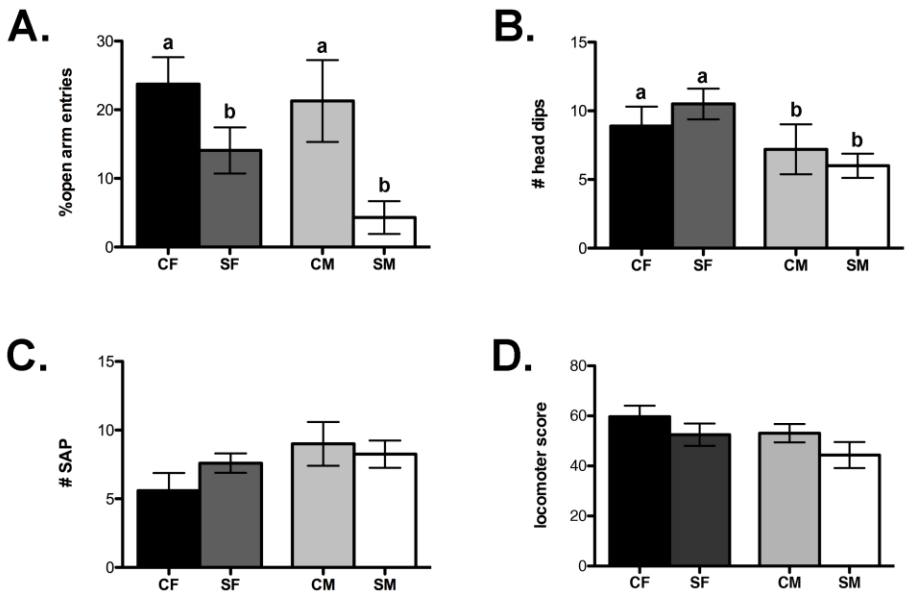
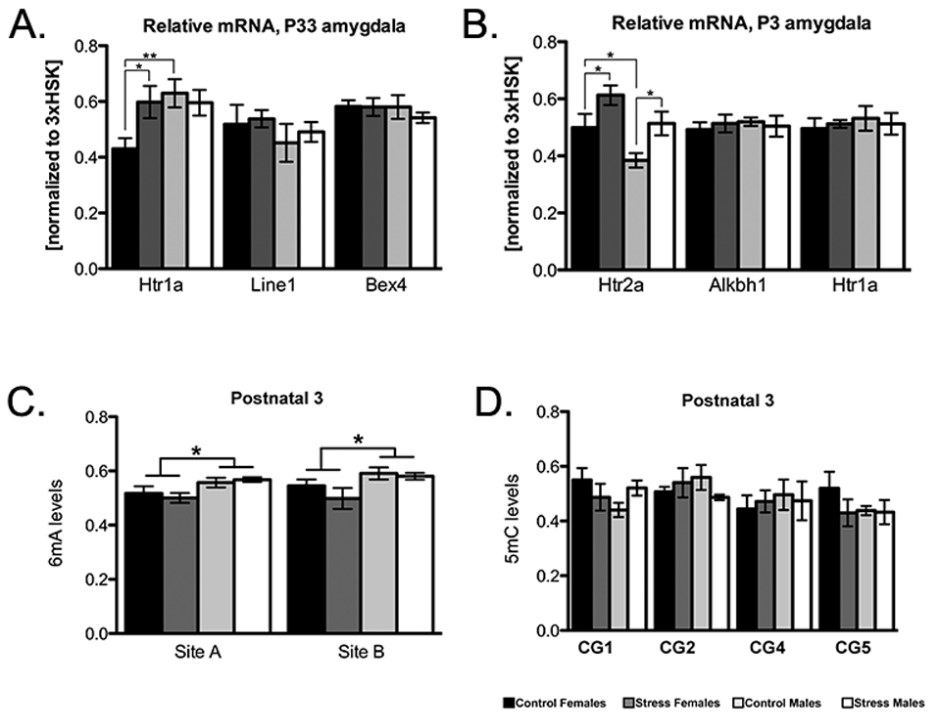
N^6-methyladenine is an epigenetic marker of mammalian early life stress

