total agonistic behaviour received) did not differ significantly between ISO-O and GH-O rats, suggesting no changes in the display of aggressive behaviour. Accordingly with behavioural data, testosterone plasma levels were similar in GH-O and ISO-O animals (2.07±0.36 and 2.11± 0.32 ng/ml, GH-O and ISO-O, respectively). In agreement with the results obtained in the STFP test, social investigation (oronasal, body and anogenital investigation, stretched approaches and attend to/approach intruder) did not differ between groups. By contrast, non social behaviours (horizontal and vertical exploration, dig, stereotypies, inactive alone, and self grooming) were significantly increased [t(28)=1.987, p=0.030] in ISO-O animals.

3.4 Offspring of socially isolated rats did not show altered novelty-induced behaviour in the novel object exploration test

Evaluation of the exploratory response towards a novel object, introduced in the rat's home cage was conducted in ISO-O and GH-O rats. Latency to the first exploration and total time spent

exploring the pencil were recorded for each individual rat; no differences were found between the two groups (Fig. 3; [t(28)=0.388, p=0.700] and [t(28)=0.532, p=0.598], for latency and time, respectively).

3.5 Offspring of socially isolated rats showed increased seizures sensitivity

To evaluate the sensitivity to isoniazid-induced seizures, all animals were injected with the drug (350 mg/Kg/2 ml) and observed for 150 minutes. As shown in table 3, all rats had seizures in response to isoniazid; however, ISO-O rats showed a reduced threshold to convulsions compared to GH-O controls (-15%, [t(34)=3.109, p=0.004]). We also observed a decrease in the number of surviving animals after convulsions in the ISO-O group (14/18 surviving) vs. the GH-O group (17/18 surviving) [χ^2(1, N=36) = 2.1, z=1.446, p=0.0741)], even though the latency to death was unchanged (Table 3).

3.6. Offspring of socially isolated rats showed altered BDNF and oxytocin levels

Figure 4 shows brain [hippocampus (panel A) and prefrontal cortex (panel B)] expression and plasma (panel C) concentrations of BDNF in adult male GH-O and ISO-O rats. Basal levels of BDNF were increased in ISO-O with respect to GH-O animals in hippocampus (+80%; [t(28)=4.112, p=0.0005]), prefrontal cortex (+23%; [t(28)=3.09, p=0.0053]), and plasma (+44%; [t(28)=2.032, p=0.035]).

Furthermore, plasma oxytocin levels were significantly decreased (-31%; t(28)=3.985, p=0.0004]) in ISO-O rats compared to GH-O controls (Figure 4, panel D).

4. Discussion

Here, we report that chronic stress, induced by juvenile social isolation, induces, in male offspring, behavioural and biochemical alterations consistent with an ASD-like phenotype. In fact, ISO-O rats showed decreased sociability, impairment in reversal learning, increased seizure susceptibility, reduced plasma oxytocin levels, and increased plasma and brain BDNF levels. These impairments came with no change in spatial learning and memory, aggression and anxiety-like behaviours. During social interaction in the STFP test, ISO-O animals showed a significant reduction in social agonistic behaviour delivered and agonistic behaviour received. In line, plasma oxytocin levels were significantly decreased in ISO-O rats. These results are in agreement with evidences of an involvement of oxytocin in social behaviour and ASD. In fact, several lines of evidence indicate

that oxytocin, released within the brain, plays a role in social bond formation (Hammmock, 2015) and, although studies examining the relationship between oxytocin levels and ASD have yielded mixed results (Taurines et al., 2014), results from genetic association studies suggest a relationship between oxytocin gene polymorphisms and ASD (Campbell et al., 2011). Moreover, clinical and preclinical studies indicate that oxytocin can be considered a potential therapeutic agent for ASD (Dromes et al., 2014; Tyzio et al., 2014).

