Neurochemistry International 129 (2019) 104510

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/neuint

Brain region specific methylation and Sirt1 binding changes in MAOA promoter is associated with sexual dimorphism in early life stress induced aggressive behavior

Arpita Konar[a,*], Mohit Rastogi[a], Aksheev Bhambri[a,b]

[a] CSIR-Institute of Genomics & Integrative Biology, New Delhi, India
[b] Academy of Scientific & Innovative Research (AcSIR), Mathura Road, New Delhi, India

ARTICLE INFO

Keywords:
Aggression
MAOA
Peripubertal stress
Sexual dimorphism
Sirt1

ABSTRACT

The maladaptive form of aggressive behavior confers risk for violence and criminal incidences with profound impact on society. Although considerable research has been devoted to elucidate the etiology of aggression, molecular correlates of sex differences remains largely unexplored. Also, little attention has been given to whether males and females respond differently to similar causal factor of aggression. Here, we show the possible association of brain region specific neural activity (c-Fos expression) and monoamine oxidase A (MAOA) epigenetic state with sexual dimorphism in peripubertal stress (PPS) induced adulthood aggression. While PPS adult males exhibited escalated aggression, females spent maximal time in social exploration. c-Fos expression was brain region and sex specific. In the PPS adult cohort, only males showed elevated c-Fos expression in the prefrontal cortex, indicative of their hyper-responsive behavior. MAOA expression and enzyme activity was reduced in hypothalamus and increased in prefrontal cortex of hyper-aggressive male mice. Investigation into the underlying mechanisms revealed hypomethylation in prefrontal cortex and hypermethylation in hypothalamus of MAOA promoter negatively correlating with the expression pattern. On the other hand, binding of Sirt1 to MAOA promoter was diametrically opposite being increased in prefrontal cortex and reduced in hypothalamus. In females, neither expression nor epigenetic state of MAOA gene was significantly altered between control and PPS adult mice. Our study revealed novel epigenetic correlates of sexual dimorphism in stress induced aggressive psychopathology. However, given the multi-factorial nature with environmental influences, further studies are warranted to uncover the biological hub.

1. Introduction

Aggression is an evolutionarily conserved social behavior expressed across the animal kingdom in response to survival threats including predator defense, dominance hierarchy for food, mate and territoriality (Waltes et al., 2016). However, aggression when escalate and becomes maladaptive often referred to as pathological, takes the shape of brutality and consequent increasing incidences of crime. A striking feature of aggressive behavior is sex differences including the type, context and magnitude (Craig and Halton, 2009). Males in general, engage in offensive aggression characterized by intended damage to the opponent with frequent bouts of physical attacks. In certain cases, these attacks are unprovoked and devoid of context. On the other hand, females are generally defensive and exhibit immense self control. They seldom initiate the encounter, inflict no deliberate attacks and are often verbal than physical (Björkqvist, 2018). Such marked sexual dimorphism indicates that the molecular mechanisms regulating aggression may be fundamentally different or diametrically opposed in pattern amongst males and females though still obscure.

In order to have mechanistic understanding, one needs to address the physiological and psychosocial factors that lead to abnormal aggressive behavior. Epidemiological studies have associated early life traumatic experiences in childhood including fear, maltreatment, domestic violence and sexual assault with adult aggression and criminality, but the neurobiological mechanisms remain unclear (Veenema, 2009). We replicated such early life adversity using a previously reported peripubertal stress (PPS) induced animal model of aggression (Márquez et al., 2013; Tzanoulinou et al., 2014).

Given the multitude of pathways in behavioral disorders with genetic x environmental influence, understanding the mechanism of sexual dimorphism is arduous and challenging. In this regard, epigenetic factors operating at genes and environment interface, enabling

* Corresponding author. CSIR-Institute of Genomics & Integrative Biology, Mathura Road, New Delhi, India.
E-mail address: arpita.konar@igib.in (A. Konar).

https://doi.org/10.1016/j.neuint.2019.104510
Received 19 March 2019; Received in revised form 21 July 2019; Accepted 23 July 2019
Available online 24 July 2019
0197-0186/

long term gene expression changes (Lester et al., 2011; Ben-Shahar, 2017) and also considered crucial for sexual orientation of the brain (Ratnu et al., 2017), captured our attention in the first place. Accumulating evidences suggest the pivotal role of DNA methylation and histone modifications in behavioral anomalies led psychiatric illness including depression, schizophrenia and autism (Bagot et al., 2014). Therefore, we hypothesized that these epigenetic machineries could contribute to sex differences in stress induced aggression. We selected prefrontal cortex (PFC) and hypothalamus for our study. PFC is strongly implicated in social behavior (Choy et al., 2018) and considered the major locus for inhibitory control of aggression. On the other hand, hypothalamus has been considered as the integral circuitry of aggression as electrical stimulation of particular hypothalamic regions rapidly induced attack behavior (Lin et al., 2011).

We focused on the epigenetic change of monoamine oxidase A (MAOA) for studying sex difference in stress induced aggression. MAOA metabolizes monoamines including norepinephrine, serotonin, and dopamine and is implicated in the etiology of stress-associated mood disorders (Schulze et al., 2000; Meyer et al., 2006; Bortolato et al., 2008; Ficks and Waldam, 2014) In particular, brain MAOA activity correlated inversely with aggression trait in humans and thus was considered as a neural substrate of aberrant aggression (Alia-Klein et al., 2008). However, brain region specific MAOA expression and epigenetic regulation has not been studied in sex variation of early life stress evoked aggression.

Addressing all the above unanswered questions, the present study was designed to i) assess whether impact of PPS on adult aggressive phenotype is similar in male and female mice ii) analyze whether sex difference if any in behavioral pattern is associated with PFC and hypothalamic specific expression of stress responsive and neuronal activity marker c-Fos and potential aggression marker MAOA iii) determine whether such expression change is regulated by epigenetic modifications particularly DNA methylation and histone deacetylase changes in MAOA core promoter. Here, we analyzed Sirt1 known to regulate MAOA in anxiety and exploratory behavior (Libert et al., 2011).

2. Materials and methods

2.1. Animals

All experimental procedures involving live animals were approved by the animal ethical committee of CSIR-Institute of Genomics and Integrative Biology (IGIB) and followed appropriate guidelines for live animal use in research. Male and female offspring of Balb/c mice bred in the animal house of CSIR-IGIB were used for the study. They were kept at 24 ± 2 °C on a 12 h light/dark cycle with *ad libitum* access to food and water. Animal handling and experiments were conducted in accordance with the institutional guidelines.

2.2. PPS stress procedure

As reported earlier (Márquez et al., 2013) male and female mice were exposed to unpredictable fear inducing stressors of synthetic fox odor (trimethylthiazoline) and elevated platform during the peri-puberty period of postnatal day (P) 28 to P42. Briefly, P28 male and female offspring were exposed to an open-field for 10 min for acclimatization in a novel environment. Thereafter, one group of mice were exposed to 9 µl fox odor (Sigma Aldrich, USA) soaked cloth kept in a filter top plastic cage and elevated platform (96 cm above ground) for 7 random days (P28, P29, P30, P34, P36, P40 and P42) across P28 to P42. Stressors were applied singly or in combination in variable schedule so that the animals do not learn and get suddenly traumatized. The duration of stress session was 25 min following which mice were kept separated for 15 min before being housed together. Control animals were handled on the days in which their counterparts were exposed to PPS. The videos of stress session were captured and total time immobile and freezing behavior was analyzed using ANY MAZE version 5.1 software (Stoelting Co, USA).

2.3. Resident intruder (RI) paradigm

Control and PPS exposed male and female mice were assessed for aggressive behavior in their adulthood (P90) using the conventional RI paradigm. The test was performed as described earlier (Márquez et al., 2013; Koolhaas et al., 2013). The resident was exposed in its home cage to a smaller and unfamiliar (10% less body weight) intruder of the same strain for 10 min for 7 consecutive days. Each day the resident was introduced to a different intruder in a latin square design. The behavioral parameters including clinch attack, move towards, social exploration, ano-genital sniffing, rearing, lateral threat, upright posture, keep down, chase, non-social explore and rest or inactivity were quantified in terms of percentage (duration) of the total observation time. Attack latency or the time between introduction of the intruder and first clinch attack was also determined. The total duration of the clinch attack, offensive upright, keeping down and lateral threat were considered as the measure of total offensive behavior. Social exploration behavior included the sum of social explore, auto and social grooming and ano-genital sniffing.

