Title: Early life trauma leads to violent behavior and its inheritance by impairing local thyroid hormone availability in brain

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Abstract

Violent behavior is an aberrant form of aggression that has detrimental impact on health and society. Early life trauma triggers adulthood violence and criminality, though molecular mechanisms remain elusive. Here, we provide brain region specific transcriptome profiles of peripubertal stress (PPS) exposed adult violent male and resilient female mice. We identify transthyretin (TTR) as a key regulator of PPS induced violent behavior and its intergenerational inheritance. TTR mediated long-term perturbation in hypothalamic thyroid hormone (TH) availability contributed to male violent behavior without affecting circulating hormone. Ttr gene ablation in hypothalamus impaired local TH signaling including levels of TH transporters (Mct8, Oatp1c1), deiodinase 2 (DIO2) and TH responsive genes (Nrgn, Trh and Hr). Violent behavior and impaired TTR-TH signaling was also inherited in F1 male progenies. Further, we deciphered Ttr promoter hyper methylation in hypothalamus of violent males across generations. Our findings reveal that trauma during puberty trigger lasting violent behavior by epigenetic programming of TTR and consequent impaired local thyroid availability in brain. TTR-TH signaling in hypothalamus can serve as potential target in reversal of violent behavior.

Introduction

Violent behavior is a complex personality trait that intersects with several psychopathologies and can lead to antisocial and criminal activities (*1*). Every year, more than 1 million people worldwide die because of assault and many more are victimized of domestic violence and other forms of physical injuries. Besides afflicting the common mass, violence poses enormous financial burden for emerging society and is a major challenge to human welfare. Such global threat to humanity necessitates identification of predisposing factors and early intervention strategies. Maladaptive form of normal aggression is considered violent, marked by an inability to conform to social norm. Normal aggression is a behavioral response to threat and competition, but when expressed out of proportion, control and context including misjudging age, sex of the opponent loses its social communicative nature. Such uncontrolled aggression devoid of inhibitory mechanisms can have injurious consequences and referred to as pathological (*2,3*). Animal aggression can also be pathological, if there is a response surpassing species-typical levels; attacks targeted on inappropriate partners, and body parts prone to serious injury; attacks not signaled by threats; or ignorance of signals of opponents (*4*) In general, these criteria resemble human aggressiveness expressed in certain psychopathologies.

The key to combat violent behavior is deciphering the triggers underlying brutal shift of normal adaptive aggression to pathological form. Mounting epidemiological evidences link early life traumatic experiences with adult aggression and criminality. A landmark study of 50 violent offenders with history of childhood abuse pioneered the concept that brain is susceptible to stress during critical periods of early life deteriorating mental health. In particular, trauma around puberty or adolescence including fear, maltreatment, physical and sexual abuse confers susceptibility to violence in adult individuals. Moreover, such behavioral anomalies are not limited parental generations and can be faithfully transmitted to progenies who have never been exposed to trauma. Although pathological aggression has emerged as a consequence of early life adversities, biological insights are obscure. Majority of research in the field of aggressive biology have focused on the adaptive form without really considering the excessive or inappropriate forms and clinical importance of targeting violent individuals (*5*). Essentially, the lacunae in biologically relevant and valid animal models for pathological aggression are the primary reason for the gap in knowing the biological roots. Recently, Tzanoulinou et al. (*6*) developed a novel animal model which showed the effect of peripubertal fearful exposures on pathological aggression at adulthood.

They primarily focused on neural circuits of aggression and on a single gene MAOA in isolation.

Considering multi-factorial etiology of violent behavior, we rationalized that unbiased genome wide investigation would decipher key molecular pathways that can be exploited further as prediction and intervention targets. We modeled PPS induced pathological aggression in laboratory bred Balb/c mice and screened the extreme violent male cohort. Female mice showed resilience towards trauma induced violent behavior as also reported previously (*7*). Next, we performed a sex specific transcriptome analysis in vulnerable brain regions of hypothalamus and prefrontal cortex (PFC). Hypothalamus is an integral brain region for expression of both normal and deviant or maladaptive form of aggressive behavior. Further, neural circuit specific manipulation experiments revealed that ventromedial hypothalamus is the key region for inter-male aggression (8,9) While hypothalamus is considered as the trigger centre for aggression, PFC plays opposite regulatory role being involved in inhibition of threat provoked aggressive behavior. More importantly, direct neuronal projections from PFC to hypothalamus have been suggested to control both type and amplitude of aggressive behavior (*10*,*11*). Therefore, we primarily focused on hypothalamic molecular culprits of abnormal violent aggression and also included PFC in our study to understand inter-brain regional molecular regulation if any.

We prioritized Ttr gene given its i) top rank in hypothalamus transcriptome analysis and unique sex specific diametrically opposite expression pattern in hypothalamus and PFC and iii) long term gene expression changes from early peripubertal age till adulthood. Next, we deciphered a molecular mechanism whereby PPS incited sustained TTR deficiency in hypothalamus resulted in altered levels of other thyroid hormone (TH) transporters (Mct8, Oatp1c1) and deiodinase (DIO2), reduced local TH availability and modulated expression of TH regulated genes (Nrgn, Trh) that eventually led to emergence of violent behavior. These behavioral and molecular deficits were also transmitted to the non-stressed F1 male progenies of PPS violent F0 males. Further, epigenetic analysis revealed that methylation mark in Ttr promoter might attribute to such long term programming of behavior.

Results

Selection of extreme violent phenotypes

The goal of the experiment was to screen the adult animals exhibiting extreme violent phenotype in response to PPS exposure. Screening parameters were optimized based on

earlier reports (12). We initially observed that in a cohort of N=60 control Balb/c mice 95% were non-aggressive (Nagg) while 5% showed minimal offensive aggression (Lagg) but devoid of any pathological signs. Amongst PPS male mice cohort of 60, 78% were hyper-aggressive (Hagg) with signs of pathological aggression including short attack latency and attack on females and anesthetized intruder in all the sessions tested, 13% were moderate-aggressive (Magg) showing signs of pathological form in some days of the RI session and 9% showed normal offensive aggression across 7 days of 10 min screening sessions. From the Hagg groups, we selected the extreme violent mice (Vio) that showed greater than 80% of attack duration and less than minute attack latency (Fig. 1B,1C) in all the session and those which attacked both females and anaesthetized intruder (Supplementary Movie S1 to S5). As reported earlier (*7*) females did not show pathological aggression.

Transcriptome analyses identify hypothalamus and PFC specific gene signatures in PPS adult violent males and resilient females

To discover unbiased molecular correlates of early life trauma induced violence and its sex differences we used RNA-sequencing to measure all polyA-containing transcripts in hypothalamus and PFC of control and PPS and male and female mice. Experiment was performed in 3 independent biological replicates for all the samples and tissues were collected 24 h after last RI session. Heatmaps of differentially expressed genes (DEG)s were constructed from the global transcriptome analysis. In hypothalamus, 49 genes were differentially expressed amongst which 28 were down-regulated, 20 were up-regulated and 1 was expressed in experimental males but not in control males. PFC of violent males showed 87 DEGs amongst which 57 were downregulated, 28 were upregulated and 2 were only expressed in experimental males but not control males (Fig. 1D, 1E)

