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Pre-weaning fluoxetine exposure perturbs social behavior at adulthood *via* altering hippocampal morphometry and PSD-95 expression in rats

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Abstract: The depression during and after pregnancy cause significant exposure of fluoxetine to the child at early life through mother. This exposure to the child, during the vulnerable window of development, can have a long lasting impact on overall mental wellbeing. Long term neurobehavioral aspect of developmental toxicity is neglected as the part of testing requirements in the process of drug developmental. In this context, the present study was designed to study the possible effect of pre-weaning fluoxetine exposure on the social behavior of rats upon adulthood followed by assessing hippocampal morphometry (hematoxylin-eosin and silver staining) and post-synaptic density protein 95 (PSD-95) expression (using qPCR). Our data showed that the fluoxetine exposure (10, 50 and 100mg/kg) caused predominant increase in the social behavior of rats; the effect more pronounced in female rats. The morphometric analysis revealed significant increase in cell population and count of dentate gyrus (DG) region of hippocampus along with enhanced dendritic arborization. Furthermore, the PSD-95 expression was found to be down regulated in the fluoxetine treated group as compared to control. In conclusion, the present study demonstrate that the early post-natal exposure to fluoxetine cause hypersociability upon attaining adulthood, which may be attributed to enhanced neuronal proliferation and decrease PSD-95 expression in the hippocampus.

Keywords: Pregnancy, depression, fluoxetine, hypersociability, brain morphometry, PSD-95.

INTRODUCTION

The communication among living being enhances their capacity to establish and maintain a social life, which is essential for survival. This social communication heavily depends upon the motivation to recognize and interact fellow beings; the phenomenon coordinated by various brain structures including prefrontal cortex, amygdala, nucleus accumbens, anterior insula, anterior cingulate cortex, hippocampus, and temporal sulcus (Gao and Mack 2021). However, under certain neurodevelopmental disorders such as Autism spectrum disorders (Keifer *et al*., 2020), Williams Syndrome (Jabbi *et al*., 2012) and Schizophrenia (Murphy *et al*., 2020), the abnormal social behavior (both hypo and hypersociability) can be observed. The basis of such social deficit is not completely understood. However, two major underlying processes have been identified as regulator of social behavior. Firstly, the discrimination between fear and friendly social signal; the process coordinated by amygdala to produce a suitable behavioral response. Secondly, the reward and aversion mechanism, which is primarily under the regulation of dopamine. This system cause the subject to approach or avoid the social contact. Hypersociability can be explained as the developmental abnormality causing failure in discrimination between familiar and stranger or increased reward or reduced aversion signals (Toth, 2019).

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Post-synaptic density protein 95 (PSD-95) is the most abundant scaffolding protein in the excitatory glutamatergic synapses in the central nervous system (Purcell *et al*., 2014, Rodzli *et al*., 2020). During neurodevelopment, it was reported to cause synaptic maturation *via* recruiting glutamatergic receptors at post-synaptic membrane (Purcell *et al*., 2014). Search of literature revealed the robust hypersocial behavior in PSD95 knock out rats, both males and females (Winkler *et al*., 2018). PSD-95 is considered as a risk gene for hypersociability due to its role in malfunction of prefrontal cortex (Barak *et al*., 2019).

Depression is the central nervous system disorder, which may victimize females during critical time of pregnancy with an estimated prevalence of 10% in a year (Gaynes *et al*., 2005, Urizar and Muñoz, 2021). In Pakistan, the estimates are far higher i.e. 37% for antenatal depression and 30% of postnatal depression (Atif *et al*., 2021). Presumably, the use of antidepressants during pregnancy is increasing with time and an estimated 1 in 10 women use them, especially fluoxetine (Cooper *et al*., 2007). It is a block buster antidepressant, which enhance the levels of serotonin in the synapse by inhibiting its reuptake through serotonin transporter (SERT) (Cipriani *et al*., 2005, Rossi *et al*., 2004). The pregnancy associated depression ultimately exposes the new born to the drugs (chemicals) during vulnerable window of development (W't Jong and Einarson, 2021). This is the time, when most of the physiological and psychological parameters are set in the

body. Any exposure (physical chemical or environmental) could have a long lasting impact on the mental wellbeing of the progeny.