2.4. qRT-PCR

Total RNA was isolated from PFC and hypothalamus of mice and 2 µg of RNA from each group was reverse transcribed to cDNA synthesis. The cDNA was used as a template for qPCR amplification using gene specific primers for c-Fos (Sense 5′ATGGGCTCTCCTGTCAACAC3′ and anti sense 5′GTCTTCACCATTCCCGCTCT3′) and MAOA (Sense 5′ GCCCAGTATCACAGGCCAC3; and anti sense 5′CGGGCTTCCAGAACCA AGA3′). The endogenous control GAPDH (Sense 5′TGTGTCCGTCGTG GATCTGA 3′ and anti sense 5′CCTGCTTCACCACCTTCTTGA3′) was used to normalize quantification of the mRNA target.

2.5. Immunoblotting

Cytosolic protein lysates (40 µg) prepared from mouse PFC and hypothalamus were resolved on to 10% SDS PAGE, transferred to PVDF membrane and used for immunoblotting using conventional method. The primary antibodies {MAOA rabbit monoclonal EPR7101 (Abcam, ab126751); anti-GAPDH mouse monoclonal (Santa Cruz Biotechnology, sc-32233)} and secondary antibodies {anti-rabbit IgG HRP (Cell Signaling Technology, 7074P2) and anti-mouse IgG HRP (Cell Signaling Technology, 7076P2)} were used at adequate dilutions.

2.6. MAO enzyme activity assay

MAO enzyme activity was measured in PFC and hypothalamus of mice using fluorometric assay kit (K795 Biovision Inc) in accordance with the manufacturer's guidelines. The total MAO as well as individual MAOA and MAOB isoenzyme activity was measured separately in the presence of respective inhibitors, clorgyline and selegiline. The assay is based on the kinetic fluorometric detection (fluorescence Ex/Em = 535/587 nm) of H_2O_2, generated during the oxidative deamination of the MAO substrate (tyramine). H_2O_2 standard curve was plotted and activity was calculated using the formula B/(ΔT X V) x D = pmol/min/ml = µU/ml where B is amount of generated H_2O_2 by MAOs from the standard curve (pmol), ΔT is reaction time (min.) V is sample volume added into the reaction well (ml) and D is sample dilution factor. The specific activity of MAO/MAOA/MAOB was expressed as µU/mg of protein.

2.7. Methylated DNA immunoprecipitation

DNA methylation was analyzed at the promoter region of MAOA by methylated DNA immunoprecipitation (MeDIP) method as mentioned earlier (Mukhopadhyay et al., 2008; Mohn et al., 2009). Briefly, 4 μg of sonicated DNA (DNA fragment size ranging from 300 to 1000 bp) isolated from PFC and hypothalamus of male and female mice was diluted in immunoprecipitation buffer and incubated with 2 μg of 5-methyl cytosine antibody (A-1014; Epigentek) at 4 °C overnight. Mouse IgG Isotype control antibody (02–6502, Thermo Fisher Scientific) was used for mock IP. Next day, 50 μL of Protein A-dynabeads was added and incubated at 4 °C for 2 h with rotation. Thereafter, it was centrifuged at 3500 × *g* at 4 °C for 10 min and the supernatant was removed carefully. After washing the pellet, the immune complex was eluted, DNA was purified and dissolved in TE buffer. Using eluted DNA as template, MAOA proximal promoter −300 to +61 bp from TSS) was amplified with specific primers (Sense 5′ CTGTATGATCAAGGGCCCGC3'; anti sense CGCCTCCAATCACGACTACG) generating a 361 bp product.

2.8. Sirt1 chromatin immunoprecipitation (ChIP)

ChIP was performed as mentioned earlier (Singh et al., 2015). Briefly, 200 μg of chromatin (DNA fragment size ranging from 300 to 1000 bp) isolated from PFC and hypothalamus of mice was pre-cleared with protein A/G-dynabeads for 2 h at 4 °C and then centrifuged at 3500 × *g* at 4 °C for 10 min. The supernatant was saved and divided into input and immunoprecipitation (IP). Sirt1 Antibody (sc-74465) (4 μg) was added to IP fraction and incubated overnight at 4 °C. Mouse IgG Isotype control antibody (02–6502, Thermo Fisher Scientific) was used for mock IP. Next day, Protein A/G-dynabeads (50 μL) was added to it and rotated for 2 h at 4 °C and then centrifuged at 3500 × *g* for 10 min at 4 °C. The pellet was washed, immune complex was eluted, reverse cross linked and DNA was isolated. The sequences of interest [ATG-proximal MAOA promoter known to show SIRT1 responsiveness (Libert et al., 2011) were amplified by qPCR using specific primers.

2.9. Statistical analyses

Each experiment was repeated three times (n = 6 mice/group and total 18 mice used per group in three experiments). In order to analyze qRT-PCR data, the 2ˆ-ΔΔCt value was used to calculate relative fold change in mRNA expression and plotted as histograms. For immunoblot analysis, the signal intensity (Integrated Density Value, IDV) of MAOA bands was measured by spot densitometry tool of AlphaEaseFC software (Alpha Innotech Corp, San Jose, CA, USA), normalized against the IDV of internal control GAPDH and histogram was plotted as relative density value. For Sirt1-ChIP and MeDIP analysis, results were represented as percentage of input using the formula 100*2ˆ{Adjusted input - Ct (IP)} and 100*2ˆ{Adjusted input - Ct (IgG)} where adjusted input is Ct Input-$\log_2$dilution factor. Here we used 10% input so the dilution factor is 10 and adjusted input is Ct Input-$\log_2$10. Histograms were represented as mean of the data (+SD) and statistical significance was calculated by Student's two tailed *t*-test or one way ANOVA followed by post hoc Student-Newman-Keuls test.

3. Results

3.1. PPS triggered immediate fear response and neuronal activity (c-Fos) in both sexes, while escalated aggression evident only in adult males

We first evaluated whether PPS evoked fear response at behavioral and molecular level both in male and female BALB/c mice. Freezing behavior, indicative of fear was evident in both sexes. PPS male and female spent 67.1% and 58.9% of total observation time (25 min) in freezing behavior, respectively. Both sexes were immobile (~85%) during maximum time of the fox odor stress session (Fig. 1A). c-Fos mRNA expression indicative of neuronal activity increased by 6.5-fold in PFC and 3.5 fold in hypothalamus in male mice 3 h after completion of last day (P41) stress session. Alterations in c-Fos mRNA in female brain regions were less drastic as compared to males, being 1.8-fold increase in PFC and 2.5-fold in hypothalamus (Fig. 1B). While male and female mice were similarly traumatized, varying only in extent at early life, they showed distinct sexual dimorphism in behavioral responses and c-Fos expression pattern in adulthood.

Resident intruder (RI) test revealed significant and heightened aggression in PPS exposed adult male mice as compared to male control counterparts. PPS males in adulthood displayed prominent offensive aggression spending maximal time (67%) in clinch attack and 8% of total time in other offensive gestures including lateral threat, keeping down and offensive upright. They spent the rest 25% of time in exploring the opponent intruder chiefly by ano-genital sniffing and chasing behavior. Control males spent 76% of observation time in social exploration; very less 5% time in attack; 15% time in other offensive behavior and rest 4% in non social explore (Fig. 2A). The aggressive male cohort showed short attack latency decreasing with days, being an average of 37.5 s on day 1 reduced to only 15 s on day 7. Attacks primarily comprised of biting at vulnerable body parts of belly and throat, irrespective of all days and intruders, characteristic of pathological aggression. Overall, significant difference was found between control males and stressed males in the total percentage of time spent in offensive aggression (Fig. 2B).

PPS adult females showed completely different RI profile as compared to males. PPS adult females did not show any sign of attack, rather they spent maximum time (80%) in social exploration, 17% time in other offensive displays like upright posture and rest 3% time they were inactive. Control females largely behaved in a similar fashion spending 82% in social exploration and 18% time in offensive behavior but no attack at all (Fig. 2A and B).