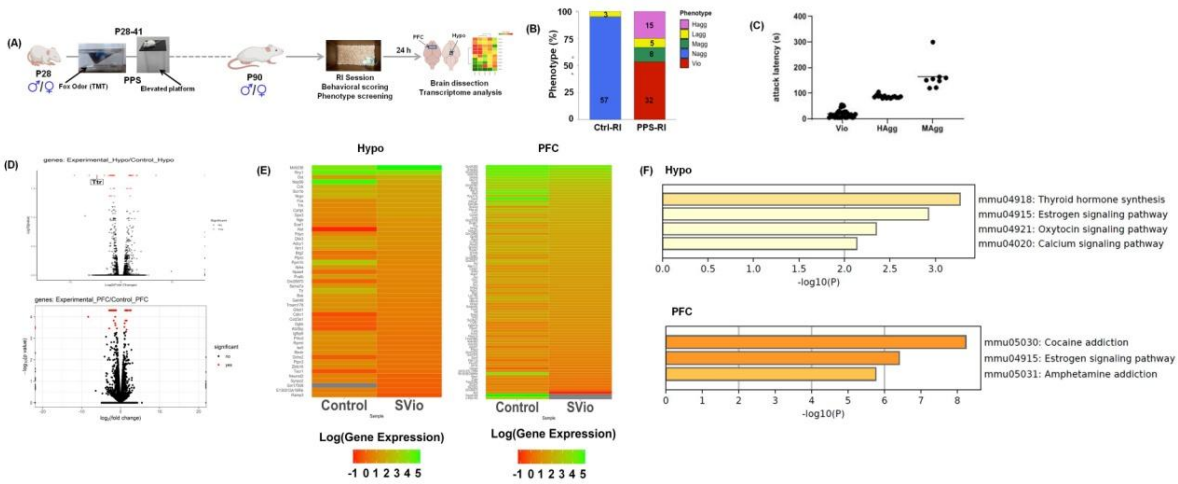


Fig. 1. Brain region specific transcriptional responses in peripubertal stress induced adult violent males. (A) Experimental timeline of peripubertal stress (PPS) exposure, resident intruder (RI) behavioural paradigm, brain dissection and trnascriptome analysis. (B) (i) Phenotypic behavioral screening post RI scoring in control mice without PPS exposure (Ctrl-RI; N=60) and Experimental mice with PPS exposure (PPS-RI; N=60). Histogram represents non-aggressive (Nagg; N=57) and less aggressive (Lagg;N=3) mice in the Ctrl-RI cohort. PPS-RI cohort comprises of violent (Vio; N=32), hyper-aggressive (Hagg; N=15), moderate-aggressive (Magg; N=8) and less-aggressive (Lagg; N=5) mice (C) Attack latency of Vio, Hagg and Lagg mice of PPS-RI cohort. (D) Volcano plot, (E) heatmap of differentially expressed genes {DEGs; Ctrl-RI (Control) vs PPS-RI Violent (SVio) males} in hypothalamus (Hypo) and prefrontal cortex (PFC) and (F) KEGG gene enrichment analysis in males. RNA sequencing libraries were prepared from three independent biological replicates each of Control and SVio group.

Resilient females showed more DEGs (hypothalamus-684;PFC-152) than violent males when compared to their respective control samples (Fig. 2A, 2B) Comparative analysis of male vs female showed both overlapping and discrete gene signatures. In hypothalamus, 16 DEGs overlapped between male and female, 12 showing expression changes in opposite direction, 4 in similar direction and 33 genes were exclusive in violent male cohort (Fig. 2D). In PFC, 15 DEGs overlapped between male and female all showing expression changes in similar direction and 72 genes were exclusive to violent males.

In order to identify the gene signatures causal for PPS induced male violent aggression, we prioritized genes of 2 categories including i) male exclusive DEGs in Hypothalamus and PFC, ii) DEGs that showed opposite pattern in both sexes. Amongst these DEGs, we selected top ranking 10 genes from each category and finally 20 DEGs got validated by RT-PCR.

Ttr, encoding for thyroid hormone (TH) transporter protein was the topmost ranking gene in hypothalamus of our transcriptome data (Fig.1D Volcano plot) that was validated by RT-PCR. Further, it was the only gene showing unique brain region and sex specific diametrically opposite pattern (Fig 3 and Supplementary Fig S1 B Volcano plot). Gene ontology enrichment analysis using KEGG tool combined with literature mining also showed TH signaling as one of the top ranking pathways (1F). TH signaling genes Nrgn and Trh was amongst the top ranking genes in hypothalamus (Supplementary Fig S1 A Volcano plot) and showed sex specific opposite pattern in hypothalamus (Fig. 4).

Amongst rest of the 17 genes (Supplementary Fig. S2), 14 were male exclusive but altered either in hypothalamus (downregulated Nrn1, Rtn4r, NeuroD2, Zbtb16, Pvalb; up-regulated Cartpt, Gm17508, Oxt or PFC (downregulated Gas5; upregulated Cyr61, Gm12840, Dcn, Man1c1, Sox2ot) but remained unaffected in females (data not shown). 3 genes (Apold btg2, ddx39b) were upregulated in both hypothalamus and PFC (Fig, S2) but unaltered in females (data not shown). We, therefore, focused on Ttr and carried out detailed functional analysis pertaining to TH signaling in our experimental regime.

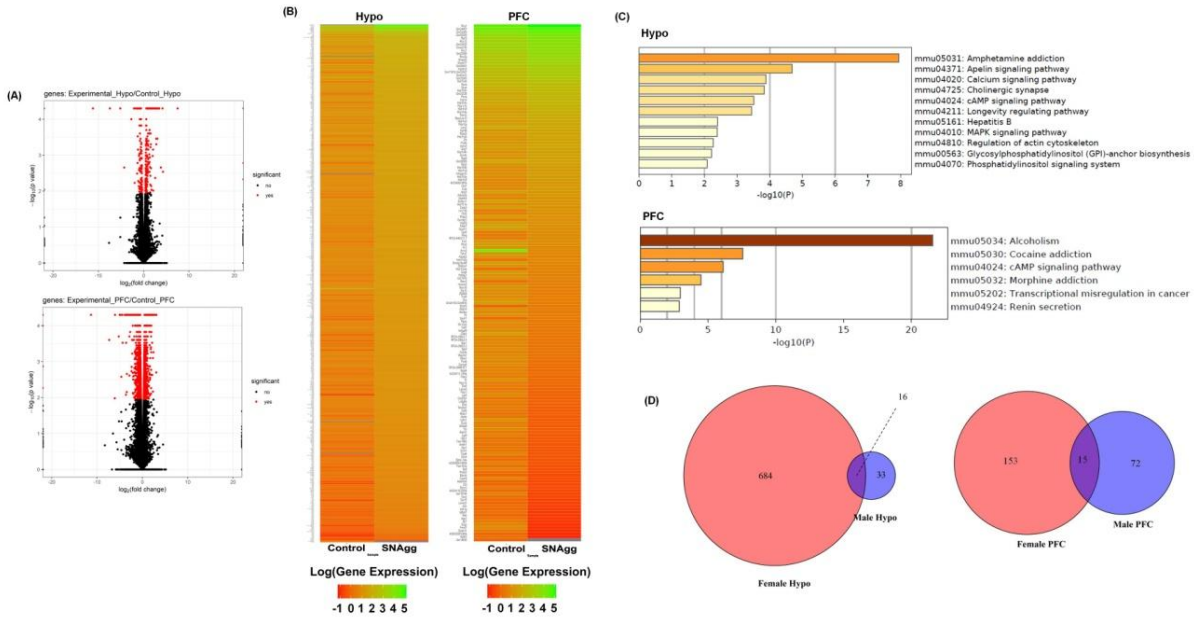


Fig. 2. Brain region specific transcriptional responses in peripubertal stress induced adult resilient females. (A) (i) Volcano plot, (B) heatmap of differentially expressed genes (DEGs; Ctrl-RI (Control) vs PPS-RI Non aggressive (SNAgg) females} in hypothalamus (Hypo) and prefrontal cortex (PFC) and (C) KEGG analysis and (D) Venn diagram of Hypo and PFC specific overlapping DEGs between SVio males and SNAgg females.RNA sequencing libraries were prepared from three independent biological replicates each of Control and SNAgg group.