In early 1960s, the thalidomide incidence led to global realization that medicines have the potential to harm fetus. Presumably, the interest in this area has led to the incorporation of guidelines for preclinical developmental toxicity testing for pharmaceutical agents. Hence, the Food & Drug Administration (FDA) has assigned various categories (A, B, C, D, X) to all drugs based on the level of risk the drug poses to the fetus (Osborne *et al*., 2020) The long-term neurobehavioral aspect of developmental toxicity is an area, which unfortunately could not catch the attraction of the policy makers in past. This is the reason why no such guidelines are available for said testing for pharmaceutical agents, a potential risk yet to be completely deciphered. However, this deficiency in testing was acknowledged in the literature (Ulbrich and Palmer, 1996). Search of literature revealed few reports which are suggestive of aforementioned behavioral deficits. One such study conclude that that a 2nd-trimester maternal influenza infection may increase risk for adult schizophrenia or major affective disorder *via* disrupting the development of the fetal brain (Watson *et al*., 1999). An important study reported higher incidence of attention deficit hyperactive disorder (ADHD) in children from the mothers administered acetaminophen (over-the-counter analgesic) during pregnancy (Liew *et al*., 2014). Another study has linked adverse developmental outcomes such as psychomotor development, externalization / internalization behavior in children exposed to acetaminophen during pregnancy (Brandlistuen *et al*., 2013). Hence, these reports are suggestive of the long term neurobehavioral toxicity of the medicines and associated ailments. In this context, the present study was designed to observe the long lasting impact of pre-weaning fluoxetine exposure on the social behavior of rats along with evaluation of potential underlying causes at expression and morphometric levels.

MATERIALS AND METHODS

Chemicals

The following chemicals were used in the study: Agarose was obtained from Sigma (Germany); Chloroform from Scharlau (Spain); DNA ladder (100bp) and ethanol from Thermo Fisher Scientific (USA); Ethidium bromide from MP Biomedical (USA); Taq green master mix from Promega (USA) and Trizol reagent from Invitrogen (USA). Fluoxetine was obtained from Lilly pharmaceuticals (Pakistan).

Animals

Female Sprague Dawley (Pregnant) Rats were obtained from Animal Resource Facility of International Center for Chemical and Biological (ICCBS), University of Karachi. They were housed separately with free access to food and water. The temperature was kept at 25°C while humidity was 60%. The 12 hour dark and light cycle was also maintained throughout the course of study. The F1 generation obtained, after weaning begins, were separated from the dams and housed gender-wise in a group of 5 rats per cage. All experiments were performed according the ethical guidelines provided the Animals Ethics Committee of the University (Approval No. 2019-004).

Experimental design

At post-natal day 1, the dams (F0 generation) were given fluoxetine at 10 (F10), 50 (F50) or 100 (F100) mg/kg daily for 3 weeks (average pre-weaning time) by dissolving the required amount of drug in the minimum drinking water (approximately 5 ml). The control group (C) received drinking water alone. After 3 weeks, the pups were fed with regular diet and allowed to reach adulthood (3 months). The males and females rats from F1 generation was subjected to behavioral, biochemical, gene expression and morphometric assessments. The schematic diagram of the experimental design is shown in fig. 1

Social behavior study

The social behavior of the rats were assessed as described earlier (Cox and Rissman, 2011). Briefly, the rat from each treatment group were placed together in the cage (50 x 50 x 40 cm, pre-cleaned with ethanol 70%) and allowed to interact with each other for 10 minutes. The following behaviors were noted:

1. Social behavior such as social interaction and Allo-grooming
2. Non-social behavior such as self-grooming and rearing with support of the walls
3. Investigative behavior such as Ano-genital sniff (sniffing the other rats' ano-genital region) Nose sniff (sniffing the other rats' nose), Body sniff (sniffing the other rats' body in any part other than nose or the ano-genital region) and Follow (walking behind and following the other animal in the cage).

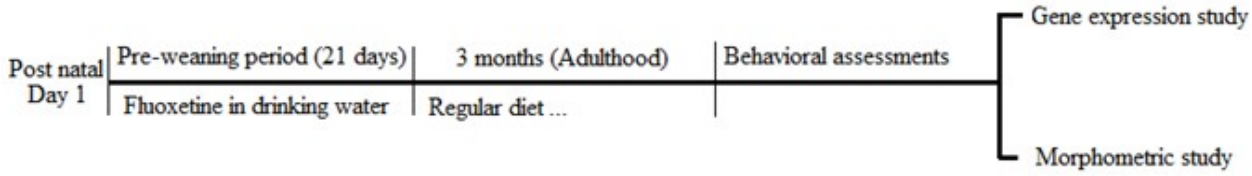

Fig. 1: Schematic diagram of the experimental design

All behavioral studies were conducted between 9am to 1pm and recorded (using Handycam) for later estimations. After behavioral estimations, the brain was harvested for morphometry gene expression studies.