PPS adult males showed elevated c-Fos mRNA in PFC (5.06-fold) and hypothalamus (3.41-fold), 3 h post RI test as compared to control males. c-Fos mRNA remained unaltered in PFC but increased by 2.5-fold in hypothalamus of PPS adult females post RI test as compared to control females (Fig. 2C).

3.2. Brain region and sex biased changes in expression and activity of MAOA in PPS adult mice

MAOA mRNA expression remained unaltered in both the brain regions and sexes 3 h after PPS (Fig. 3A i and ii). However, MAOA mRNA and protein levels and enzyme activity showed marked regional and sex specificity in adulthood. PPS adult males showed 2.1 fold upregulation in MAOA mRNA in PFC and 0.27-fold downregulation in hypothalamus, 3 h post RI test as compared to control males. In PPS adult females, MAOA mRNA decreased to a small extent in PFC (0.76-fold) as compared to control females but the changes were not significant. Hypothalamus MAOA mRNA remained unaltered in PPS adult females (Fig. 3A iii and iv). Expression pattern of MAOA protein (60 kDa) corresponded to that of mRNA showing 30% increase in PFC and 42% decrease in hypothalamus in PPS exposed adult male mice as compared to respective control animals (Fig. 3B).

In PPS adult females, no significant change in MAOA protein was observed in any brain region as compared to control without stress exposure (Fig. 3B). Further, we measured total MAO, MAOA and MAOB enzyme activity in PFC and hypothalamus of male mice (Fig. 3C). The specific activity of total MAO enzyme increased from 19.82 μU/mg protein in PFC of control males to 24.79 μU/mg protein in PPS adult males. MAOA activity in PFC also increased from 11.07 μU/mg protein in control adult males to 14.75 μU/mg protein in PPS adult males. MAOB specific activity showed insignificant change in PFC (control adult males- 8.75 μU/mg protein; PPS adult males-10.04). In hypothalamus, opposite pattern was observed as the specific activity of total MAO enzyme decreased from 21.85 μU/mg protein control adults

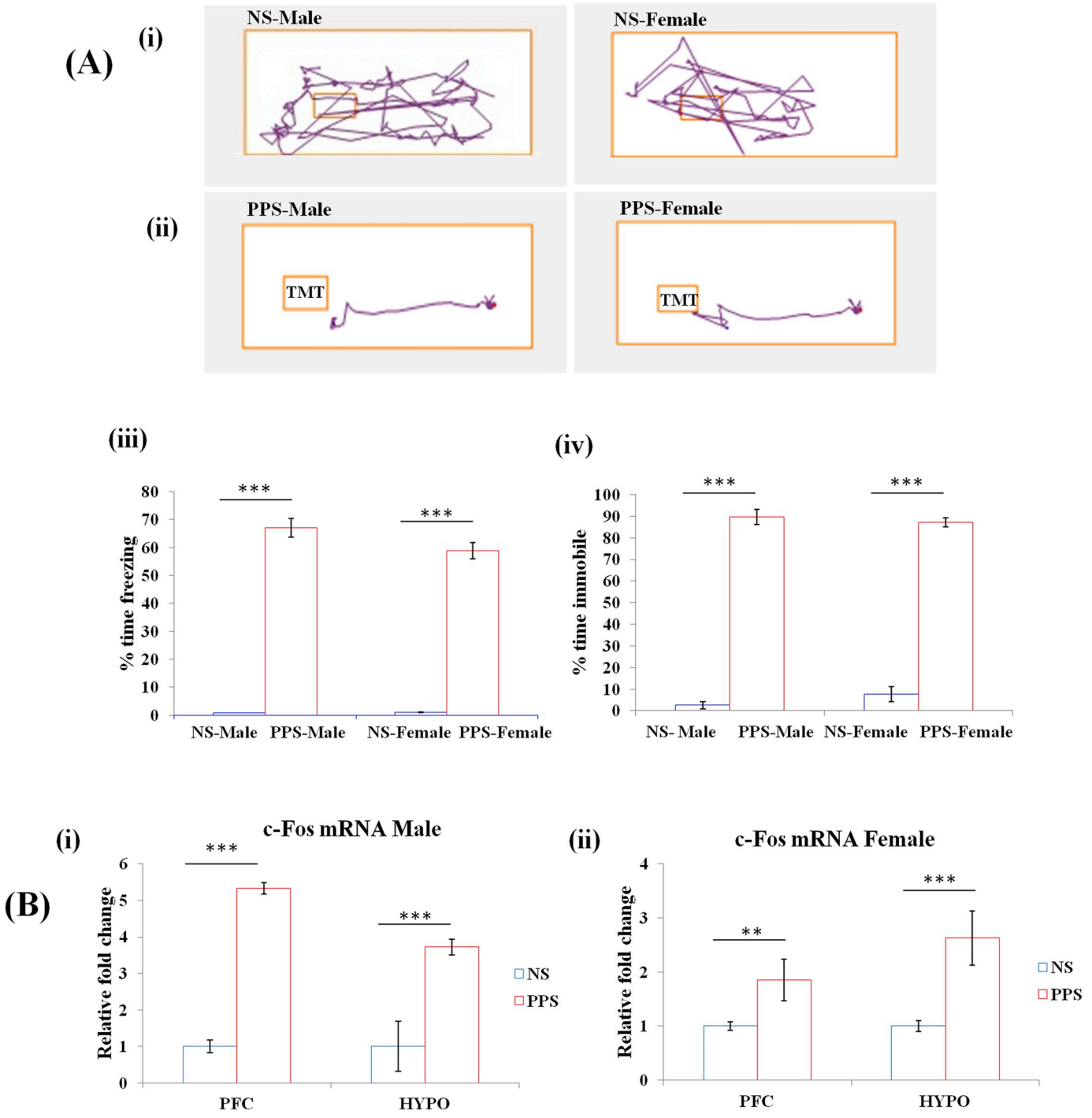

Fig. 1. Peripubertal stress (PPS) induced fear response and c-Fos expression in both sexes. (A) Track plot of NS (i) and PPS (ii) male and female mice during the stress session highlighting the zone of fox odor (Trimethylthiazoline-TMT) soaked cloth. NS mice were exposed to cloth soaked in water. Percentage of time spent in (iii) freezing behavior, (iv) immobility and (B) c-Fos mRNA expression in prefrontal cortex (PFC) and hypothalamus (HYPO) of (i) male and (ii) females. Histogram represents mean of the data from three independent experiments (± SD). Statistical analyses were performed using two tailed Student's t-test. Asterisk '**' denotes significant difference P < 0.01, '***' denotes significant difference P < 0.001 between NS- No stress exposure and PPS- Peripubertal stressed groups (n = 18 animals used for each group).

to 17.33 μU/mg protein in PPS adult males. MAOA activity also decreased from 12.15 μU/mg protein in control mice to 7 μU/mg protein in PPS adult male mice. MAOB specific activity again showed insignificant change in PFC (control adult males- 9.7 μU/mg protein; PPS adult males-10.33uU/mg protein).

3.3. Brain region and sex biased DNA methylation and Sirt1 binding changes in MAOA promoter of PPS adult mice

With the rationale that brain region and sex specific effects on MAOA expression may be controlled epigenetically, we first analyzed methylation status of MAOA proximal promoter (Fig. 4A) in our experimental groups. Methylation state of MAOA promoter as assessed by MedIP PCR, showed a marked brain region specific differential pattern.