Stacey L. Kigar[1], Liza Chang[2], Candace R. Guerrero[3], Jacqueline R. Sehring[2], Amelia Cuarenta[2], Laurie L. Parker[3], Vaishali P. Bakshi[4], Anthony P. Auger[2,5]

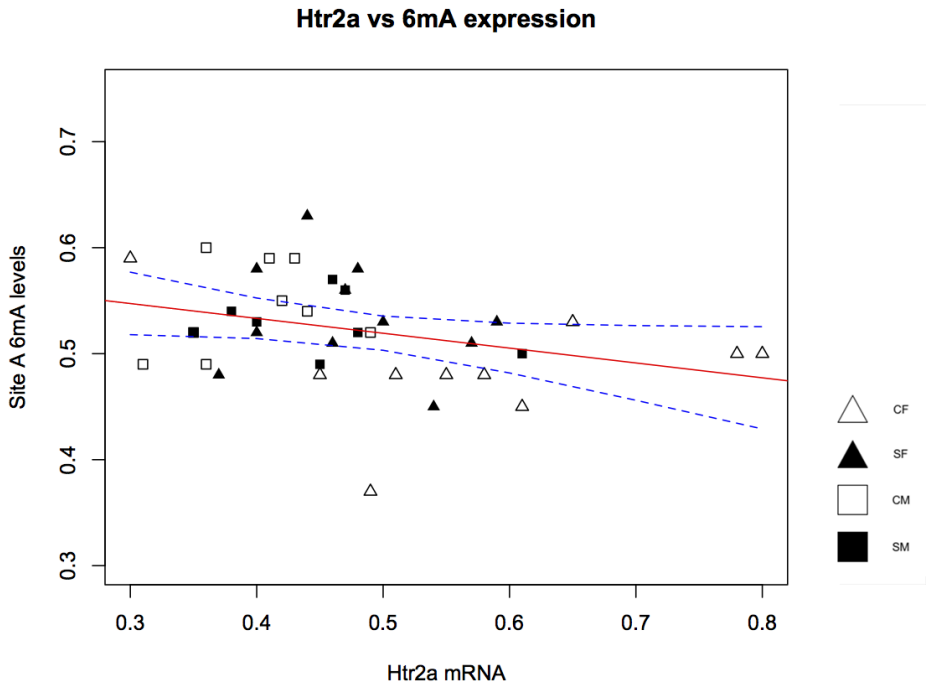
[1.]Molecular and Cellular Pharmacology Training Program, [2.]Department of Psychology, University of Wisconsin-Madison, [3.]Department of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota, Minneapolis, MN 55455, [4.]Department of Psychiatry, University of Wisconsin-Madison, [5.]Neuroscience Training Program, University of Wisconsin-Madison, Madison, WI 53706.



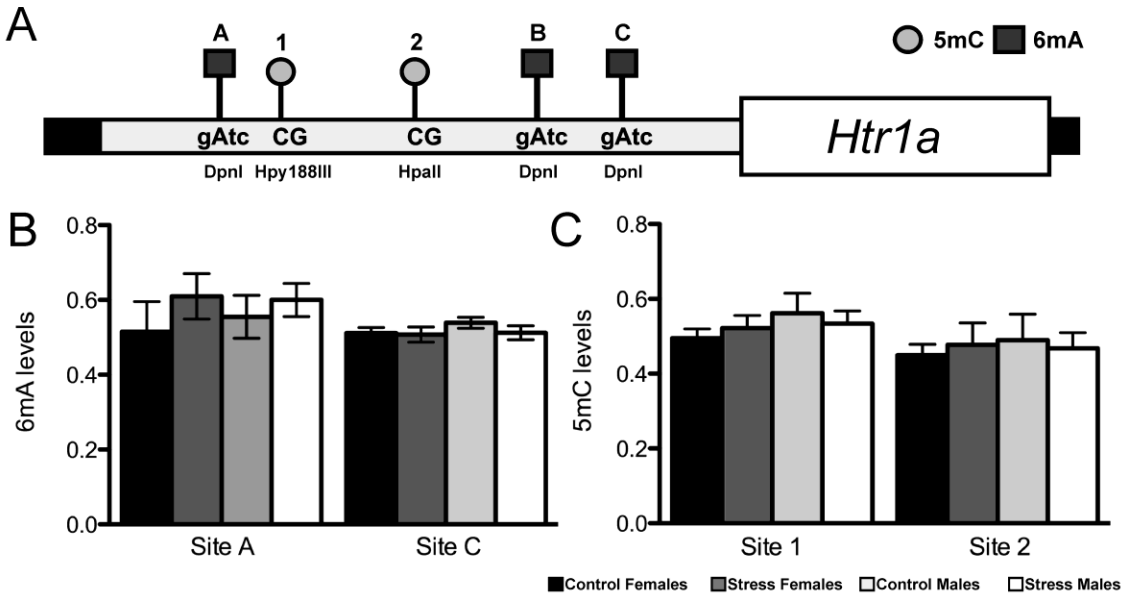
Supplementary Figure 1: Anxiety-like behavior testing in the elevated plus maze (EPM). *(A)* A significant treatment effect on number of crosses into the open arm as a percentage of all crosses was observed *(B)* A significant sex difference in the number of head dips was observed (two-way ANOVA; $F_{(1,34)} = 4.843$, $p = 0.035$). *(C)* There were no effects on the number of times animals took the stretch attend posture. *(D)* We assessed general activity in these rats by combining the number of total crosses in the EPM, with the number of approaches to an object in a separate behavioral task; there was no significant effect of stress or sex on movement.