Surprisingly, we found not effect of parent's isolation stress on social investigation (oronasal, anogenital and body investigation) displayed by their offspring in the STFP paradigm; nonetheless, ISO-O rats exhibited impaired social learning in the STFP test. These results seem to be in disagreement; in fact, it has been demonstrated that the oronasal interaction with the demonstrator's breath is essential in order to acquire preference for food, as the interaction with surrogate demonstrators is not effective in changing the observer's food preference (Galef, 2012). Thus, the learning impairment observed in ISO-O animals in the STFP test could be due to factors other than the oronasal interaction with the demonstrator, which may include deficits in olfaction or in learning and memory. We found that parent's social isolation did not affect, in the offspring, spatial learning and memory in the Morris water maze test. However, this result does not rule out the possibility that ISO-O rats might have an impairment in the associative olfactory memory, which does not have an explicit spatial memory component (Alvarez et al., 2002). Thus, the learning deficit of ISO-O rats in the STFP test is not in contrast with the results obtained assessing memory performance in the Morris water maze test; rather it strengthens another result obtained through the same test, that is the impairment in reversal learning showed by these animals. In fact, the STFP paradigm, which exhibits some of the key features of human declarative memory (Bessieres et al., 2017), involves the capability to recall information in circumstances different from those existing during the initial learning, and this process requires cognitive flexibility. Reversal learning is a form of behavioural flexibility and represents the ability to shift the response according to spatial position (Birrel and Brown, 2000). Impairments in cognitive flexibility have been used to characterize the neuropsychological profile in people with ASD (Leung and Zakzanis, 2014), and a deficit in cognitive flexibility has also been demonstrated in rodent models of autism (Crawley, 2007; Micheau et al., 2014). Compelling evidence suggests that impairment in reversal learning is the result of impairment in neural networks of the frontal lobes but also in the dysregulation of BDNF signalling in the brain (McCarty et al., 2016; Izquierdo et al, 2017).

BDNF plays an important role in maintaining trophism in the adult brain, and in controlling long-term potentiation (LTP) with important implications for learning and memory and emotional behaviour (Azogu and Plamondon, 2017; Famitafreshi et al., 2016; Li and Li, 2015). BDNF has

also been implicated in pain, inflammation and convulsive activity (Smith, 2014). In reward-related regions, BDNF levels are increased following cocaine exposure (Russo et al., 2009), and in a mouse model of prenatal cocaine exposure impaired reversal learning has been associated with excessive BDNF-TrkB signalling in the frontal cortex (McCarty et al., 2016). Our finding of increased BDNF expression in the frontal cortex, hippocampus and plasma of ISO-O rats is in line with these evidences.

Although conflicting results are reported in the literature, most studies have shown that BDNF levels were increased in the blood of ASD patients (Qin et al., 2016; Armeanu et al., 2017), with higher levels observed in children with intellectual disability (Bryn et al., 2015), suggesting that BDNF may be part of a biochemical continuum underlying autistic traits in the general population (Brondino et al., 2018). Increased BDNF levels have also been reported in several animal models of ASD (Reim and Schmeisser, 2017; Sungur et al., 2017). Nonetheless, our results are in contrast with the reduction in both hippocampal BDNF protein levels and in behavioural flexibility reported in the BTBRT+*tf*/J mouse model of autism (Scattoni et al., 2013).

It has been proposed that progesterone and its 3α,5α–pregnane derivative, allopregnanolone, increase BDNF mRNA and protein expression (Kaur, 2007; Nin et al., 2011). Given that we previously reported an increase in brain allopregnanolone levels in ISO-O rats (Pisu et al., 2013), it is tempting to speculate that such increase might correlate with the increased BDNF levels observed in the same brain areas of these animals (present data). The constitutively high basal cerebrocortical allopregnanolone levels found in these animals might also normalize their emotional state, as previously demonstrated in the Vogel's and elevated plus maze tests (Pisu et al., 2013), and further confirmed by the present results using the novel object exploration test. Allopregnanolone is one of the most potent and efficacious positive allosteric modulators of $GABA_A$ receptor function (Lambert et al., 1995; Porcu et al., 2016), which induces a potent anxiolytic-like effect (Brot et al, 1997). In agreement, ISO-O rats also display less reactivity to stress (Pisu et al., 2013).

The elevated basal allopregnanolone levels in the brain of ISO-O rats may also be correlated with the lack of changes in aggression observed in the resident-intruder test. In fact, in a mouse model of chronic stress it has been demonstrated that pharmacological treatment with allopregnanolone dose-dependently decreased aggression in a manner that correlated with an increase in corticolimbic allopregnanolone content (Pinna et al., 2003). Our observation is also in line with the evidence that ISO-O rats have plasma testosterone levels similar to that of GH-O animals.