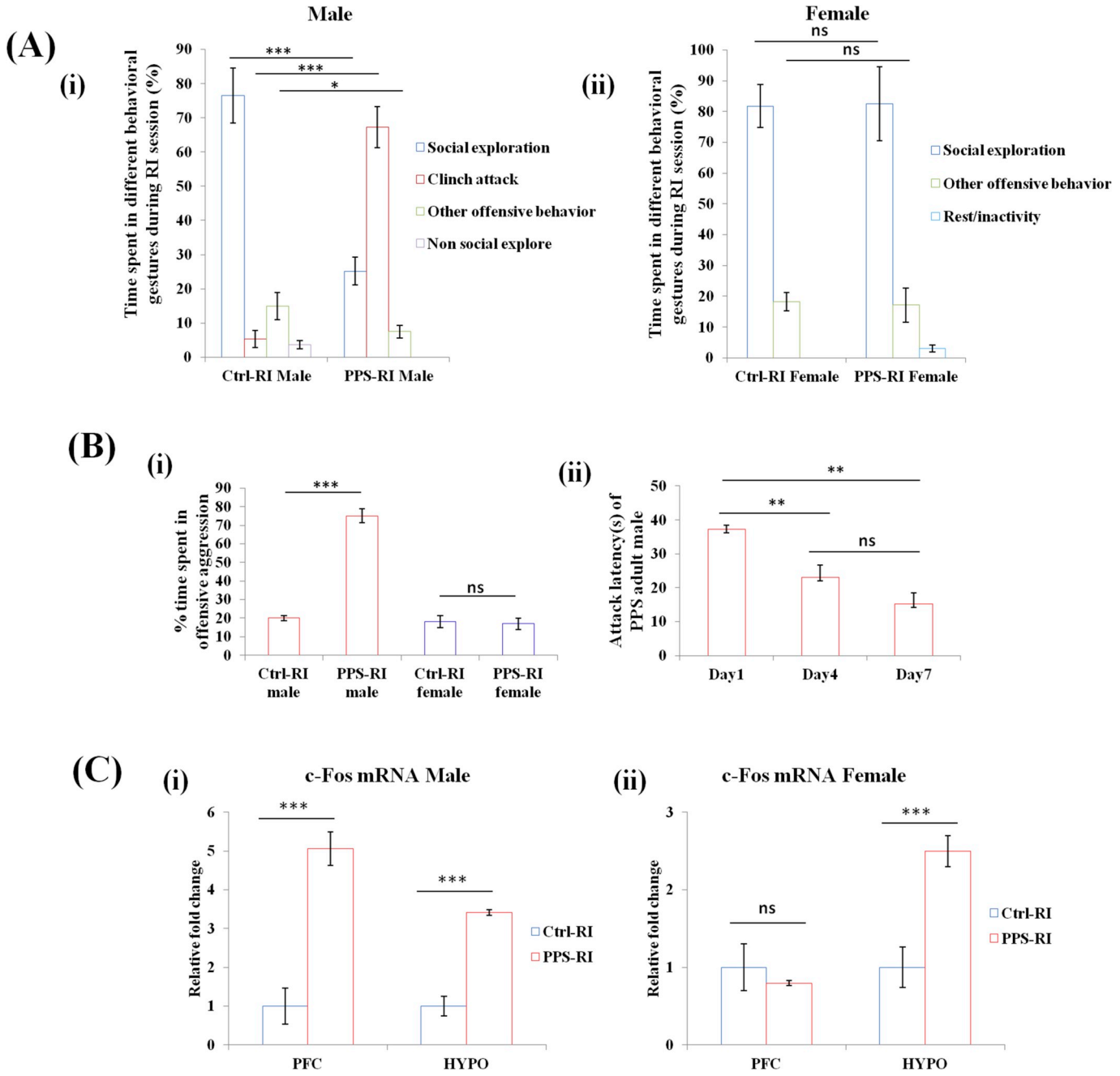

Fig. 2. Sexually dimorphic aggressive behaviors with concomitant changes in c-Fos expression in PPS adult mice (A) Bar plot representing the time spent in different behavioral gestures during the resident intruder (RI) session by (i) male and (ii) female mice, (B) (i) Percentage of time spent in offensive aggression and (ii) Day wise comparison of attack latency of RI test and (C) c-Fos mRNA expression post RI test in (i) male and (ii) female mice. Histogram represents mean of the data from three independent experiments (± SD). Statistical analyses were performed using two tailed Student's t-test except 2B (ii) where one way ANOVA followed by post hoc Student-Newman-Keuls was used. '*' denotes significant difference $P < 0.05$, '**' denotes significant difference $P < 0.01$, '***' denotes significant difference $P < 0.001$ between Ctrl-RI (control adult mice not exposed to PPS) and PPS-RI (PPS exposed adult mice) groups ($n = 18$ animals used for each group). In Fig. 2B (ii) '**' denotes significant difference ($P < 0.05$) as compared to Day1. 'ns' represents no significant difference $P > 0.05$.

In PFC, MAOA promoter was hypomethylated, 1.94% of input recovery in PPS adult males as compared to 7.4% of input recovery in control. In hypothalamus, MAOA promoter was hypermethylated, 6.15% of input recovery in PPS adult males as compared to 3.7% of input recovery in control. In females, the extent of methylation was not statistically significant between control and PPS adult mice. In PFC, MAOA promoter showed 4.8% input recovery in PPS adult females as compared to 5.3% input recovery in control. In hypothalamus, MAOA promoter showed 3.55% input recovery in PPS adult females as compared 3.96% input recovery in control (Fig. 4B). DNA methylation interfering with transcriptional machinery corresponded well with earlier mentioned increase in MAOA mRNA in PFC and reduction in hypothalamus in PPS adult males.

Besides DNA methylation, histone deacetylases work in concert or independently to regulate brain specific gene expression. In this regard, Sirt1, a member of HDAC6 family has been reported to regulate MAOA transcription in brain. Here, Sirt1 binding to MAOA promoter showed brain region and sex specific differential pattern (Fig. s1). Interestingly, the pattern was diametrically opposite to that of methylation. In PFC of PPS adult males, MAOA promoter showed 5.07% input recovery as