Morphometric analysis

The brains of rats were used for morphometric analysis as follows:

Collection, Preservation & Processing

After decapitation, one hemispheres of brain was dissected, washed and quickly placed in the fixative i.e. neutral buffered formalin (20 times greater than tissue volume). The samples were kept in fixative overnight and processed on next day. The tissues were dehydrated in graded alcohol followed by embedding in paraffin and tissue blocks were made using tissue molds and tissue embedding cassettes. The prepared tissue blocks was stable at room temperature and was subsequently used for sectioning (5 mm) with the help of a microtome (Thermo Shannon Microtome, China). The sections were collected on the slides for staining as follows:

H&E Staining

After rehydration of sections, the sections were placed in hematoxylin (3 minutes) followed by eosin (30 sec) after washing with distill water. At the end, the slides were mounted with xylene soluble DPX mounting media and kept on a clean tissue paper for 30 minutes for drying. These were used to calculate total dentate gyrus (DG) area and granular cells count in the DG region present in the hippocampus of the brain.

Silver Staining

After rehydration of sections, the sections were placed in permanganate ($KMNO_4$ for 5 minutes), potassium nitrate sulphate (2 minutes), iron alum (10 minutes). silver nitrate (10% for 1 minutes) and formalin (1 minute) followed by mounting with DPX and stored at room temperature for further analysis. These were used for morphological changes in the neuronal dendritic tree using sholl's analysis (und Halbach, 2013) as shown in fig. 2. After the neuronal image was taken, it was placed on the trace paper containing circles. The number of branches and counting of ring intersections were noted to prepare Sholl's profile; which gives an idea about dendritic alterations among various treatment groups.

Microscopy

The aforementioned stained slides were examined under bright field microscope (Nikon Eclipse Inverted Microscope, Japan). Images were captured by using NIS-Elements D software and processed using Adobe Photoshop software.

Gene expression studies

The expression levels of post-synaptic density protein (PSD-95) was estimated using qPCR. GAPDH was used as housekeeping gene. Briefly, the total RNA was isolated using TRIzol reagent followed by assessment of its concentration and purity using Multi Scan Sky spectrophotometer, Thermo Fisher Scientific, USA. Afterwards, the cDNA was synthesized using Revert Aid First Strand cDNA synthesis Kit according to the manufacturer's protocol (Thermo Fisher, USA) (Macedo and Ferreira, 2014). The synthesized cDNA was stored at -20˚C till further analysis.

Fig. 2: A representative diagram of Sholl's analysis

Primer designing

Primers have been designed using the primer3 design program at http://frodo.wi.mit.edu/primer3/ (Untergasser *et al*., 2012, Koressaar and Remm, 2007) and the sequences are given below. Each primer was reconstituted in 10mM Tris-HCl/EDTA (TE) buffer (pH 8). The primer stocks (100µM) was prepared from master primer vials and further diluted to 10µM in TE buffer (pH 8.0). Melting temperature (Tm) for each primer was calculated by using the following formula:

$$Tm = 4(G + C) + 2 (A + T)$$

qPCR

Expression level of genes of interest were analyzed by quantitative PCR. Experiment was carried out in triplicates for each gene of interest. The cDNA (0.4µl)

S No.	Gene	Primer	Sequence	Annealing Temperature	Product size
1	GAPDH	F	GTGGACCTCATGGCCTACAT	57°C	157
		R	GGATGGAATTGTGAGGGAGA		
2	PSD-95	F	TGGGATGAGGTAGGATGAGG	57°C	154
		R	AGAAACAGAGCAGGGAGATG		