Finally, ISO-O rats display the most frequently reported endophenotype across ASD models, that is, increased seizures susceptibility. Given the elevated brain levels of allopregnanolone (Pisu et al., 2013), this result was somehow unexpected. In fact, this neuroactive steroid, as a positive

modulator of the GABAergic transmission, has a powerful anticonvulsant activity in humans and in various animal models (Reddy, 2010). However, in certain conditions, such as overactivation of glutamate receptors, it can paradoxically potentiate seizure activity (Salazar and Tapia, 2012). It has been postulated that an imbalance in the excitation/inhibition ratio might contribute to some forms of autism (Rubenstein and Merzenich, 2003); in agreement, electrophysiological and biochemical investigations in animal models of ASD have revealed brain region- and model-specific imbalances in excitatory/inhibitory function (Horder et al., 2018). Moreover, high seizures susceptibility has been reported in a BDNF overexpression model (Weidner et al., 2014).

Altogether, the results suggest that preconceptional stressful experiences should be considered as crucial elements for the aetiology of ASD, although further studies are warranted in order to prove a causal association between preconceptional stress and ASD in the offspring, and to elucidate the underlying mechanisms. Dams of ISO-O rats show a marked decrease in the quality of maternal care, as well as in the brain and plasma concentrations of progesterone and its metabolite allopregnanolone (Pisu et al., 2017). Such altered hormone levels may play a role in the development of an ASD-like phenotype; in fact, it has been suggested that low maternal progesterone may be responsible for both obstetrical complications and neurodevelopmental changes in the fetal brain associated to autism (Whitaker-Azmitia et al., 2014). Accumulating evidences demonstrate that the developing brain is sensitive to progesterone during critical periods of maturation and that the male fetal brain expresses progesterone receptors, whereas the female fetal brain has very few (Wagner, 2008). Lower progesterone in the mother leading to autism in the offspring could explain the clinical and epidemiological studies on ASD that indicate that boys are affected more frequently than girls (ratio 4:1) (Fombonne, 1999, 2003; Volkmar et al., 1993). In agreement, preliminary data collected in our lab show that female ISO-O rats do not show any of the behavioural and biochemical alterations observed in males and here reported (unpublished results).

5. Conclusion

Social deficits, behavioural perseveration, HPA hyperactivity, low oxytocin and high BDNF levels and seizures sensitivity are peculiar characteristics of ASD patients and of some established genetic animal models of ASD. Thus, offspring of socially isolated animals meets the attribute of face validity for an ASD animal model. In addition, this novel animal model could allow studies on the molecular mechanisms underlying ASD, avoiding the adaptations that may occur following genetic or pharmacologic manipulation, which may lead to changes in brain circuits not directly involved in the pathology.

Contributors

PMG assisted with study design, performed part of the biochemical experiments and the statistical analysis, and wrote the first draft of the manuscript. BG and BF performed part of the biochemical experiments. GA, CC, CS and ME performed the behavioural experiments. FP and CA contributed to the interpretation of the data. PP assisted with interpretation of the data and edited the revision of the manuscript. SM conceived the study's design, supervised the experiments and critically revised the manuscript. All authors contributed to and have approved the final manuscript.

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Conflicts of interest

All authors declare that they have no conflict of interest.

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Table 1. ***Social interaction during the taste reactivity test in the STFP paradigm.***

Groups	GH-O	ISO-O	GH-O	ISO-O
	Frequency (n)		Duration (s)	
Agonistic behaviour delivered				
Follow demonstrator	4.8 ± 0.6	1.57 ± 0.4[b]	8.07 ± 1.3	2.77 ± 0.7[b]
Dominant behaviour	4.2 ± 0.8	0.97 ± 0.3[b]	18.29 ± 3.8	4.10 ± 1.5[b]
Attacks delivered	8.1 ± 1.1	2.50 ± 0.7[b]	-	-
Agonistic behaviour received				
Avoid demonstrator	1.6 ± 0.4	0.53 ± 0.2[a]	3.71 ± 1.1	1.90 ± 0.8
Submissive behaviour	4.3 ± 0.9	1.23 ± 0.4[b]	23.86 ± 6.6	8.03 ± 2.8[a]
Attacks received	8.2 ± 1.9	2.47 ± 0.5[b]	-	-
Defensive upright posture	3.3 ± 0.5	0.80 ± 0.2[b]	8.75 ± 1.9	2.23 ± 0.6[b]
Social behaviour				
Oronasal investigation	14.5 ± 1.0	15.6 ± 1.1	38.7 ± 4.7	39.6 ± 3.4
Anogenital investigation	4.2 ± 0.7	3.0 ± 0.6	13.7 ± 3.1	13.8 ± 3.0
Body investigation	26.3 ± 1.3	23.2 ± 1.2	109.5 ± 7.4	101.5 ± 7.1