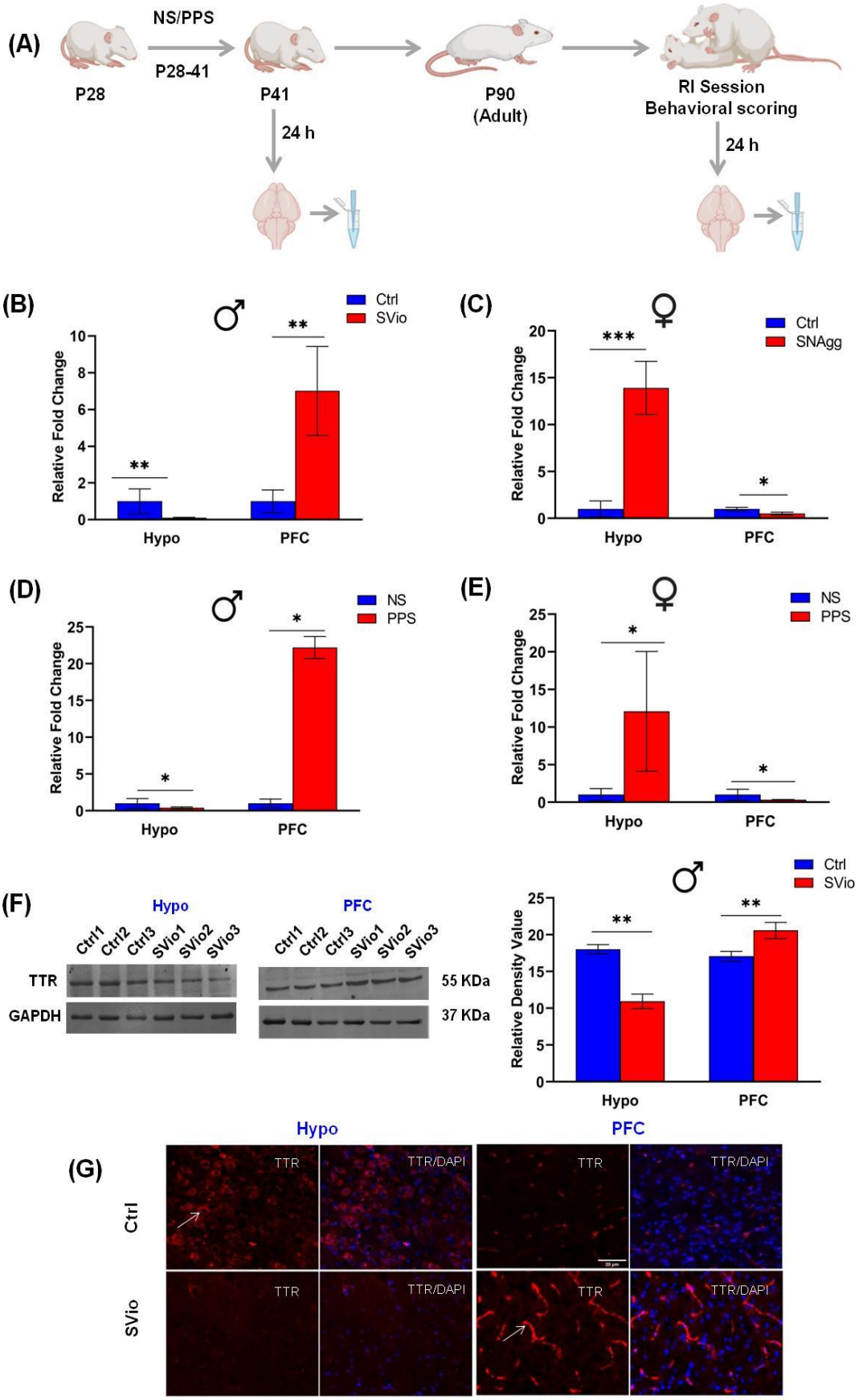
PPS incites persistent changes in Ttr gene expression in both sexes

RT-PCR validation of the transcriptome data revealed unique brain region and sex biased diametrically opposite expression pattern of the only gene Transthyretin (Ttr) in adult mice cohort. Ttr was also amongst the topmost DEGs based on fold change and p value (Fig. 1D volcano plot). In PPS induced adult violent males (SVio), Ttr mRNA showed a decrease of 0.1-fold in hypothalamus and a robust increase of 7-fold in PFC relative to control (Ctrl) males (Fig. 3B). On the contrary, adult females that did not show violent phenotype (SNAgg), Ttr mRNA expression pattern was opposite to males, being increased in hypothalamus (13.9-fold) and drastically reduced (0.5-fold) in PFC relative to control counterparts (Fig. 3C)

In order to understand whether this gene expression changes was persistent from peripubertal age, we analyzed Ttr mRNA in brain regions post 24h after PPS exposure. The direction of Ttr mRNA changes was similar at peripuberty in both the brain regions and sexes although there were minor differences in the extent. PPS caused drastic reduction in hypothalamus (0.40-fold) and increase in PFC of Ttr mRNA expression (22-fold) of males (Fig. 3D). In females, the changes were reverse being upregulated (12-fold) in hypothalamus and reduced (0.31-fold) in PFC of PPS mice relative to unstressed (NS) controls (Fig. 3E)

TTR protein alters in spatial and cell type specific manner

Immunoblot analysis of TTR protein levels corresponded to its transcript pattern in both the sexes. TTR protein was reduced to 0.37fold in hypothalamus and upregulated by 1.36- fold in PFC in PPS induced adult violent males (SVio), relative to control (Ctrl) animals (Fig. 3F). Until now we were considering the changes in bulk tissue, therefore, we performed immunofluorescence to elucidate spatial and cell type specificity if any. In SVio males, TTR protein intensity was significantly reduced in hypothalamus and was exclusive to specialized glial cells, tanycytes in ventromedial region. In PFC, TTR protein intensity was markedly increased in the endothelial cells. (Fig. 3G). TTR was found unaffected in choroid plexus region, referred to as the main site of the protein synthesis (Supplementary Fig. S3). Of note, TTR showed changes in cells involved in uptake of blood borne substances in the brain which further intrigued us to check the TTR mediated uptake of thyroxine in the brain regions.


(A)
NS/PPS
P28-41
P28
P41
24 h
P90
(Adult)
RI Session
Behavioral scoring
24 h
(B)
Ctrl
SVio
Relative Fold Change
Hypo
PFC
(C)
Ctrl
SNAgg
Relative Fold Change
Hypo
PFC
(D)
NS
PPS
Relative Fold Change
Hypo
PFC
(E)
NS
PPS
Relative Fold Change
Hypo
PFC
(F)
Hypo
PFC
Ctrl1
Ctrl2
Ctrl3
SVio1
SVio2
SVio3
TTR
GAPDH
55 KDa
37 KDa
Ctrl
SVio
Relative Density Value
Hypo
PFC
(G)
Hypo
PFC
Ctrl
SVio
TTR
TTR/DAPI

172

Fig. 3. Peripubertal stress induced long term changes in TTR expression in brain region and sex specific diametrically opposed pattern. (A) Experimental timeline for transthyretin (TTR) expression analysis. Ttr mRNA expression profile in Hypo and PFC of peripubertal stress exposed (PPS) adult (B) male (SVio) and (C) female (SNAgg) mice with control (Ctrl) counterparts 24 h after RI session (N=9 mice/group). Ttr mRNA expression profile in Hypo and PFC of peripubertal (D) male and (E) female mice 24 h after stress exposure (PPS) with control [no stress exposure (NS)] counterparts (N=3 mice/group). Ttr protein expression profile (F) immunoblot and (G) immunofluorescence analysis in Hypo and PFC of Ctrl and SVio males (N=3 mice/group). Immunoblot showing three independent biological replicates of Ctrl and SVio groups. Histogram represents mean of the data (+ SD). Statistical analysis were performed using unpaired Student's t-test [* ($p< 0.05$), ** ($p< 0.01$) and *** ($p< 0.001$)] between NS vs PPS groups, Ctrl vs SVio or Ctrl vs SNAgg.

TTR perturbation affects long term availability of thyroid hormone in brain with concomitant changes in transporters, deiodinases and target gene expression

To explore the functional consequences of perturbed TTR expression, we measured peripheral as well as brain region specific T4 and T3 content in both sexes. Circulating TH including total T4 (Fig 4B, 4D) and T3 in serum (Fig 4F, 4H) was neither altered in adulthood nor at peripubertal age in both sexes. Interestingly, brain TH content was remarkably altered corresponding to TTR gene expression right from peripuberty till adulthood. In adult violent males (SVio), total T4 and T3 was reduced in hypothalamus but increased in PFC as compared to control samples (Ctrl) (Fig. 4C, 4G). These changes of hypothalamic and PFC T4 and T3 content was persistent from early peripubertal (NS vs PPS males) age (Fig. 4E, 4I). Details of T4 and T3 concentration have been tabulated in Supplementary Table S2 and S3.

Besides TTR, local TH availability in the brain is dependent upon TH transporters and deiodinase enzymes that determine the intracellular conversion of T4 to T3. Further, TH mediates its action by regulating expression of target genes. Therefore, we addressed possible changes in brain local TH signalling by analyzing levels of TH transporters, Mct8 and Oatp1c1 and deiodinase 2 (Dio2). We also explored TH responsive genes that was differentially expressed in our transcriptome data (Trh, Nrgn).Both Mct8 and Oatp1c1 transcript levels were reduced in hypothalamus of SVio males to 0.59-fold and 0.80-fold respectively, but remained unaltered in PFC as compared to Ctrl males (Fig. 4J, 4K). On the other hand, Dio2 mRNA showed a subtle increase of 1.89-fold in hypothalamus but did not show significant change in PFC (Fig. 4L). DIO2 enzyme concentration was also increased in hypothalamus (Ctrl- 11.1 ng/ml ; Svio- 14.73 ng/ml) but was reduced in PFC of SVio males (3.96 ng/ml) as compared to Ctrl (11.55 ng/ml) males (Fig.4M).