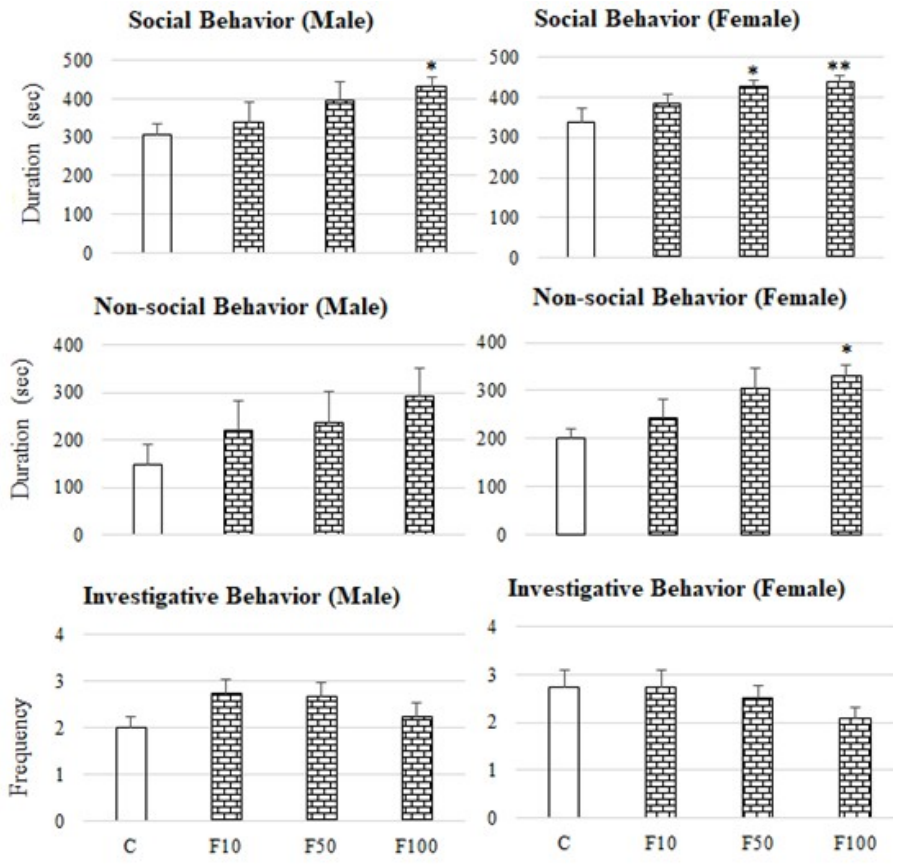

Fig. 3: Effect of pre-weaning fluoxetine exposure on social behavior of rats upon adulthood. The fig. depicts the effect of pre-weaning fluoxetine (10, 50 and 100mg/kg) on the social behaviors (social, non-social and investigative) of rats. Both social and non-social behavior either showed elevated trend or significant rise. Data is presented as mean ± SEM (n = 5). Asterisks showed the significant difference as compared to control. *p<0.05 and **p<0.005

was added in 10µl of 1X SYBR green Master in a PCR tube. In this mixture, the 9.6µL of a particular primer in diluted form (1:100) was added to make up total volume of 20µl. The 40 cycles of denaturation, annealing and extension was run to obtain the CT values (Schmittgen and Livak, 2008). Relative gene expression and relative fold change was calculated for each gene in all groups. GAPDH was used to normalize the expression.

STATISTICAL ANALYSIS

The data is presented as mean ± SEM of n = 5, 3 and 3 per group for behavioral, morphometric and expression studies, respectively. Differences among various means were computed using one-way ANOVA followed by post-hoc analysis (Least significant difference) using SPSS software (Version 20, SPSS Inc, Chicago, IL, USA)

RESULTS

Social behavioral study

Our data showed that pre-weaning fluoxetine exposure significantly enhanced the social behavior in both genders in dose dependent manner (fig. 3). The effect appeared to be more pronounced in female rats. The non-social behavior also exhibit increasing trend in male rats while it became significantly elevated in case of female rats at highest tested dose. However, the investigative behavior remained unaltered.

Morphometric study

The fluoxetine treatment (10, 50 and 100 mg/kg) caused significant increase in the dentate gyrus morphometry (cell count and area) in dose dependent manner in both genders (table 1). The sholl's analysis also revealed significant rise in neuronal branching and dendritic arborization in both genders (fig. 4).

Gene expression study

The pre-weaning fluoxetine exposure caused down regulation of PSD-95 gene in dose dependent manner in both genders as compared to control (fig. 5). The difference was found to be significant at the dose of 10 and 50 mg/kg in female rats only.