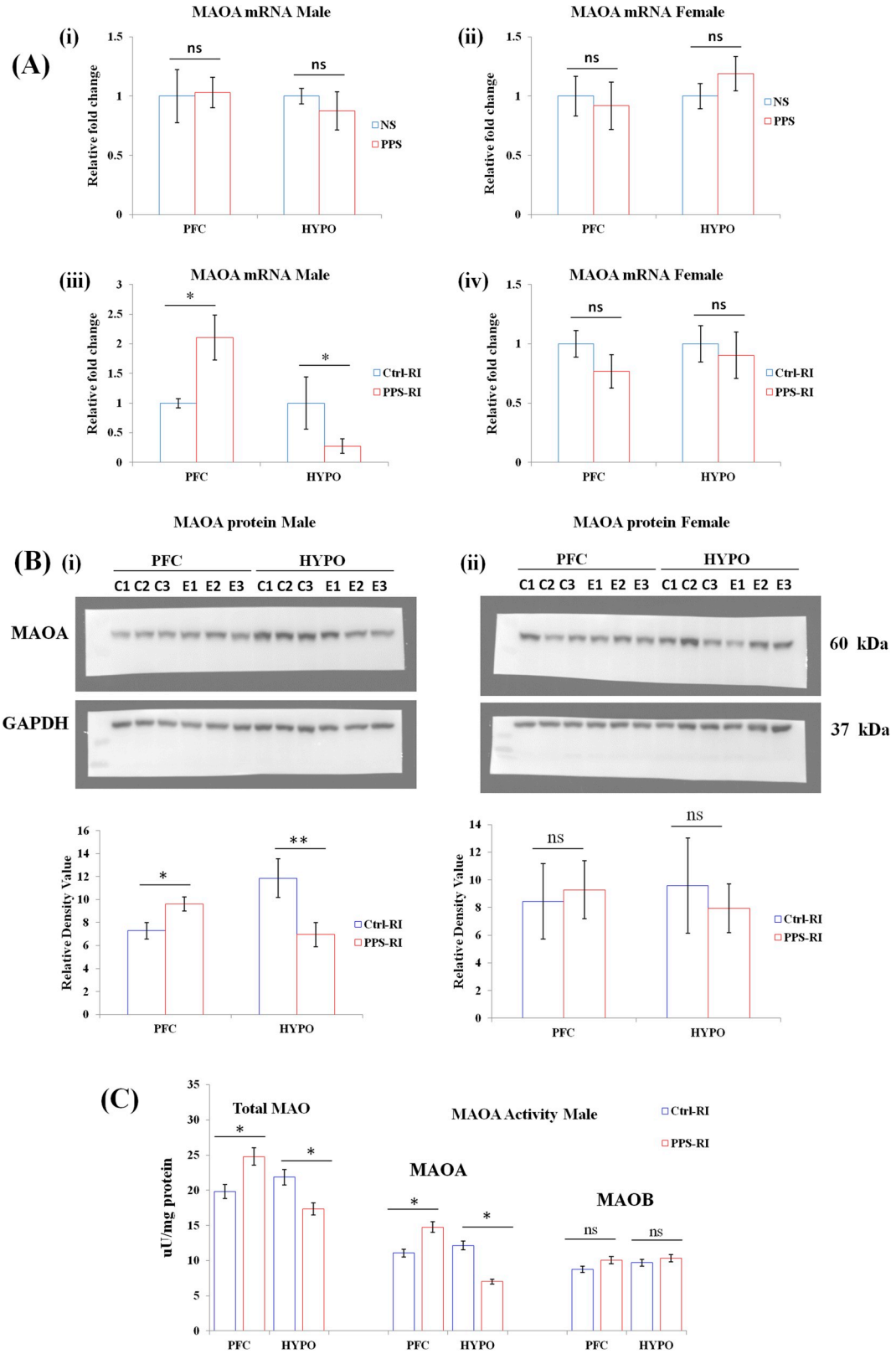

Fig. 3. Brain region and sex-biased, MAOA expression and activity changes in PPS adult mice (A) MAOA mRNA expression 3 h after PPS exposure (i,ii) and in adulthood at postnatal day 90 (iii,iv) (B) MAOA protein expression and (C) enzyme activity in PPS exposed adults. Histogram represents mean of the data from three independent experiments (± SD). Statistical analyses were performed using two tailed Student's t-test. Asterisk '*' denotes significant difference $P < 0.05$, '**' denotes significant difference $P < 0.01$ between NS and PPS or Ctrl-RI and PPS-RI groups ($n = 18$ animals used for each group). Western blot showing representative image of 3 individual mice (biological replicates) of Ctrl-RI group (C1, C2, C3) and 3 individual mice (biological replicates) of PPS-RI group (E1, E2, E3). 'ns' represents no significant difference $P > 0.05$.

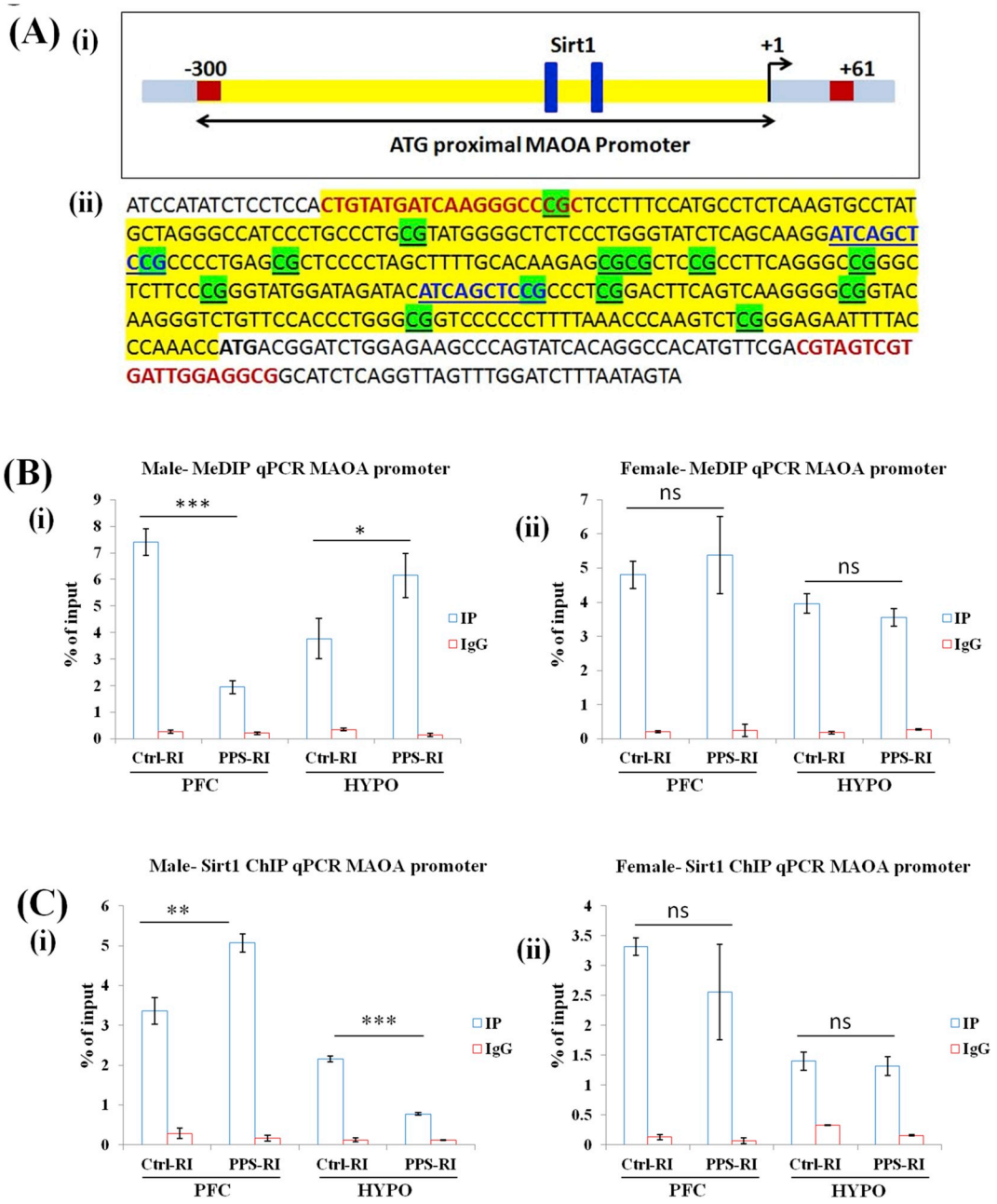

Fig. 4. Brain region and sex specific opposed methylation and Sirt1 binding pattern in PPS adult mice (A) (i) Schematic representation of MAOA promoter showing −300 to +1 ATG proximal promoter (yellow) having Sirt1 binding sites (Blue) Primers used for MedIP and Sirt1 ChIP qPCR amplified the region (red) between −300 and +61 and (ii) Details of the MAOA proximal promoter sequence (−300 to +1) highlighted with yellow showing CG sites (green), putative for methylation and Sirt1 binding region (blue), (B) MedIP qPCR showing percentage of input of 5 methyl cytosine (5MeC) in MAOA proximal promoter in (i) male and (ii) female mice and (C) ChIP qPCR showing percentage of input of Sirt1 in MAOA proximal promoter in (i) male and (ii) female mice. Histogram represents mean of the data from three independent experiments (± SD). Statistical analyses were performed using two tailed Student's t-test. Asterisk '**' denotes significant difference $P < 0.01$, '***' denotes significant difference $P < 0.001$ between Ctrl-RI and PPS-RI groups ($n = 15$ animals used for each group). 'ns' represents no significant difference $P > 0.05$.

compared to 3.36% in control males. In hypothalamus, MAOA promoter showed 0.78% input recovery in PPS adult males as compared to 2.15% in control. In females although there was a subtle decrease of Sirt1 binding evident by 2.55% input recovery in PFC of PPS animals as compared to 3.32% control, individual variation was large and the difference was not statistically significant. In hypothalamus, Sirt1 binding extent was similar in control and PPS adult females (Fig. 4C).

4. Discussion

Early life stress around puberty, critical developmental window for organization of brain (Blakemore et al., 2010) and remodeling of neural circuits has been instrumental in increasing vulnerability for late life behavioral disorders (Paus et al., 2008). In particular, aberrant social behavior has been attributed to adverse experiences during adolescence. Here, we demonstrate that male and female Balb/c mice subjected to same peripubertal stressors exhibit dramatic qualitative and quantitative differences in adulthood aggressive behavior. While male

mice show conspicuous signs of violent aggression, the minimal offensive behavior that was observed in females were not maladaptive. Such behavioral responses correlated with PFC and hypothalamus specific c-Fos and MAO levels along with altered methylation and Sirt1 binding in MAOA promoter.