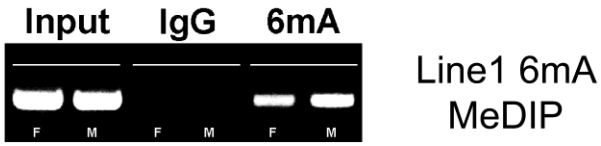
Supplementary Figure 2: RT-qPCR data showing the effects of neonatal POE and biological sex on gene expression and DNA methylation in the amygdala. *(A)* Significant difference in the mRNA expression of Htr1a that are opposite that of Htr2a in the juvenile amygdala. There were no group differences in mRNA expression of the retrotransposon Line1 or the X-linked gene Bex4. *(B)* mRNA expression in the P3 amygdala shows sex and treatment effects on Htr2a, but not Htr1a or Alkbh1. There were no changes in Line1 or Bex4 (data not shown). *(C)* Sex differences in 6mA abundance are present at both sites in the *Htr2a* promoter neonatally. *(D)* No differences in 5mC were observed (levels of methylation were below the limit of detection at CG site 3). Values shown as mean ± SEM, $^{**}p < 0.01$, $^{*}p < 0.05$.



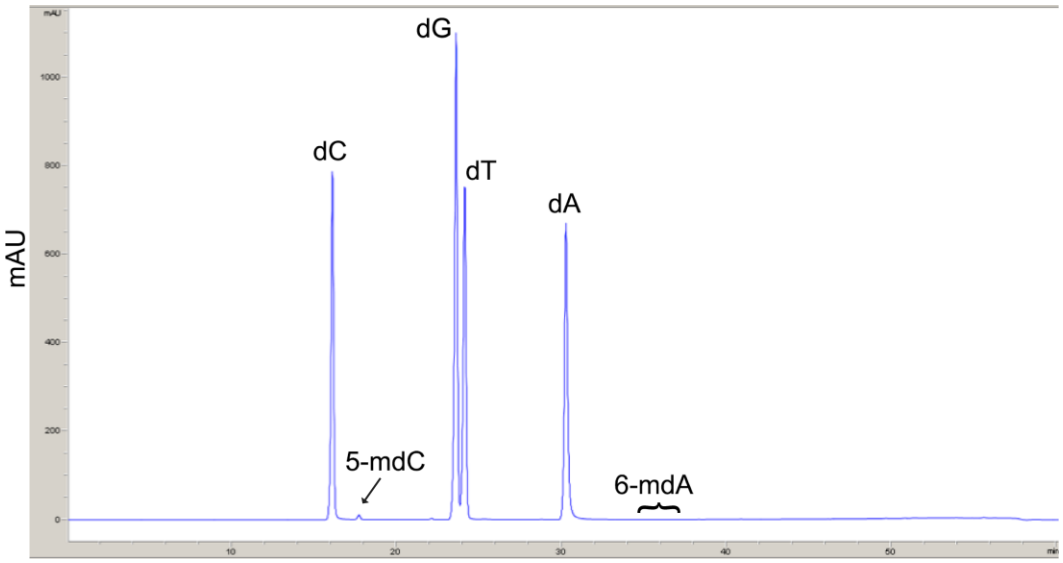
Supplementary Figure 3: Regression analysis showing trending correlation between relative levels of 6mA abundance and Htr2a mRNA expression levels in the P33 amygdala. $p = 0.0509$, $F_{1,36} = 4.079$.