Hypothalamic reduction in T4 and T3 content and consequent impaired TH signaling was clearly evident from expression of downstream target genes. TH responsive Nrgn mRNA expression showed significant downregulation of 0.5-fold in SVio males compared to Ctrl males (Fig. 4O) in hypothalamus similar to Ttr mRNA. NRGN protein level was also reduced to 0.6-fold in hypothalamus of SVio males (Fig.4P). Uncropped gel images of NRGN western blot has been included in supplementary Fig. S4 B). Another TH regulated gene, Trh showed a robust increase of 5-fold in hypothalamus of SVio males while remained unaltered in PFC (Fig. 4N). Both Nrgn and Trh mRNA levels showed similar expression profile in early life peripubertal age (Supplementary Fig. S4 A) indicating a long term changes in gene

expression. In females, direction of changes was reverse being increased in hypothalamus and reduced in PFC (Supplementary Fig. S4 C-F). Details of T4 and T3 concentration have been tabulated in Supplementary Table S4 and S5.

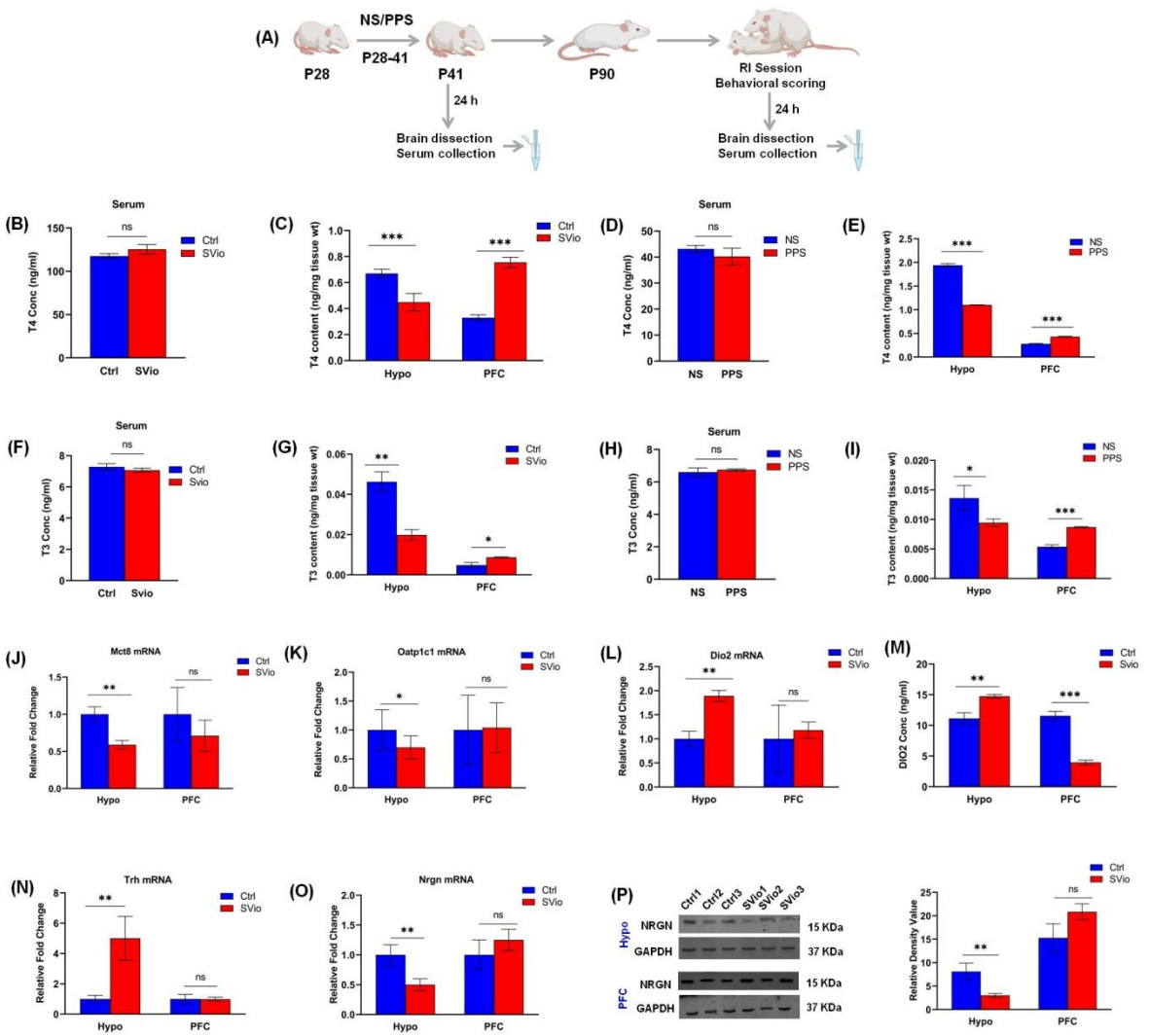


Fig. 4. Peripubertal stress induced long term perturbation of thyroid hormone availability in the brain of violent males with concomitant changes in transporters, deiodinase and target gene expression. (A) Experimental timeline. T4 level in (B) serum and (C) brain regions (Hypo and PFC) of peripubertal stress (PPS) exposed (SVio) and control (Ctrl) adult males 24 h after RI session (N=3 mice/group). T4 level in (D) serum and (E) brain regions (Hypo and PFC) of peripubertal males 24 h after stress exposure (PPS) with control [no stress exposure (NS)] counterparts (N=3 mice/group). T3 level in (F) serum and (G) brain regions (Hypo and PFC) of peripubertal stress (PPS) exposed (SVio) and control (Ctrl) adult males 24 h after RI session (N=3 mice/group). T3 level in (H) serum and (I) brain regions (Hypo and PFC) of peripubertal males 24 h after stress exposure (PPS) with control [no stress exposure (NS)] counterparts (N=3 mice/group). Mct8 mRNA (J), Oatp1c1 mRNA (K) and Dio2 mRNA(L) expression profile in Hypo and PFC of Ctrl and SVio males (N=3 mice/group). DIO2 enzyme (M) concentration in Hypo and PFC of Ctrl and SVio males (N=3

mice/group). Trh mRNA (N) and Nrgn mRNA (O) expression profile in Hypo and PFC of Ctrl and SVio males (N=3 mice/group). NRGN protein expression (P) profile in Hypo and PFC of Ctrl and SVio males (N=3 mice/group). The immunoblot is showing three independent biological replicates of Ctrl and SVio groups. Histogram represents mean of the data (+ SD). Statistical analysis were performed using unpaired Student's t-test [ns ($p > 0.05$),* ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$)] between NS vs PPS groups or Ctrl vs SVio.

Drug induced TH manipulation in hypothalamus perturbed local TH signaling and evoked violent behavior in males without stress exposure

To more directly assess the thyroid hormone manipulation on violent behavior, control males not subjected to PPS were stereotaxically injected with T3 depleting drug methimazole (MMI) in hypothalamus and synthetic levothyroxine (LT4) in PFC. Interestingly, intra-hypothalamic administration of MMI by stereotaxy triggered violent behavioural phenotypes in adult male mice even without stress exposure (Supplementary Movie S6). Average attack latency of MMI treated male mice were less than a minute (32 seconds) and spent 34.6% time of total RI session (average) in clinch attack as compared to 3.8% time (average) of saline (SA) treated controls (Fig.5B) .The extent of behavioral response was similar to that of PPS exposed adult males (SVio) in earlier experiments (Fig.1). However stereotaxic injection of LT4 in PFC did not produce any change in behavioral phenotype (Fig. 5C). This implicated that hypothalamic deficiency in thyroxine could be causal in manifesting violent phenotype while increase in PFC could be a consequence.

Local determinants of TH availability including Mct8, Oatp1c1, Dio2 and target genes Nrgn and Trh were also affected upon MMI treatment in hypothalamus indicating perturbed TH signaling. Both Mct8 and Oatp1c1 transcript levels were drastically diminished in hypothalamus of MMI treated males to 0.29-fold and 0.20-fold respectively, as compared to SA treated control males. On the other hand, Dio2 mRNA showed increase of 1.86-fold in MMI hypothalamus as compared to SA treated control males (Fig. 5D). DIO2 enzyme concentration was also increased in MMI hypothalamus (16.86 ng/ml) as compared to SA (12.6 ng/ml) males (Fig. 5E). TH target genes Trh were upregulated (3.8-fold) and Nrgn mRNA was downregulated (0.8-fold) in hypothalamus of MMI treated animals as compared to SA treated control counterparts (Fig.5F).