Male offspring of socially isolated and group-housed rats (ISO-O and GH-O, respectively) were assessed for social interaction during the taste reactivity test in the STFP paradigm. Rats (n = 30 per experimental group) were videotaped and, for the first 10 min, the frequency (n) and duration (s) of each behaviour has been recorded. Data were analyzed by Student's t-test. [a]$p<0.05$, [b]$p<0.01$ vs.GH-O rats.

Table 2. ***Aggressive behaviour in the resident-intruder test in offspring of socially isolated and group-housed rats.***

Groups	GH-O	ISO-O
	Frequency (n)	
Agonistic behaviours delivered	7.07 ± 2.15	4.07 ± 0.73
Dominance score	2.43 ± 1.11	0.93 ± 0.08
Total social behaviours	720 ± 0.68	725 ± 0.67
Social investigation	40.1 ± 3.14	40.5 ± 2.41
Total activity	101.5 ± 5.67	103.8 ± 3.31
Non social behaviours	47.6 ± 2.46	58.5 ± 3.60[a]

Male offspring of socially isolated and group-housed rats (ISO-O and GH-O, respectively) were assessed for aggressive, social and non-social behaviours during the resident-intruder paradigm. Rats (n = 15 per experimental group) were videotaped for 10 min and the frequency (n) of each behaviour has been recorded. Behaviours were analyzed from the time the intruder was introduced into the home cage. Data are presented as the mean ± SEM and were analyzed by Student's t-test. [a]$p<0.05$ vs. GH-O animals.

Table 3. ***Isoniazid-induced seizures in offspring of socially isolated and group-housed rats.***

Groups	Seizures		Death	
	latency (min)	n° of animals	latency (min)	n° of animals
GH-O	67.0 ± 2.8	18/18	86.1	1/18
ISO-O	56.6 ± 1.7[a]	18/18	88.9 ± 15.2	4/18

Male offspring of socially isolated and group-housed rats (ISO-O and GH-O, respectively) were injected with isoniazid (350 mg/Kg/2 ml, s.c., dissolved in physiological saline), and observed for at least 150 min for the appearance of seizures. Data are presented as the mean ± SEM of 18 animals per group and represent the latency (min) to appearance of seizures, the number of animals showing seizures, the latency to death (min), and the number of animals that died from seizures. Latency data were analyzed by Student's t-test; frequency data were analysed by the $\chi 2$ test. [a]$p<0.01$ vs. GH-O animals.

Figure captions

Figure 1: Offspring of socially isolated rats showed impaired social learning in the STFP test
Panels A, B: Anise preference ratio at 1, 2, 4 and 24 h of testing in observers whose demonstrators have been fed with an anise- (black squares) or a mint-flavoured solution (open triangles).
Panel C: Total fluid intake in offspring of group-housed (GH-O, white circles) and socially isolated (ISO-O, black circles) rats.
Data are mean ± SEM of 30 animals per group and were analyzed by Student's t-test. $^{a}p<0.01$ vs. the respective time point whose demonstrator was fed with mint-flavoured milk.