Majority of studies on aggressive behavior originated from male rodents and only a few studies which explored females have never directly compared the two sexes in similar experimental environment. Moreover, sex differences in long lasting impact of stress on behavior have often been linked with anatomical differences in brain and gonad hormones without really determining the neural molecular correlates (McCarthy et al., 2012). Investigating sex specific molecular differences with emphasis on epigenetic changes add to the major strength of the work. Another distinct feature of the work lies in inclusion of both PFC and hypothalamus and comparing the molecular differences. PFC inactivity in animal models and humans has been positively correlated with aggression and antisocial personality disorders, though causal role is still elusive (Choy et al., 2018). However, studying PFC independently can partially decipher the causal mechanism in etiology of aggression but it is equally important to consider the connection it holds with other systems. Therefore, we were also intrigued to see concomitant alterations in hypothalamus, critical for manifestation of aggression and more importantly prefronto-hypothalamic projections elegantly shown to regulate type and magnitude of aggressive behavior (Biro et al., 2018).

Sex difference in behavioral disorders could be due to differential response to psychosocial threats. Here, we observed that the immediate response of predator fear and elevated platform was largely similar in males and females. c-Fos mRNA was induced in PFC and hypothalamus of both sexes after completion of stress session. Immediate early genes, primarily c-Fos expression has been considered a molecular measure of neuronal activity induced by an acute physical or psychological stress (Lin et al., 2018). c-Fos induction pattern in different brain regions has been utilized to decipher the neural circuits and adaptive stress responses in rodent models (Kovács, 2008).

The extent of c-Fos upregulation was drastic in PFC as compared to hypothalamus and more in stressed males than females. PFC required decision making and moral judgment is very important in adolescents with social and cognitive burden and audacious behavior. Therefore, PFC has been the most vulnerable brain region for acute as well as chronic emotional stress during adolescence (Raine and Yang, 2006). Neural architecture and cognitive functions associated with PFC gets compromised even with milder and single stress exposure while other brain regions are threatened after several days or weeks (Brown et al., 2005; Izquierdo et al., 2006; Arnsten, 2009). With regard to the higher magnitude of c-Fos increase in males, earlier studies have also reported such sex specificity in response to acute restraint stress. Acute stress evoked similar neural circuitry in both males and females though magnitude varied depending on the brain region and specifically the neuronal populations being studied (Babb et al., 2013). The variable responses are independent of circulating sex steroid hormones. On the other hand nature of stressor has been attributed to sex differences in c-Fos expression based neural activation patterns in medial PFC, lateral septum, habenula and hippocampus (Sood et al., 2018).

PPS male mice displayed offensive escalated aggression in adulthood with signs of pathological form as was also reported for Wistar rats (Márquez et al., 2013). However, female resident mice exhibited social exploratory behavior with no intention to harm the intruder. Although the type of traumatic experiences generally vary between men and women, sex differences has been prevalent even in same kind of trauma including natural calamities, parental loss or accidental insults (Shansky, 2015; Tolin and Foa, 2006). Recently, it has been reported that with the same stressor, male rats were hyper-responsive while females showed distinct depression like phenotype, independent of gonad hormones (Pooley et al., 2018). They suggested that rather being resilient, females respond differently to traumatic stress causative of behavioral differences.

Our findings partially do not correspond to an earlier report (Cordero et al., 2013) where female Wistar rats subjected to peripuberty stress were more aggressive than the control animals. However, they have not compared the RI profile of males and females subjected to same experimental regime. In our laboratory conditions, PPS adult females also showed certain offensive gestures like moving towards and chasing but no attack. **S**exually dimorphic extent and pattern of c-Fos induction was again noted in PPS adult mice. Especially, in PFC the increase in c-Fos mRNA was limited to only PPS aggressive males. A selection study between short and long attack latency mice revealed that consistent c-Fos activation in the medial PFC is required for violent attacks (Haller et al., 2006). It is possible that c-Fos increase in PFC was persistent right after PPS till adulthood and indicative of the frequent clinch attack behavior shown by stressed adult males.

After evaluation of behavioral and c-Fos associated neural activity pattern, next we investigated expression and epigenetic state of MAOA in sex differences of aggression. Earlier studies demonstrated that MAOA null mice with elevated serotonin and norpepinephrine were more aggressive than normal and MAOB null counterparts (Meyer-Lindenberg et al., 2006; Narvaes and de Almeida, 2014). Serotonin receptor agonists also inhibited inter-male and social instigation associated aggression.(Centenaro et al., 2008). Effect of complete MAOA gene ablation on aggression was determined, but its role in sexual dimorphism and correlation with brain region specific expression pattern was not known.

We observed PFC elevation and hypothalamic downregulation of MAOA mRNA and protein levels as well as enzyme activity only in adult aggressive mice, being unaffected in females. PFC has connections to brain stem monoamine cells bodies (Santana and Artigas, 2017) and can regulate catecholamine inputs as well as suppress stress response of brain stem (Diorio et al., 1993). Since hypothalamus is involved in the way aggression is expressed, it is possible MAOA reduction is required for offensive attack gestures and as a feedback mechanism; MAOA got induced in PFC to suppress the hypothalamic response. PFC rise in MAOA could also be required to dampen the inhibitory influence on aggression and warrants investigation.

In humans, trauma exposed children with MAOA-L polymorphism were predisposed to develop antisocial behavior as adults. However, environmental modulations were more crucial than genetic variation suggesting involvement of other gene × environment interaction pathways (Weder et al., 2009). These evidences attributed the adjective of “warrior gene” to MAOA, though it is not clear as to whether it drives aggression or not. We also did not find significant change in MAOA levels at peripubertal age post stress exposure, indicating that it might be required for manifestation of aggression and does not act as a trigger existing from early life.

We hypothesized that brain region and sex specific expression of MAOA in PFC and hypothalamus correlating with distinct differences in stress induced aggression phenotype could be due to altered epigenetic regulation. An *in silico* study determined two CpG islands, one overlapping with MAOA core promoter and the other located upstream; both sensitive to differential methylation (Shumay and Fowler, 2010). Later individual differences in brain MAOA level and enzyme activity was associated with methylation status of white blood cells (Shumay et al., 2012). They suggested that differential methylation of MAOA gene warrants investigation inter individual variation in behavioral phenotypes. Following this study, other groups have shown hyper or hypomethylated state of MAOA promoter in blood samples of psychiatric disorders including schizophrenia (Chen et al., 2012), panic disorder (Domschke et al., 2012), PTSD (Ziegler et al., 2018) and antisocial personality disorder (Checknita et al., 2015). However all these studies have focused on peripheral tissue and methylation state of MAOA in brain is largely unexplored in these disorders. Moreover correlation of sex differences in brain MAOA promoter methylation with any behavioral phenotype is also not known. Here, we have shown

for the first time both brain region and sex specific methylation changes in MAO could attribute to differences in magnitude and type of aggressive behavior. Detailed investigation of the CpG sites and putative transcription factors binding to those sites would make the picture more conspicuous.

Chromatin remodeling mechanisms particularly post translational modifications of histones catalyzed by HATs and HDACs play crucial role in gene regulation. DNA methylation machinery including DNMTs and methyl binding proteins are also known to interact with some of these HDACs for transcriptional control. Sirt1 is a unique HDAC6 that directly deacetylate histones as well as wide range of transcription factors thus capable of both activating and silencing gene expression (Zhang and Kraus, 2010; Yamamoto and Takahashi, 2018). More importantly, Sirt1 has been reported to decetylate and activate NHLH2, a transcription factor of MAOA gene (Libert et al., 2011). Also, Sirt1 is a direct target of miR-142-5p, which reduced MAOA transcription in neurons (Chaudhuri et al., 2013). Intrigued by these reports, we explored Sirt1 and MAOA link in sex differences of aggression and its dependence on brain regions. In accordance with our hypothesis, Sirt1 showed more binding in PFC and reduced in hypothalamus correlating with PFC increase and hypothalamic MAOA mRNA reduction in aggressive males. In females, both MAOA expression and Sirt1 binding was not altered. Here, Sirt1 regulation of MAOA expression can be mediated through NHLH2 or even other transcription factors that have binding sites on the differentially methylated regions of MAOA promoter. Earlier studies have demonstrated plausible association of Sirt1 (Lo et al., 2015) polymorphisms with substance abuse and its blood levels with childhood stress induced depression (Hou et al., 2018). However, for the first time we have reported Sirt1 in trauma evoked aggression and its sex variation. Our findings highlight the importance of brain region specific gene expression and differential epigenetic states in sex differences in stress induced behavioral disorders. However the study is correlative and warrants further investigation on genome wide causal factors.