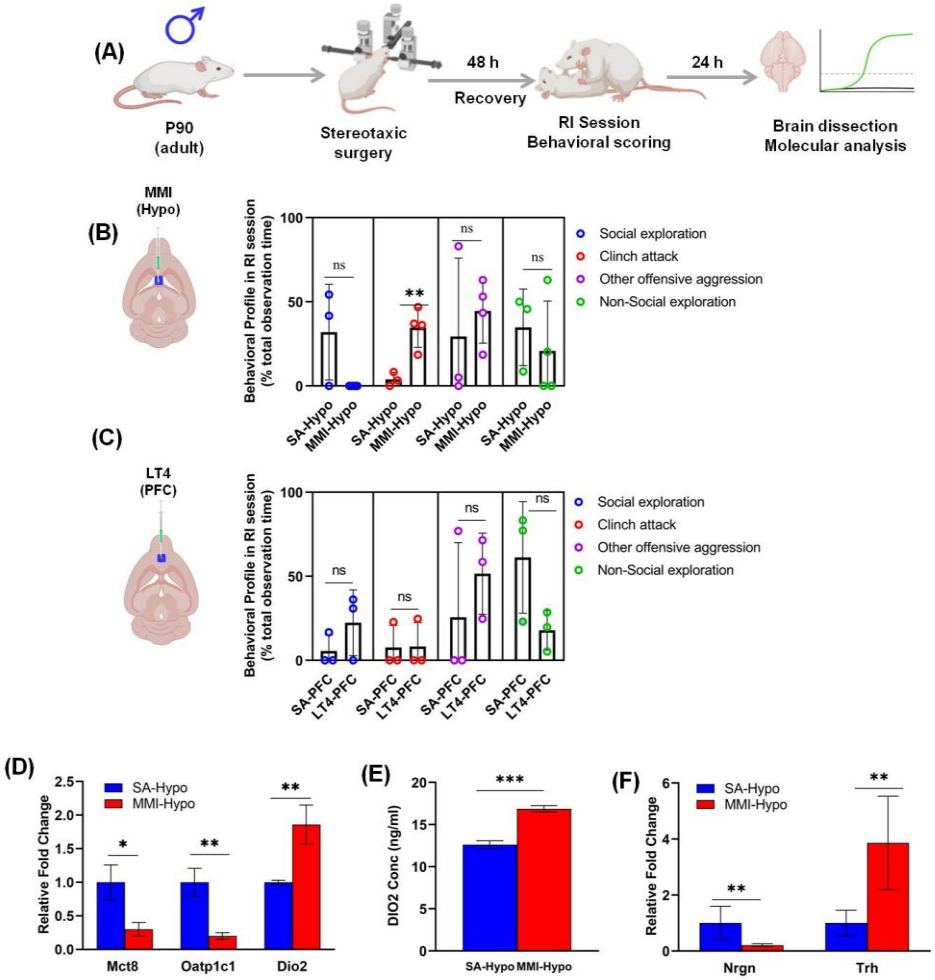


Fig. 5. Drug impaired local TH signaling in hypothalamus and resulting violent behavior in males without stress exposure (A) Experimental timeline of stereotaxic surgeries followed by behavioural and molecular experiments. (B) (i) Diagrammatic rep-resentation of injection site in hypothalamus for methimazole (MMI) and vehicle (Saline-SA) delivery. (ii) Comparative analysis of behavioural profile during RI session between SA (N=3) and MMI (N=4) administered males. (C) (i) Diagrammatic representation of injection site in prefrontal cortex (PFC) for levothyroxine (LT4) and vehicle (Saline-SA) delivery. (ii) Comparative analysis of behavioural profile during RI session between SA and LT4 administered males (N=3 mice/group). (D) Mct8 mRNA, Oatp1c1 mRNA and Dio2 mRNA expression profile in Hypo of MMI and SA males (N=3 mice/group). (E) DIO2 enzyme (M) concentration in Hypo of MMI and SA males (N=3 mice/group). (F) Trh mRNA and Nrgn mRNA expression analysis in Hypo of SA and MMI administered males (N=3 mice/group). Histogram represents mean of the data (+ SD). Statistical analysis were performed using unpaired Student's t-test [ns ($p > 0.05$),* ($p<0.05$) ** ($p< 0.01$) and *** ($p< 0.001$)] between SA vs MMI groups, SA vs LT4 groups.

Hypothalamus targeted TTR gene ablation impaired TH signaling and induced violent behavior in males without stress exposure

We checked the direct causal role of TTR by blocking its gene expression through jet-PEI mediated Ttr esiRNA injection in hypothalamus. Hypothalamus targeted Ttr knockdown to 0.1-fold (90% reduction) in adult unstressed males mirrored the violent behavioural phenotype induced by PPS. Hypothalamus specific Ttr deficient males showed very short attack latency of 15 seconds and spent 48% of total behavioural RI session in clinch attack while none of the scrambled control animals showed signs of attack (Fig. 6B). Such behavioural profile mirrored the phenotype of PPS exposed violent male (Vio) cohort as shown in Fig1 and Supplementary Movie S7.

Ttr gene silencing dramatically reduced mRNA expression of other transporters Mct8 and Oatp1c1 to 0.2 and 0.18-fold respectively. Dio2 mRNA showed an increase of 2-fold upon Ttr gene deficiency in hypothalamus (Fig. 6C). DIO2 enzyme levels showed pronounced increase from 11.4 ng/ml in scramble treated group to 39.48 ng/ml in Ttr esiRNA treated group (Fig. 6D).T3 content in hypothalamus was decreased from 0.06 ng/mg tissue wt in scramble treated group to 0.028 ng/mg tissue wt in Ttr esiRNA treated group (Fig.6E).TH regulated Trh mRNA was also markedly increased by 6.15-fold and Nrgn mRNA got reduced to 0.6-fold upon Ttr gene silencing. Here we included another well established TH responsive gene hairless (Hr) that showed maximal downregulation to 0.1-fold upon Ttr gene silencing in hypothalamus (Fig.6F).