DISCUSSION

Pre-natal and early post-natal life is the vulnerable window of development in the life of a developing being. Any trauma (physical, chemical or environmental) during this stage can have long lasting consequence on the overall wellbeing of an individual. In this context, the long term neurobehavioral impact of pharmaceuticals is ignored as the component of developmental toxicity studies. Emerging concepts revealed that this lack of testing is jeopardizing the mental health of humans by exposing them to harmful pharmaceuticals. Keeping this in view, the present study was designed to study the impact of pre-weaning fluoxetine exposure on the social behavior upon attaining adulthood.

Deficit in social behavior, either hypo and hyper, has been attributed to developmental abnormalities and is observed in certain indications such as autism spectrum disorder. Our data showed that the early post-natal fluoxetine exposed rats predominantly demonstrated hypersociability upon adulthood (fig. 3). In similar lines, the fluoxetine treatment was reported to enhance social behavior in pre-existing social deficit gerbils (Hendrie *et al*., 2003). On the contrary, the reduced social behavior was reported in prairie voles adults exposed early to fluoxetine (Lawrence *et al*., 2020). Keeping aforementioned in view, one possible explanation could be the decisive role of specie in defining the outcome of fluoxetine on social behavior. However, the experiment on prairie voles involved subcutaneous painful injections to the mother and higher anxiety levels were observed in the animals too, which may have contributed to reduce socialization as described earlier (Toth, 2019). On the contrary, our experimental design does not involve any painful procedure for the administration of fluoxetine, which may result in opposite outcome. Our data further showed that female rats are more sensitive towards hypersociability induce ability of fluoxetine, which is suggestive of the role of gender in defining the intensity of effect. In similar lines, the vulnerability of female gender towards behavioral alterations following chemical and environmental stressors has been reported earlier (Dagh, 2013, Zaman *et al*., 2017). It is of note that non-social behavior demonstrated increasing trend, which became mildly significant at the highest testes dose in case of female. One possibility for this outcome is enhanced locomotor activity, which may have contributed in enough physical activity to do self-grooming along the grooming of other rats at higher doses.

Fluoxetine was reported to enhance neurogenesis and dendritic arborization; the effect attributed to its antidepressant action (Zavvari *et al*., 2020, de Oliveira *et al*., 2020). Dentate gyrus is an area in the hippocampus of the brain, which was reported to possess the stem cell and has been extensively studied in neurogenesis studies (Licht *et al*., 2020, Abbott and Nigussie, 2020). Keeping this in view, the hippocampal morphometry of rats were performed. In line with the existing literature, the developmentally fluoxetine exposed rats revealed significantly higher area and cells of DG along with superior dendritic arborization (fig. 4, table 1). The abnormally enhanced neurogenesis has been attributed to underlie pathogenesis of social disorder (such as autism) during developmental stage (Packer, 2016, Kaushik and Zarbalis, 2016). Although, the connection between neurogenesis and social disorder is yet to be established, our study provides the direct evidence reporting co-existence of enhanced hippocampal density (cellular count, area and neuronal arborization) and hypersociability in same animals, an outcome worthy of further investigations to delineate the underlying mechanisms of social disorders.

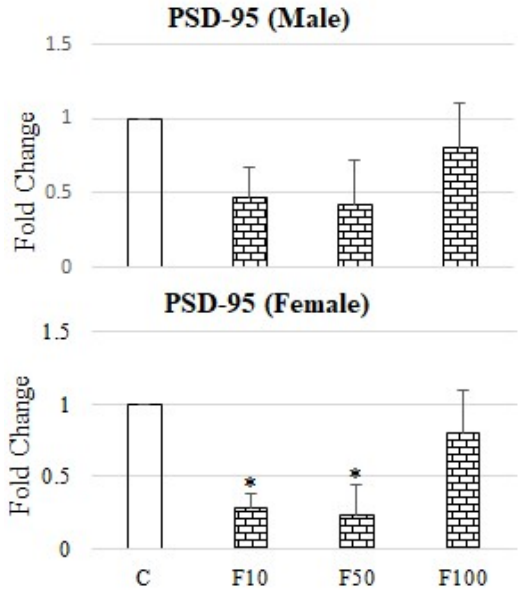

Fig. 5: Effect of pre-weaning fluoxetine exposure on PSD-95 expression in rat brain. The fig. depicts the PSD-95 expression in the brains of rats subjected to fluoxetine (10, 50 and 100 mg/kg) during pre-weaning time period and compared to control. The general down regulation of the gene was observed, which was found to be statistically significant in female rats at 10 and 50 mg/kg. The data is presented as mean ± SEM of relative fold change as compared to control. Asterisks represent significant difference as compared to control (*$p<0.05$).