Supplementary Figure 4: *(A)* Schematic of *Htr1a* gene promoter, showing individual methylation sites analyzed. *(B)* MDRE analysis shows no differences in relative 6mA methylation at two distinct GATC sites in the *Htr1a* promoter. Note: no methylation was detected at Site B. *(C)* MSRE analysis shows no differences in 5mC methylation at two distinct CG sites. Sites A, C, 1, and 2 were explored using MeDIP analysis; there were no significant differences (data not shown). Values shown as mean ± SEM.



Supplementary Figure 5: DNA gel for 6mA MeDIP of Line1 showing robust enrichment, as well as input and IgG controls.



Supplementary Figure 6: Representative enrichment chromatogram of enzymatically digested DNA from P35 amygdala showing separation of nucleoside peaks and their relative abundance.

Primer	Accession	Forward	Reverse
Ywhaz	NM_013011	TTGAGCAGAAGACGGAAGGT	GAAGCATTGGGGATCAAGAA
Hprt	NM_012583	GCAGACTTTGCTTTCCTTGG	CCGCTGTCTTTTAGGCTTTG
Rpl13a	NM_173340	AGCAGCTCTTGAGGCTAAGG	GGGTTCACACCAAGAGTCCA
Alkbh1	NM_001108718	GCGGAGACCCCGAAGTTTAC	TGGCGACTTGCTCTTACTGT
Htr2a	NM_017254	AACGGTCCATCCACAGAG	AACAGGAAGAACACGATGC
Htr1a	NM_012585	CCGCACGCTTCCGAATCC	TGTCCGTTCAGGCTCTTCTTG
Bex4	NM_001037554	GAAAACAAGAAGCCTGGGGG	GCGTGTAAGGCCTCATCTGT

Supplementary Table 1: mRNA Primer sequences and Pubmed accession numbers for RT-qPCR.

Promoter	Location:	Forward	Reverse
Htr2a	Site #1 – Hpy188III	AAAGTAAGCTAGTTGCGAGATGTA	GTCAGGAAGTCTGTGGCAATAA
Htr2a	Site A - DpnI	CCTGACTCCTCTGAACGTGT	GTCCTGGGATCTAGGGGCAT
Htr2a	Site #2 - HpaII	CCTAGGCTGTCTCCCCATTC	TGCCTCCTTCCTCAGACCTC
Htr2a	Site #3 - HhaI	TGCGGCTCTTTTGTGTGACT	AGCAGCCCAGGAACTTACAT
Htr2a	Site #4 - HpaII	TGCGGCTCTTTTGTGTGACT	AGCAGCCCAGGAACTTACAT
Htr2a	Site B - DpnI	CTCCCTCCTCGTTTGGATCT	GCTGTAAGTTCTCACGGAAGC
Htr2a	Site #5 - HpaII	CTCCCTCCTCGTTTGGATCT	GCTGTAAGTTCTCACGGAAGC
Htr1a	Site A - DpnI	AGGAGGCGGGGTTTAATCTG	CCCCCACCACCATCTAACAC
Htr1a	Site #1 – Hpy188III	AGGAGGCGGGGTTTAATCTG	CCCCCACCACCATCTAACAC
Htr1a	Site #2 - HpaII	GAGAGAAGCAACCAGGAGATG	GGATTCTCCCGCCTAACAAA
Htr1a	Site B - DpnI	TGAGTGCTCTTCTCAGATGCC	TTTTCTGGGGAGTTTCAGAGGG
Htr1a	Site C - DpnI	TCCCTCTGAAACTCCCCAGAA	AGGTCACGTCGGAGATGCTA
Line1	----	GAAAGCACCAAATGCCACTGG	GTAGTCTGCTATCGGGCGTT

Supplementary Table 2: Genomic DNA promoter primer sequences for RT-qPCR. *Htr2a* accession number: L31546. *Htr1a* accession number: AF217200.