Conflicts of interest

The authors declare no potential conflicts including financial, personal or academic interests.

Acknowledgements

A. Konar and M. Rastogi are supported by grants from Department of Science and Technology, Ministry of Science and Technology, Government of India, under the project head DST/INSPIRE/04/2014/002261/GAP0125. A. Bhambri is supported by GATE Senior Research Fellowship [31/GATE/43(6)/2014-EMR-I] from Council of Scientific and Industrial Research, Govt. of India.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2019.104510.

References

Alia-Klein, N., Goldstein, R.Z., Kriplani, A., Logan, J., Tomasi, D., Williams, B., Telang, F., Shumay, E., Biegon, A., Craig, I.W., Henn, F., Wang, G., Volkow, N.D., Fowler, J.S., 2008. Brain monoamine oxidase A activity predicts trait aggression. J. Neurosci. 28 (19), 5099–5104.

Arnsten, A.F., 2009. Stress signalling pathways that impair prefrontal cortex structure and function. Nat. Rev. Neurosci. 10 (6), 410–422.

Babb, J.A., Masini, C.V., Day, H.E., Campeau, S., 2013. Sex differences in activated CRF neurons within stress-related neurocircuitry and HPA axis hormones following restraint in rats. Neuroscience 234, 40–52.

Bagot, R.C., Labonté, B., Peña, C.J., Nestler, E.J., 2014. Epigenetic signaling in psychiatric disorders: stress and depression. Dialogues Clin. Neurosci. 16 (3), 281–295.

Ben-Shahar, Y., 2017. Epigenetic switch turns on genetic behavioral variations. Proc. Natl. Acad. Sci. U.S.A. 114 (47), 12365–12367.

Biro, L., Sipos, E., Bruzsik, B., Farkas, I., Zelena, D., Balazsfi, D., Toth, M., Haller, J., 2018. Task division within the prefrontal cortex: distinct neuron populations selectively control different aspects of aggressive behavior via the hypothalamus. J. Neurosci. 38 (17), 4065–4075.

Björkqvist, K., 2018. Gender differences in aggression. Curr. Opin. Psychol. 19, 39–42.

Blakemore, S.J., Burnett, S., Dahl, R.E., 2010. The role of puberty in the developing adolescent brain. Hum. Brain Mapp. 31 (6), 926–933.

Bortolato, M., Chen, K., Shih, J.C., 2008. Monoamine oxidase inactivation: from pathophysiology to therapeutics. Adv. Drug Deliv. Rev. 60 (13–14), 1527–1533.

Brown, S.M., Henning, S., Wellman, C.L., 2005. Mild, short-term stress alters dendritic morphology in ratmedial prefrontal cortex. Cerebr. Cortex 15 (11), 1714–1722.

Centenaro, L.A., Vieira, K., Zimmermann, N., Miczek, K.A., Lucion, A.B., de Almeida, R.M., 2008. Social instigation and aggressive behavior in mice: role of 5-HT 1A and 5-HT1B receptors in the prefrontal cortex. Psychopharmacology (Berlin) 201 (2), 237–248.

Chaudhuri, A.D., Yelamanchili, S.V., Fox, H.S., 2013. MicroRNA-142 reduces monoamine oxidase A expression and activity in neuronal cells by downregulating SIRT1. PLoS One 8 (11), e79579.

Checknita, D., Maussion, G., Labonté, B., Comai, S., Tremblay, R.E., Vitaro, F., Turecki, N., Bertazzo, A., Gobbi, G., Côté, G., Turecki, G., 2015. Monoamine oxidase A gene promoter methylation and transcriptional downregulation in an offender population with antisocial personality disorder. Br. J. Psychiatry 206 (3), 216–222.

Chen, Y., Zhang, J., Zhang, L., Shen, Y., Xu, Q., 2012. Effects of MAOA promoter methylation on susceptibility to paranoid schizophrenia. Hum. Genet. 131 (7), 1081–1087.

Choy, O., Raine, A., Hamilton, R.H., 2018. Stimulation of the prefrontal cortex reduces intentions to Commit aggression: a randomized, double-blind, placebo-controlled, stratified, parallel-group trial. J. Neurosci. 38 (29), 6505–6512.

Cordero, M.I., Ansermet, F., Sandi, C., 2013. Long-term programming of enhanced aggression by peri-puberty stress in female rats. Psychoneuroendocrinology 38 (11), 2758–2769.

Craig, I.W., Halton, K.E., 2009. Genetics of human aggressive behaviour. Hum. Genet. 126 (1), 101–113.

Diorio, D., Viau, V., Meaney, M.J., 1993. The role of the medial prefrontal cortex (cingulate gyrus) in the regulation of hypothalamic-pituitary-adrenal responses to stress. J. Neurosci. 13, 3839–3847.

Domschke, K., Tidow, N., Kuithan, H., Schwarte, K., Klauke, B., Ambrée, O., Reif, A., Schmidt, H., Arolt, V., Kersting, A., Zwanzger, P., Deckert, J., 2012. Monoamine oxidase A gene DNA hypomethylation - a risk factor for panic disorder? Int. J. Neuropsychopharmacol. 15 (9), 1217–1228.

Ficks, C.A., Waldman, I.D., 2014. Candidate genes for aggression and antisocial behavior: a meta-analysis of association studies of the 5HTTLPR and MAOA-uVNTR. Behav. Genet. 44 (5), 427–444.

Haller, J., Tóth, M., Halasz, J., De Boer, S.F., 2006. Patterns of violent aggression-induced brain c-fos expression in male mice selected for aggressiveness. Physiol. Behav. 88 (1–2), 173–182.

Hou, Y., Su, B., Chen, P., Niu, H., Zhao, S., Wang, R., Shen, W., 2018. Association of SIRT1 gene polymorphism and its expression for the risk of alcoholic fatty liver disease in the Han population. Hepatol. Int. 12 (1), 56–66.

Izquierdo, A., Wellman, C.L., Holmes, A., 2006. Brief uncontrollable stress causes dendritic retraction ininfralimbic cortex and resistance to fear extinction in mice. J. Neurosci. 26, 5733–5738.

Koolhaas, J.M., Coppens, C.M., de Boer, S.F., Buwalda, B., Meerlo, P., Timmermans, P.J., 2013. The resident-intruder paradigm: a standardized test for aggression, violence and social stress. J. Vis. Exp. 4 (77), e4367.

Kovács, K.J., 2008. Measurement of immediate-early gene activation- c-fos and beyond. J. Neuroendocrinol. 20 (6), 665–672.

Lester, B.M., Tronick, E., Nestler, E., Abel, T., Kosofsky, B., Kuzawa, C.W., Marsit, C.J., Maze, I., Meaney, M.J., Monteggia, L.M., Reul, J.M.H.M., Skuse, D.H., Sweatt, J.D., Wood, M.A., 2011. Behavioral epigenetics. Ann. NY Acad. Sci. 1226, 14–33.

Libert, S., Pointer, K., Bell, E.L., Das, A., Cohen, D.E., Asara, J.M., Kapur, K., Bergmann, S., Preisig, M., Otowa, T., Kendler, K.S., Chen, X., Hettema, J.M., van den Oord, E.J., Rubio, J.P., Guarente, L., 2011. SIRT1 activates MAOA in the brain to mediate anxiety and exploratory drive. Cell 147, 1459–1472.

Lin, D., Boyle, M.P., Dollar, P., Lee, H., Lein, E.S., Perona, P., Anderson, D.J., 2011. Functional identification of an aggression locus in the mouse hypothalamus. Nature 470 (7333), 221–226.