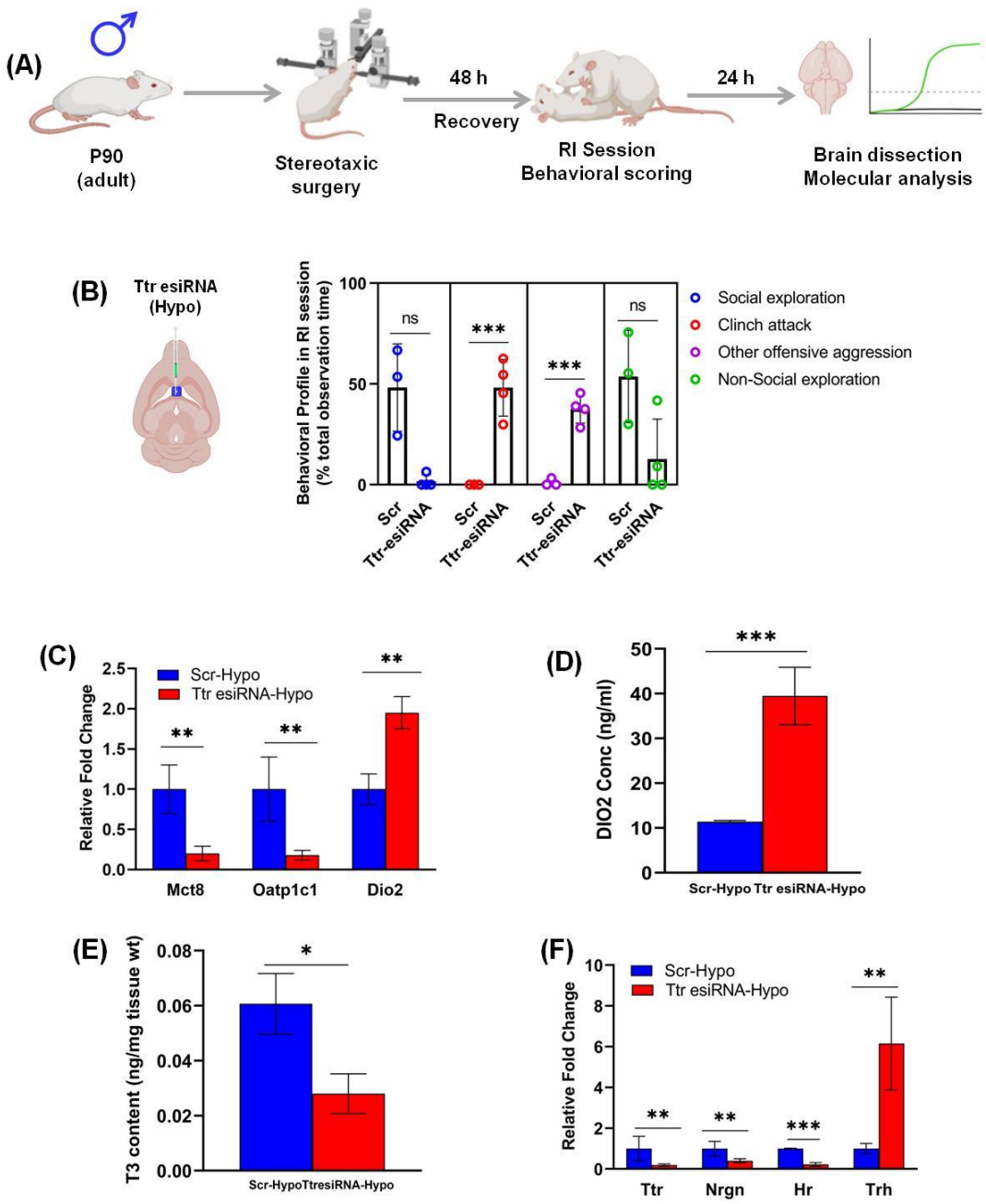


Fig.6. (A) Experimental timeline of stereotaxic surgeries followed by behavioural and molecular experiments. (B) (i) Diagrammatic representation of injection site in hypothalamus for JetPEI mediated Ttr esiRNA and scrambled siRNA (Scr) delivery. (ii) Comparative analysis of behavioural profile during RI session between Ttr esiRNA (N=4) and Scr (N=3) administered males. (C) Mct8 mRNA, Oatp1c1 mRNA and Dio2 mRNA expression profile in Hypo of Ttr esiRNA and Scr males (N=3 mice/group). (D) DIO2 enzyme concentration in Hypo of Ttr esiRNA and Scr males (N=3 mice/group). (E) T3 content in Hypo of Ttr esiRNA and Scr males (N=3 mice/group). (F) Ttr mRNA, Nrgn mRNA, Hr mRNA and Trh mRNA expression analysis in Hypo of Ttr esiRNA and Scr males (N=3 mice/group).

Histogram represents mean of the data (+ SD). Statistical analysis were performed using unpaired Student's t-test [ns ($p > 0.05$),* ($p<0.05$) ** ($p < 0.01$) and *** ($p < 0.001$)] between Scr vs Ttr esiRNA group.

Violent behaviour is inherited in F1 males with impairment in Ttr gene expression and local TH signaling in hypothalamus

We investigated whether PPS-triggered violent behavioral phenotype of mouse strain Balb/c is propagated in next generation. Adult violent males that were exposed to PPS paradigm were mated with non-stressed females to generate the F1 progenies. F1 male and female progenies were examined at their adulthood. F1 male progenies of F0 violent males showed similar behavioral phenotype that characterized the parental generation including short attack latency, attack towards anesthetized and female intruder. F1 male progenies of F0 violent males spent 45% of RI observation time in clinch attack with extremely short attack latency of 11 seconds while males from control F0 did not exhibit attack (Fig.7B, 7C).However, female siblings of F1 males did not display any prominent sign of aggression. (Supplementary Movie S8 and S9).

Next, we checked whether molecular changes in parental generation (F0 violent father) including impaired Ttr gene expression and local TH signaling in brain were also perpetuated in the next generation. Similar to F0 violent father, F1 males showed deficiency in hypothalamic T3 content while that of PFC was not altered (Fig.7E). DIO2 enzyme levels also increased in hypothalamus but remained unchanged in PFC (Fig.7D). Ttr expression reduced to 0.35-fold in the hypothalamus of F1 violent males without any significant change in the PFC. Nrgn (reduction to 0.35-fold) and Trh (upregulation by 4.3-fold) were also altered similarly in hypothalamus (Fig.7F)

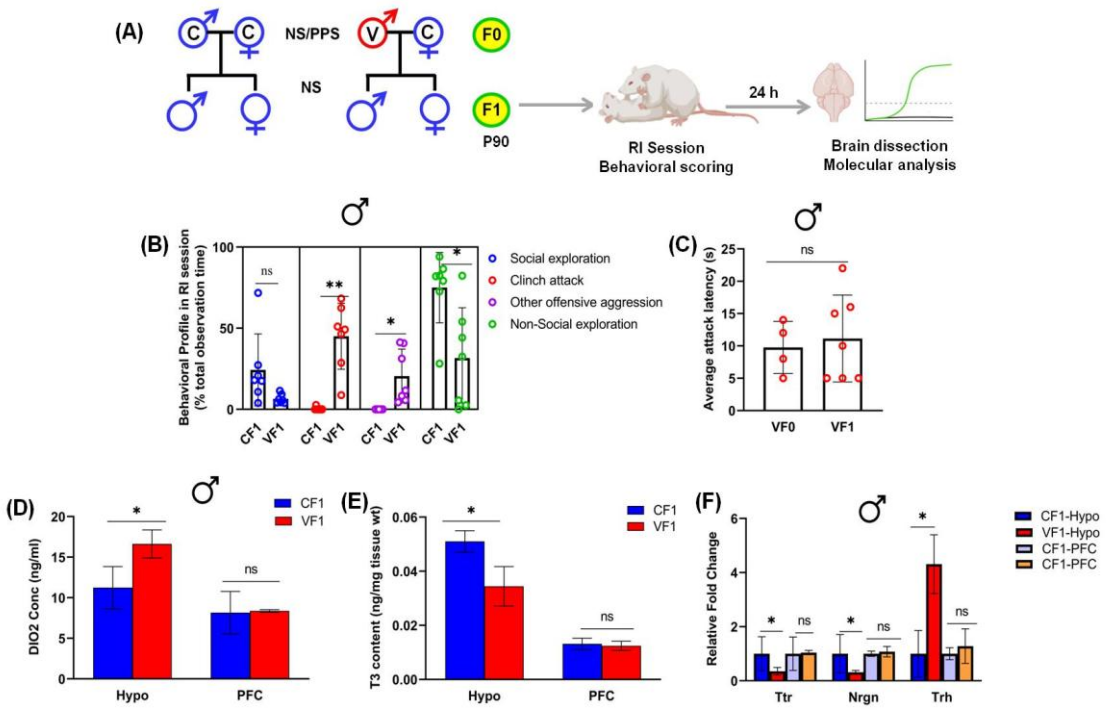


Fig.7. Intergenerational inheritance of PPS induced violent behaviour with concomitant changes in Ttr and thyroid hormone signaling. (A) Breeding pairs and experimental timeline. (B) Comparative analysis of behavioural profile during RI session between F1 progenies of control male crossed with control female (CF1) and F1 progenies of peripubertal stress exposed violent males crossed with control female (VF1) administered males (N=7 mice/group). (C) Attack latency comparison between parent violent male (VF0) and violent F1 male (VF1). (D) DIO2 enzyme concentration in Hypo and PFC of CF1 and VF1 males (N=3 mice/group). (E) T3 content in Hypo and PFC of CF1 and VF1 males (N=3 mice/group).(F) Ttr, Nrgn and Trh mRNA expression analysis in Hypo and PFC and of CF1 and VF1 males (N=3 mice/group). Histogram represents mean of the data (+ SD). Statistical analysis were performed using unpaired Student's t-test [ns ($p > 0.05$),* ($p < 0.05$) and ** ($p < 0.01$)] between CF1 and VF1 groups or VF0 vs VF1.

PPS elicits long lasting change in Ttr DNA methylation in the brain of violent males

Next, we examined whether epigenetic regulation of Ttr could explain the sustained molecular and behavioral changes invoked by PPS exposure. To address this question, we analyzed DNA methylation state of Ttr proximal promoter in the PFC and hypothalamus of male mice. As anticipated, MedIP PCR demonstrated that peripubertal traumatic experiences trigger changes in Ttr DNA methylation within the PFC and hypothalamus in adulthood and even inherited in next generation. Ttr promoter showed brain region specific differential methylation state in opposite direction to that of its expression pattern. Hypermethylation was induced in hypothalamus (11-fold) where as hypomethylation (0.27-fold) was evident in PFC of adult violent males relative to control. Hypermethylation in hypothalamus even persisted in the brain of F1 males (5.66-fold) as compared to controls. In an independent study on DNA methylome of violent male cohort (data not shown), we found Ttr as one of the topmost differentially methylated gene. As animals used for behavioral experiments were same as those for epigenetic studies, we inferred that Ttr promoter methylation could serve as a predictor of early life trauma induced gene expression and behavioral deficits (Fig. 7)

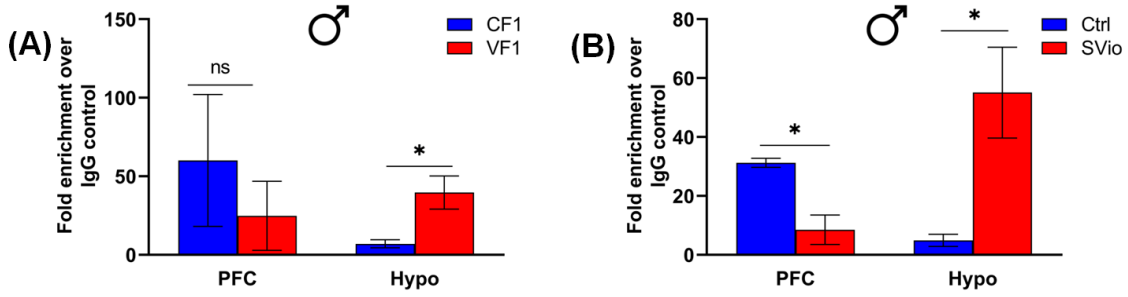


Fig. 8. Ttr promoter methylation changes in F0 violent males perpetuated in F1 generation. Methylated DNA immunoprecipitation analysis showing 5-methylcytosine fold enrichment in Ttr promoter in PFC and Hypo of (i) Ctrl and SVio males (F0) (ii) CF1 and VF1 (N=3 mice/group). Statistical analysis were performed using unpaired Student's t-test [ns ($p > 0.05$) and * ($p < 0.05$)] between Ctrl and SVio or CF1and VF1 males.