Figure 2: Performances in the Morris water maze test during training (A), probe trial (B) and reversal training (C) in male offspring of socially isolated and group-housed rats.
Panel **A**: Cumulative search error was calculated in offspring of socially isolated (ISO-O, open circles) and group-housed (GH-O, filled squares) rats from days 1 to 5 of training in the Morris Water maze test. Cumulative search error represents the corrected cumulative distance from the platform (deviation from an optimal search, that is, from a direct path to the goal). Data are mean ± SEM of 20 animals per group and were analyzed by repeated measures ANOVA followed by Bonferroni post-hoc test. $^{a}p<0.05$, $^{b}p<0.01$ vs. the respective day 1 performance.
Panel **B**: Average proximity was calculated in offspring of socially isolated (ISO-O) and group-housed (GH-O) rats during a 60s probe trial on day 6, 24 h after the last training session in the Morris Water maze test. Average proximity for target refers to the average distance from the target location; average proximity for opposite refers to the average distance from a comparable location in the opposite quadrant of the maze, that is, 180° from the initial target location. Data are mean ± SEM of 20 animals per group and were analyzed by two-way ANOVA followed by Bonferroni post-hoc test. $^{a}p<0.01$ vs. the respective opposite location.
Panel **C**: Cumulative search error was calculated in offspring of socially isolated (ISO-O) and group-housed (GH-O) rats from days 7 to 10 of the reversal training in the Morris Water maze test. Data are mean ± SEM of 20 animals per group and were analyzed by repeated measures ANOVA followed by Bonferroni post-hoc test. $^{a}p<0.05$, $^{b}p<0.01$ vs. the respective day 1 of training; $^{c}p<0.05$ vs. the respective time point in the GH-O group.

Figure 3: Novel object exploration test in male offspring of socially isolated and group-housed rats.
A) Latency to the first exploration and B) total time spent exploring the pencil for each individual rat were recorded in a novel object exploration test. Data represent the mean ± SEM of 15 animals

per group and were analyzed by unpaired Student's t-test. GH-O = offspring of group-housed rats; ISO-O = offspring of socially isolated rats.

Figure 4: BDNF and oxytocin levels in offspring of socially isolated and group-housed rats.
Panels A, B: Basal expression of BDNF in hippocampus (A) and prefrontal cortex (B) in male offspring of socially isolated (ISO-O) and group-housed (GH-O) rats. Densitometric quantitation of BDNF was normalized by the corresponding amount of GAPDH; data are expressed as percentage vs. the GH-O control group and are the mean ± SEM of 15 values per group.
Panel C: Basal plasma levels of BDNF in male offspring of socially isolated (ISO-O) and group-housed (GH-O) rats. Data are expressed as pg/ml and are the mean ± SEM of 15 values per group.
Panel D: Basal plasma oxytocin levels in male offspring of socially isolated (ISO-O) and group-housed (GH-O) rats. Data are expressed as ng/ml and are the mean ± SEM of 15 values per group.
All data were analyzed by Student's t-test. $^{a}p<0.05$, $^{b}p<0.01$ vs. the respective GH-O group.