Lin, X., Itoga, C.A., Taha, S., Li, M.H., Chen, R., Sami, K., Berton, F., Francesconi, W., Xu, X., 2018. c-Fos mapping of brain regions activated by multi-modal and electric foot shock stress. Neurobiol. Stress. 8, 92–102.

Lo, Iacono L., Visco-Comandini, F., Valzania, A., Viscomi, M.T., Coviello, M., Giampà, A., Roscini, L., Bisicchia, E., Siracusano, A., Troisi, A., Puglisi-Allegra, S., Carola, V., 2015. Adversity in childhood and depression: linked through SIRT1. Transl. Psychiatry 5, e629. https://doi.org/10.1038/tp.2015.125.

Márquez, C., Poirier, G.L., Cordero, M.I., Larsen, M.H., Groner, A., Marquis, J., Magistretti, P.J., Trono, D., Sandi, C., 2013. Peripuberty stress leads to abnormal aggression, altered amygdala and orbitofrontal reactivity and increased prefrontal MAOA gene expression. Transl. Psychiatry 3, e216. https://doi.org/10.1038/tp.2012.144.

McCarthy, M.M., Arnold, A.P., Ball, G.F., Blaustein, J.D., De Vries, G.J., 2012. Sex differences in the brain: the not so inconvenient truth. J. Neurosci. 32 (7), 2241–2247.

Meyer, J.H., Ginovart, N., Boovariwala, A., Sagrati, S., Hussey, D., Garcia, A., Young, T., Praschak-Rieder, N., Wilson, A.A., Houle, S., 2006. Elevated monoamine oxidase a levels in the brain: an explanation for the monoamine imbalance of major depression. Arch. Gen. Psychiatr. 63 (11), 1209–1216.

Meyer-Lindenberg, A., Buckholtz, J.W., Kolachana, B., Hariri, A.R., Pezawas, L., Blasi, G., Wabnitz, A., Honea, R., Verchinski, B., Callicott, J.H., Egan, M., Mattay, V., Weinberger, D.R., 2006. Neural mechanisms of genetic risk for impulsivity and violence in humans. Proc. Natl. Acad. Sci. U.S.A. 103 (16), 6269–6274.

Mohn, F., Weber, M., Schübeler, D., Roloff, T.C., 2009. Methylated DNA immuno-precipitation (MeDIP). Methods Mol. Biol. 507, 55–64.

Mukhopadhyay, A., Deplancke, B., Walhout, A.J., Tissenbaum, H.A., 2008. Chromatin immuno-precipitation (ChIP) coupled to detection by quantitative real-time PCR to study transcription factor binding to DNA in *Caenorhabditis elegans*. Nat. Protoc. 3 (4), 698–709.

Narvaes, R., de Almeida, R.M., 2014. Aggressive behavior and three neurotransmitters: dopamine, GABA, and serotonin-A review of the last 10 years. Psychology Neuroscience 7 (4), 601–607.

Paus, T., Keshavan, M., Giedd, J.N., 2008. Why do many psychiatric disorders emerge during adolescence? Nat. Rev. Neurosci. 9 (12), 947–957.

Pooley, A.E., Benjamin, R.C., Sreedhar, S., Eagle, A.L., Robison, A.J., Mazei-Robison, M.S., Breedlove, S.M., Jordan, C.L., 2018. Sex differences in the traumatic stress response: the role of adult gonadal hormones. Biol. Sex Differ. 9, 32–44.

Raine, A., Yang, Y., 2006. Neural foundations to moral reasoning and antisocial behavior. Soc. Cogn. Affect. Neurosci. 1 (3), 203–213.

Ratnu, V.S., Emami, M.R., Bredy, T.W., 2017. Genetic and epigenetic factors underlying sex differences in the regulation of gene expression in the brain. J. Neurosci. Res. 95 (1–2), 301–310.

Santana, N., Artigas, F., 2017. Laminar and cellular distribution of monoamine receptors in rat medial prefrontal cortex. Front. Neuroanat. 11, 87.

Schulze, T.G., Müller, D.J., Krauss, H., Scherk, H., Ohlraun, S., Syagailo, Y.V., Windemuth, C., Neidt, H., Grässle, M., Papassotiropoulos, A., Heun, R., Nöthen, M.M., Maier, W., Lesch, K.P., Rietschel, M., 2000. Association between a functional polymorphism in the monoamine oxidase A gene promoter and major depressive disorder. Am. J. Med. Genet. 96 (6), 801–803.

Shansky, R.M., 2015. Sex differences in PTSD resilience and susceptibility: challenges for animal models of fear learning. Neurobiol. Stress. 1, 60–65.

Shumay, E., Fowler, J.S., 2010. Identification and characterization of putative methylation targets in the MAOA locus using bioinformatic approaches. Epigenetics 5 (4), 325–342.

Shumay, E., Logan, J., Volkow, N.D., Fowler, J.S., 2012. Evidence that the methylation state of the monoamine oxidase A (MAOA) gene predicts brain activity of MAO A enzyme in healthy men. Epigenetics 7 (10), 1151–1160.

Singh, P., Konar, A., Kumar, A., Srivas, S., Thakur, M.K., 2015. Hippocampal chromatin modifying enzymes are pivotal for scopolamine- induced synaptic plasticity gene expression changes and memory impairment. J. Neurochem. 134 (4), 642–651.

Sood, A., Chaudhari, K., Vaidya, V.A., 2018. Acute stress evokes sexually dimorphic, stressor-specific patterns of neural activation across multiple limbic brain regions in adult rats. Stress 21 (2), 136–150.

Tolin, D.F., Foa, E.B., 2006. Sex differences in trauma and posttraumatic stress disorder: a quantitative review of 25 years of research. Psychol. Bull. 132 (6), 959–992.

Tzanoulinou, S., Riccio, O., de Boer, M.W., Sandi, C., 2014. Peripubertal stress-induced behavioral changes are associated with altered expression of genes involved in excitation and inhibition in the amygdala. Transl. Psychiatry 4, e410. https://doi.org/10.1038/tp.2014.54.

Veenema, A.H., 2009. Early life stress, the development of aggression and neuroendocrine and neurobiological correlates: what can we learn from animal models? Front. Neuroendocrinol. 30 (4) 497-418.

Waltes, R., Chiocchetti, A.G., Freitag, C.M., 2016. The neurobiological basis of human aggression: a review on genetic and epigenetic mechanisms. Am J. Med. Genet. B Neuropsychiatr. Genet. 171 (5), 650–675.

Weder, N., Yang, B.Z., Douglas-Palumberi, H., Massey, J., Krystal, J.H., Gelernter, J., Kaufman, J., 2009. MAOA genotype, maltreatment, and aggressive behavior: the changing impact of genotype at varying levels of trauma. Biol. Psychiatry 65 (5), 417–424.

Yamamoto, M., Takahashi, Y., 2018. The essential role of SIRT1 in hypothalamic-pituitary axis. Front. Endocrinol. (Lausanne). 9, 605.

Zhang, T., Kraus, W.L., 2010. SIRT1-dependent regulation of chromatin and transcription: linking NAD+ metabolism and signaling to the control of cellular functions. Biochem .Biophys. Acta. 1804 (8), 1666–1675.

Ziegler, C., Wolf, C., Schiele, M.A., Feric Bojic, E., Kucukalic, S., Sabic Dzananovic, E., Goci Uka, A., Hoxha, B., Haxhibeqiri, V., Haxhibeqiri, S., Kravic, N., Muminovic Umihanic, M., Cima Franc, A., Jaksic, N., Babic, R., Pavlovic, M., Warrings, B., Bravo Mehmedbasic, A., Rudan, D., Aukst-Margetic, B., Kucukalic, A., Marjanovic, D., 13, Babic, D., Bozina, N., Jakovljevic, M., Sinanovic, O., Avdibegovic, E., Agani, F., Dzubur-Kulenovic, A., Deckert, J., Domschke, K., 2018. Monoamine Oxidase A gene methylation and its role in posttraumatic stress disorder: first evidence from the south eastern Europe (SEE)-PTSD study. Int. J. Neuropsychopharmacol. 21 (5), 423–432.