Discussion

In the present study, we fill in the existent gap of knowledge about the molecular roots of violent behavior. Based on unbiased transcriptome screening, we identify novel role of TTR in long-term programming of violent aggression induced by PPS. More importantly, we show that TTR regulated thyroid hormone availability in hypothalamus contributes to abnormal behavioral response.

TTR is a 55 kDa protein that is involved in the uptake of T4 from blood to CSF and local distribution of the hormone in brain (*13*). TTR has also been assigned other functions including proteolysis of Neuropeptide Y (*14*), neuroprotection and regeneration of damaged neurons (*15*). However, we focused on TH based on multiple reasons. Our transcriptome data showed significant expression change in TH genes primarily Trh and Nrgn in hypothalamus. Several other genes in our list (PFC-Rasd2, Fosl2, Nr4a3, Inf2, Arl4d, Dcn, Pcp4l1, Drd2, Syndig1l, Hspa1a, Spock3; Hypothalamus- Oxt, Cdhr1, Col23a1, Dgkk, Dkk3, Cck, Ptpro;) showed overlap with literature available on T3 responsive genes in primary cultured neurons (*16,17*). Moreover, we observed persistent TTR expression change right from peripubertal age and perturbation of TH action in developing brain rather than adult stage have been considered as critical determinants of multiple neurological deficits (*18*). Finally, we observed TTR protein changes in PFC endothelial cells and hypothalamic specialized glial cells known as tanycytes, both of which are involved in uptake of blood borne substances into brain (*19,20*). This clearly indicated that transport associated functions of TTR might be hindered in our experimental paradigm. It is important mentioning here that we show TTR expression in these cells for the first time. Earlier studies report TTR synthesis in choroid plexus epithelial cells of the brain until recently it was identified in neurons (*21,22*) and astrocytes indicating wide expression of the protein in CNS.

In clinical settings, thyroid hormone abnormalities are diagnosed by serum parameters. However, we show that alterations in TTR gene expression paralleled perturbation in T4 and T3 content in brain tissues of hypothalamus and PFC without affecting the circulating levels of the hormone. Determining the effective concentration of T4 and T3 in brain tissues is difficult owing to multiple factors driving their synthesis, transport across blood-brain and blood-CSF barrier, intracellular distribution and activation/inactivation (23,24). Therefore brain specific alterations in TH intrigued us to explore factors besides TTR including transporters Mct8, Oatp1c1 and enzyme Dio2 that determine the local TH availability in brain (25, 26) .

We noted significant downregulation of Mct8 and Oatp1c1 mRNA in hypothalamus of PPS induced violent males that corresponded well with reduced hypothalamic T4 and T3 content. Previously, it was reported that mutation in thyroid transporter MCT8 gene or decrease in its expression hindered intracellular T3 distribution and neuro-glia communication eventually leading to neurological ailments like multiple sclerosis (*27*). Also, Mct8 knockout mice showed pronounced deficiency in brain T3 content and was region specific particularly hypothalamus but not in other areas (28)

Mct8 deficiency in rodents is considered to be compensated by T4 transport through Oatp1c1. Oatp1c1 was also reduced in our model system suggesting significant local hypothyroidism in hypothalamus owing to lack of both the transporters (25, 29). In particular, tissues such as brain that depend only on these transporters for TH uptake and distribution, become hypothyroid upon their deficiency whereas other tissues including liver and kidney can still maintain levels of T3 (30).

Dio2 levels showed subtle increase in hypothalamus of PPS induced violent males. In brain, T3 is derived partly from circulation and is also formed locally by Dio2 mediated 5'-deiodination of T4. Dio2 action is integral to ascertain optimal intracellular concentrations of T3 (31). Therefore, Dio2 increase is suggested to be a compensatory response to deficiency of both Mct8 and Oatp1c1 and maintain TH levels (32). Dio2 is known to be negatively regulated by TH and was increased upon hypothyroidism in aging rodent brain (33). This literature information indicated that DIO2 increase in hypothalamus in our model system strongly suggests reduction in local TH availability.

Any change in local TH status of brain has a direct influence on expression of TH responsive genes. T4 and T3 action initiates with formation of ligand-receptor complexes with TH nuclear receptors (TRs) which in turn binds to TH response element (TRE) on the promoter of target genes. A broad spectrum of TH responsive genes are critical for plethora of TH action in varied cellular processes such as cell proliferation, differentiation, metabolism and homeostasis (34,35). TH deficiency during postnatal brain development causes irreversible neurological manifestations through target gene expression changes (36).

Nrgn is one such brain specific TH responsive gene that was also amongst the top ranking differentially expressed gene in our transcriptome data containing TRE elements in promoter and its tracription is dependent on TH in brain (37,38). Nrgn regulates synaptic plasticity by activating calmodulin kinase II (CaMKII) protein and spine density. We observed significant

reduction in Nrgn transcript and protein levels in concordance with hypothalamic decrease in T4 and T3 levels. Another top ranking gene of our transcriptome data, Trh known to be negatively regulated by T3 was robustly upregulated in hypothalamus of violent males. Overall these findings implicated the role of reduced local thyroid hormone availability in hypothalamus in PPS induced violent aggression

We further strengthened our hypothesis by employing drug induced brain region targeted TH manipulation approach and assessment of behavioural consequences. As anticipated, hypothalamic administration of antithyroid drug MMI impaired local TH signaling and produced violent phenotype in normal unstressed male mice. MMI administration in hypothalamus drastically downregulated levels of TH transporters Mct8 and Oatp1c1 and increased Dio2 mRNA and DIO2 enzyme. T3 regulated genes also showed significant alteration in hypothalamus of MMI treated animals. Earlier reports showed that systemic MMI treatment reduced Mct8 mRNA, increased Trh and Dio2 mRNA in hypothalamus of rats (39). Cumulative all these data pointed towards effect of MMI on reduced local T3 availability as also supported by earlier literature (25, 29, 39).

Until now we found a strong link between reduced local TH availability in hypothalamus and emergence of violent phenotype but whether it was mediated by Ttr or was independently regulated was not clear. We showed that intra-hypothalamic Ttr gene knockdown led to similar reduction in T3 availability, decrease in Mct8 and Oatp1c1, increase in Dio2 and alteration in expression of TH target genes as found in our earlier experiments. Besides Trh and Nrgn, Ttr silencing also reduced hairless (Hr), a universal TH responsive gene that is studied to monitor the local TH status in brain, expression (40). Therefore, we included Hr mRNA expression analysis in our study. Ttr gene ablation also evoked violent aggression in unstressed males to a similar extent to that of PPS induced males. These data clearly indicated that behavioural and molecular consequences in our experimental regime was downstream of Ttr, though detailed studies are required to delineate the precise mechanism of action of Ttr in hypothalamic cells.

Interestingly, TTR showed expression changes in both hypothalamus and PFC whereas violent behavior was triggered only by hypothalamic TTR knockdown or T4 depletion by methimazole. T4 increase by levothyroxine in PFC did not produce any behavioral changes. It is likely, that TTR mediated thyroxine reduction in hypothalamus have direct causal role in violence and consequently PFC endothelial cells express more TTR to uptake hormone and alleviate the deficiencies. Interestingly, we did not observe significant change in other

transporters Mct8 and Oatp1c1 in PFC further indicating that TTR mediated TH uptake in PFC might result in the compensatory increase of T4 and T3 in response to hypothalamic reduced TH availability. We explored intergenerational inheritance of PPS induced violent behavior and observed paternal transmission of behavior in F1 males with concomitant reduction in, hypothalamic TTR and Nrgn expression and T3 availability. Previous studies suggest that thyroid hormone changes in neonatal brain can elicit neuroendocrine abnormalities in their F1 progenies. Also, developmental exposure of thyroxine disrupting chemicals can affect gene expression and behavior in later generations (*41*). Further, mechanistic investigation revealed lasting methylation mark in TTR promoter which was inherited in F1 generation. DNA methylation plays crucial role in the inheritance of traumatic memories (*42,43*). Therefore, we speculate that epigenetic inheritance at the TTR methylation locus controls long-term programming of the hypothalamic-thyroid axis which in turn modulates thyroid hormone availability and function throughout life and in subsequent generations. Future epigenetic manipulation at TTR locus during PPS can shed light on life-long vulnerability to violence and its inheritance.