Post-synaptic density protein (PSD-95) has been considered as an important gene predisposing subject to social deficits, especially hypersociability (Barak *et al*., 2019). Therefore, its expression was also studied in the brain of rats. Our data showed its general down regulation in the brains of both gender, with slight significant changes in females (fig. 5). In similar lines, the PSD-95 knockouts were previously shown to demonstrate hypersocial behavior in both males and females rats

Table 1: Effect of pre-weaning fluoxetine exposure on brain morphometry

Group	Dentate Gyrus (DG) Morphometry		Sholl's Analysis	
	DG cells (count)	DG area (µm)	Average neuronal branches (count)	Dendritic arborization (ring intersections)
MALE				
Control	161±3	418580±21858	4±1	19±10
F10	187±2***	607932±15275***	5±1	31±3
F50	244±3***	668226±17638***	6±2	36±9
F100	265±3***	704599±8819***	8±1*	44±2*
FEMALE				
Control	161±5	411913± 26458	4±1	22±6
F10	182±2***	674599±14530***	6±1	33±8
F50	245±4***	684798±18559***	7±2	38±5
F100	290±2***	886681±37118***	9±2*	52±11*

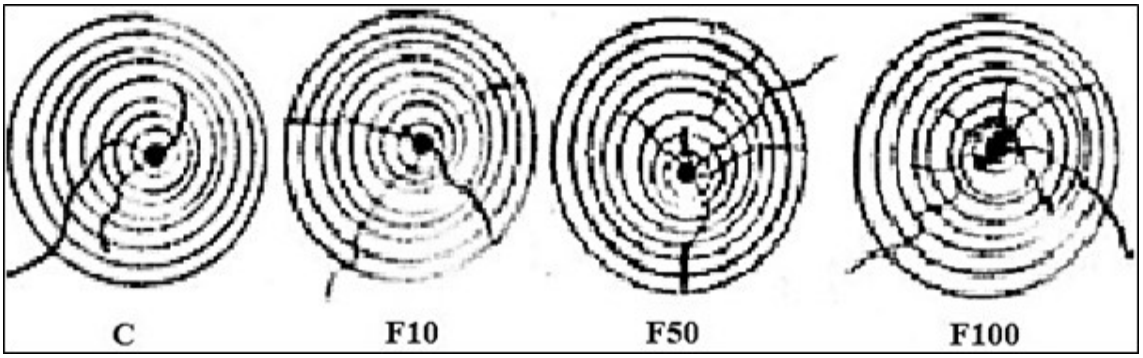

Fig. 4: A representative diagram of Sholl's analysis. The fig. depicts the representative sholl's analysis for morphometric assessment of neurons following silver staining. The "C" represents control, while F10, F50 and F100 represents the treatment groups who have received fluoxetine at the doe of 10, 50 and 100 mg/kg during pre-weaning time period. Enhanced neuronal branching and arborization was observed in test group in a dose dependent manner.

(Winkler *et al*., 2018). Furthermore, the early life fluoxetine exposure was also reported to reduce the expression of PSD-95 in the hippocampus (Millard *et al*., 2019). It is of note that our data revealed slightly increasing trend in PSD-95 expression at highest testes dose of 100 mg/kg. Search of literature also revealed that fluoxetine was reported to slightly increase the expression of PSD-95 (O'Leary *et al*., 2009) and was reported to underlie its antidepressant action too in adult animals (Reinés *et al*., 2008). The aforementioned literature is suggestive of age dependent effect of fluoxetine on the expression of PSD-95. This is in line with emerging concept that immature brain incorporates information into its structure and function differently than the mature brain (Andersen, 2003). Hence, age dependency can possibly be explained in a manner that extensive neuronal growth and proliferation at pre-synaptic level may be balanced by reduction in post-synaptic density at early age. However, the adult predominantly post mitotic brain does not need such homeostatic manipulation of the PSD-95 gene. In this regard, the decisive role of strong homeostasis in brain has been reported earlier (Abbas *et al*., 2012, Abbas *et al*., 2011). However, further work is required to delineate this explanation.

CONCLUSION

the present study demonstrate that pre-weaning fluoxetine exposure predominantly caused hypersociability upon adulthood, which can be attributed to enhanced hippocampal morphometry and reduced expression of PSD-95. Hence, the present study highlights the potential underlying role of fluoxetine in setting the social behavior observed within the society.

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