Figure 1

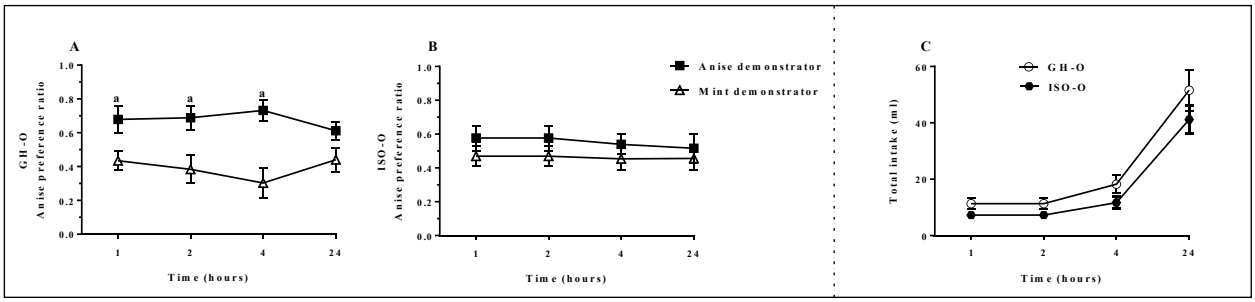


Figure 2

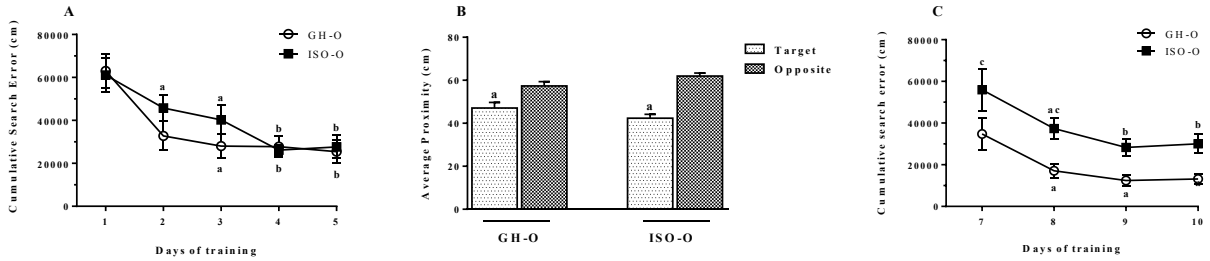


Figure 3

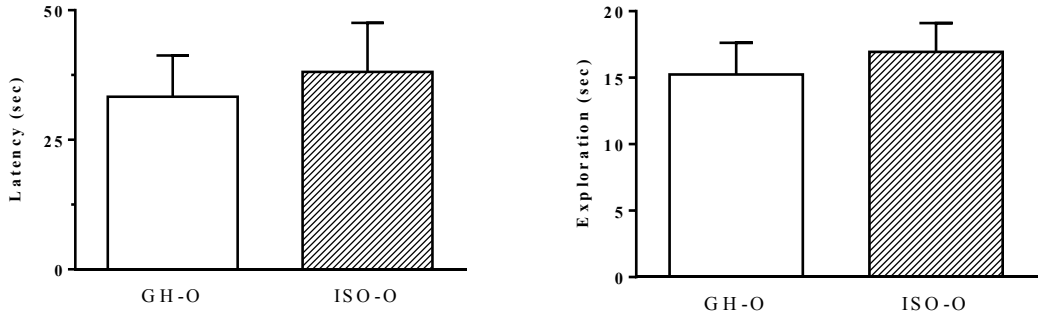
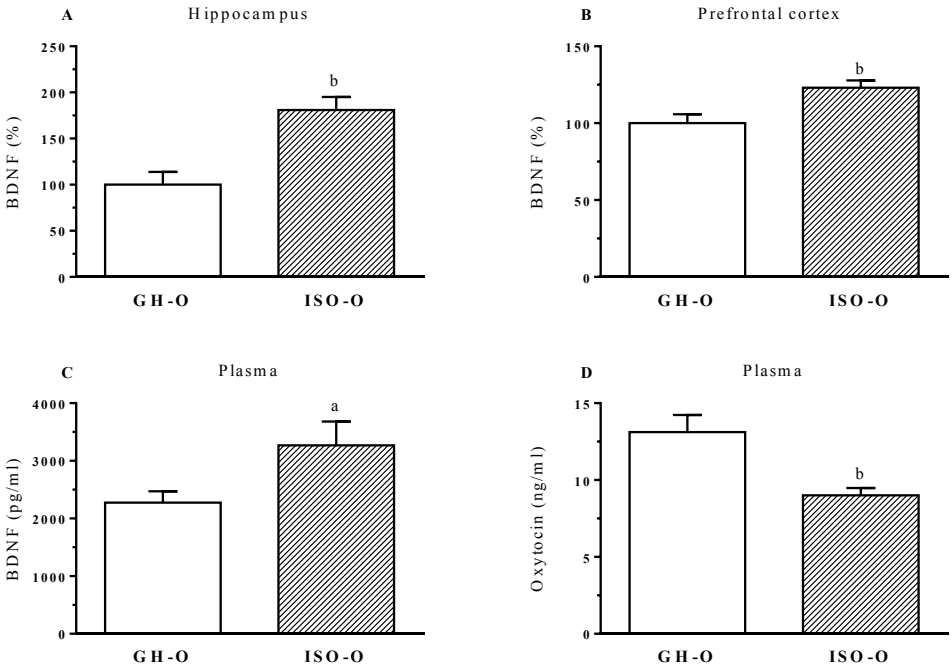


Figure 4


A
Hippocampus
BDNF (%)
250
200
150
100
50
0
b
GH-O
ISO-O
B
Prefrontal cortex
BDNF (%)
150
100
50
0
b
GH-O
ISO-O
C
Plasma
BDNF (pg/ml)
4000
3000
2000
1000
0
a
GH-O
ISO-O
D
Plasma
Oxytocin (ng/ml)
15
10
5
0
b
GH-O
ISO-O

Highlights

- Preconceptional long lasting social isolation produces male offspring with a phenotype consistent with autism spectrum disorders

- Offspring of socially isolated parents shows decreased sociability, impairment in reversal learning and increased seizures susceptibility

- Offspring of socially isolated parents shows reduced plasma oxytocin and increased plasma and brain BDNF levels