In conclusion, we provide molecular evidence for emergence, gender vulnerability and inheritance of post traumatic violent behavior. We delineate novel role of brain TTR-thyroid signaling in manifestation of early life trauma induced violence and its intergenerational inheritance that could be mediated at least in part by epigenetic mechanisms. Brain TTR-thyroid signaling can also serve as valid molecular predictors as well as intervention targets in violence. Our findings have inherent limitations of investigations in animal models and therefore further studies are warranted in relevant human cohort to establish role of TTR-thyroid pathway in violence and criminality. Our work also provides resource for investigating sexual dimorphism in behavioral disorders and deciphering susceptibility as well as protective pathways.

Materials and Methods

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±2ºC on a 12h light/dark cycle with ad libitum access to food and water. Animal handling and experiments were conducted in accordance with the institutional guidelines.

PPS stress procedure

Male and female mice were exposed to unpredictable fear inducing stressors of synthetic fox odor (trimethylthiazoline) and elevated platform during the peripuberty period of postnatal day (P) 28 to P42. Briefly, P28 male and female offspring were exposed to an open-field for 10 minutes for acclimatization in a novel environment. Thereafter, one group of mice were exposed to 9 µl of fox odor (Sigma) 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 minutes following which mice were kept separated for 15 minutes 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).

Resident intruder (RI) paradigm

Control and PPS exposed mice of both sexes were assessed for aggression in their adulthood (P90) using the conventional RI paradigm. The test was performed as described in our previous report (*7*). The resident was exposed in its home cage to various category of intruders including a smaller and unfamiliar (10% less body weight) size, larger size (10% more body weight), anesthetized, opposite sex and intruder of different strain for 10 minutes 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. Behavioural screening of animals into non-aggressive, hyper-aggressive and violent was done based on conventional parameters as published in earlier reports (12, 44)

RNA-sequencing

RNA was isolated from hypothalamus and PFC of male and female mice using Trizol reagent. Approximately, 1µg of RNA was taken per sample and RNA sequencing libraries were made using TruSeq v2 Library Prep Kit as per manufacturer's protocol. Briefly, the RNA was polyA selected using OligodT magnetic beads followed by shearing into 200-500 bp fragments. This sheared RNA was then used to generate cDNA. The cDNA was end-repaired to blunt ends. These blunt ends were then A-tailed i.e. an "A" overhang was added so as to ligate the adapters in the next step. The adapter-ligated cDNA was then amplified by PCR and purified by AMPure XP beads. The prepared library was quantified using Qubit Fluorometer, and validated for quality on High Sensitivity Bioanalyzer Chip (Agilent) and sequenced on Illumina HiSeq 2500. The FASTQ sequencing reads were adapter-trimmed along with a minimum length cut-off of 50 bases using Prinseq-lite. The reads were aligned to mouse genome assembly using TopHat (v.2.0.11) followed by reference-based assembly using Cufflinks (v.2.2.1). Then differentially expressing transcripts were identified using Cuffdiff (v.2.2.1).

RT-PCR

Total RNA was isolated from hypothalamus and PFC of mice and 2 µg of RNA from each group was reverse transcribed to cDNA synthesis. RT-PCR was carried out using SYBR Green master mix for detection in Light cycler LC 480 (Roche). All primers used for qRT-PCR are given in Supplementary Table S1.The endogenous control GAPDH was used to normalize quantification of the mRNA target.

Immunoblotting

Cytosolic protein lysates (40 µg) prepared from mouse hypothalamus and PFC were resolved on to 10% SDS PAGE, transferred to PVDF membrane and used for immunoblotting using conventional method. The primary antibodies {anti-TTR rabbit polyclonal, anti-Nrgn rabbit polyclonal; anti-GAPDH mouse monoclonal} 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.

Immunohistochemistry

Mice were anaesthetized with thiopentone (40 mg/kg) and perfused with cold 4% paraformaldehyde in PBS. Brains were removed, post-fixed, cryoprotected in PBS + 15% sucrose for 2–3 hours followed by immersion in PBS + 30% sucrose for 24 h, and then sectioned coronally (7 μm) on a cryotome. Free-floating sections were permeabilized with blocking buffer (PBS + 3% normal donkey serum, 0.3% Triton X-100) for 2 hours and then incubated with TTR primary antibody overnight at 4°C. Slices were then washed 4×15 min with PBS, incubated with corresponding secondary antibodies for 2 hours, washed 4 × 15 min with PBS, mounted on microscope slides followed by counterstaining with DAPI and photomicrographs were captured by FLoid fluorescence microscope.

Thyroid hormone measurement

Mouse blood samples were collected from heart to test serum levels of total tetraiodothyroxine (T4) and total tri-iodothyroxine (T3). Thyroid hormone content in brain regions was determined by dissecting hypothalamus and PFC and individually homogenizing them in artificial cerebral spinal fluid by the ratio of 1 μg/4 μl (brain tissues/ACSF) and centrifuged at 14,000 rpm for 15 min at 4°C. The resulting supernatant was collected and used for ELISA based determination of total T4 and T3 (EliKineTM Thyroxine (T4) ELISA Kit KET007 and EliKineTM Triiodothyronine (T3) ELISA Kit KET006).

Deiodinase 2 (DIO2) measurement

We performed an in vitro quantitative measurement of DIO2 in mouse brain tissue (hypothalamus and PFC) homogenates using a sandwich enzyme immunoassay kit (Reddot Biotech INC., Mouse Deiodinase, Iodothyronine, Type II (DIO2)ELISA Kit RDR-DIO2-Mu). Briefly, mouse hypothalamus and PFC tissues were isolated from control and experimental groups, homogenized in 1XPBS. The resulting suspension was subjected to 2 freeze/thaw cycles to break the cell membranes and centrifuged for 5 minutes at 5000 × g. The supernatant was removed and used for DIO2 ELISA assay as per manufacturer's instructions. The concentration of DIO2 was measured spectrophotometrically using a microplate reader at a wavelength of 450 nm.

Stereotaxic surgeries and gene manipulation

Mice were anesthetized with 40 mg/kg BW thiopentone i.p. and positioned on a stereotaxic frame. Methimazole and levothyroxine (1 μg/μl, 1 μl/side) were bilaterally administered into the hypothalamus and PFC respectively. The specific coordinates for injection were relative

to bregma (mediolateral, dorsoventral, and rostrocaudal axes: PFC = ± 0.35, _2.1, +2.2 mm; hypothalamus = ± 0.5, _1.5, +5.8 mm). For brain targeted gene manipulation TTR esiRNA (esiRNA targeting mouse Ttr- EMU030721, Sigma Aldrich)–jetPEI complex a was infused into hypothalamus and PFC. Injection rate for all the surgeries, was kept at 100 nl/min and the system was left in place for an additional 1 min and then gently withdrawn. Mice were allowed to recover individually over heating pads until they recovered from anesthesia and thereafter returned to their home cages. RI test for aggression was performed 48h after surgery followed by molecular experiments after an additional 24 h.

Methylated DNA immunoprecipitation

DNA methylation was analyzed at the promoter region of Ttr by methylated DNA immunoprecipitation (MeDIP) method as mentioned earlier (45,46). Briefly, 4 µg of sonicated DNA (DNA fragment size ranging from 300 to 1000 bp) isolated from hypothalamus and PFC of F0 and F1 male mice was diluted in immunoprecipitation buffer and incubated with 2 µg 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 2h with rotation. Thereafter, it was centrifuged at 3500xg 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, Ttr proximal promoter -184 to -33 bp from TSS) was amplified with specific primers (Supplementary Table 1) generating a 151 bp product.

Statistical analyses

In order to analyze RT-PCR data, the 2^-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 TTR and Nrgn 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 MeDIP analysis, results were represented as fold enrichment normalized to IgG control. Histograms were represented as mean of the data (+SD) and statistical significance was calculated by Student's unpaired two tailed t-test.

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