Pet-1 is required across different stages of life to regulate serotonergic function

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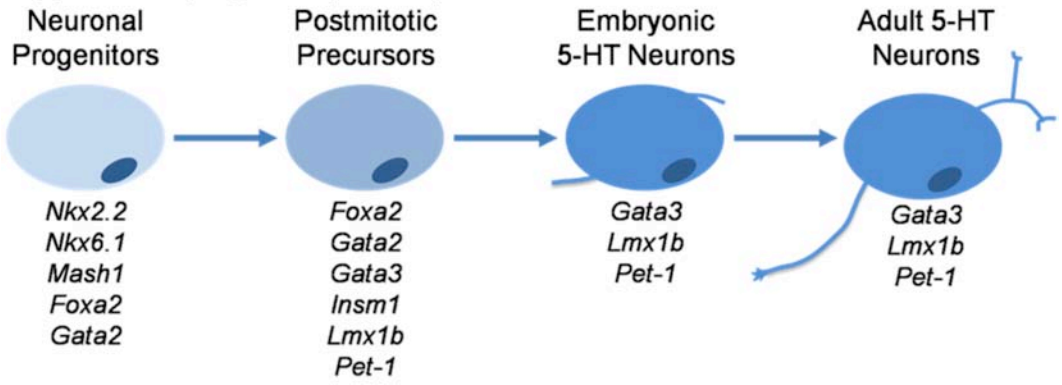
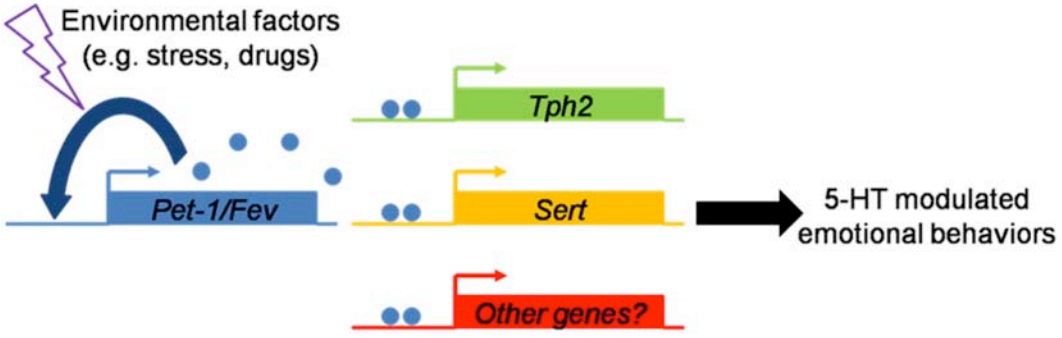
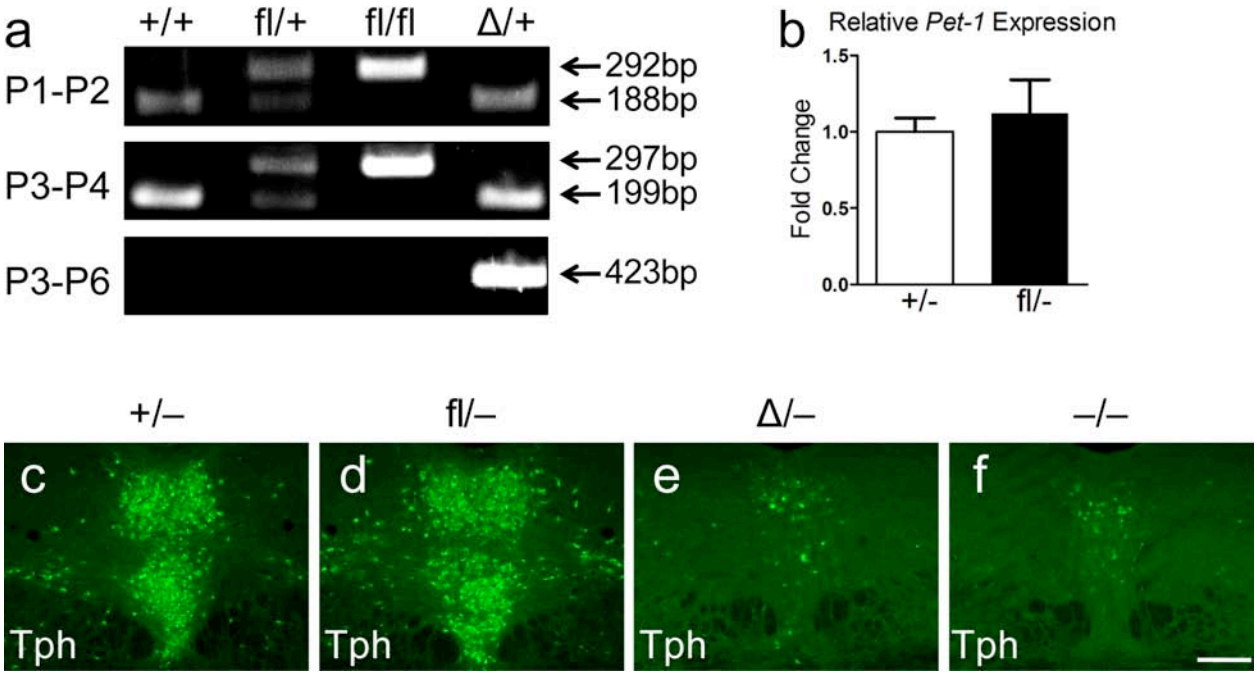
Supplementary Figures 1-12, Tables 1-2



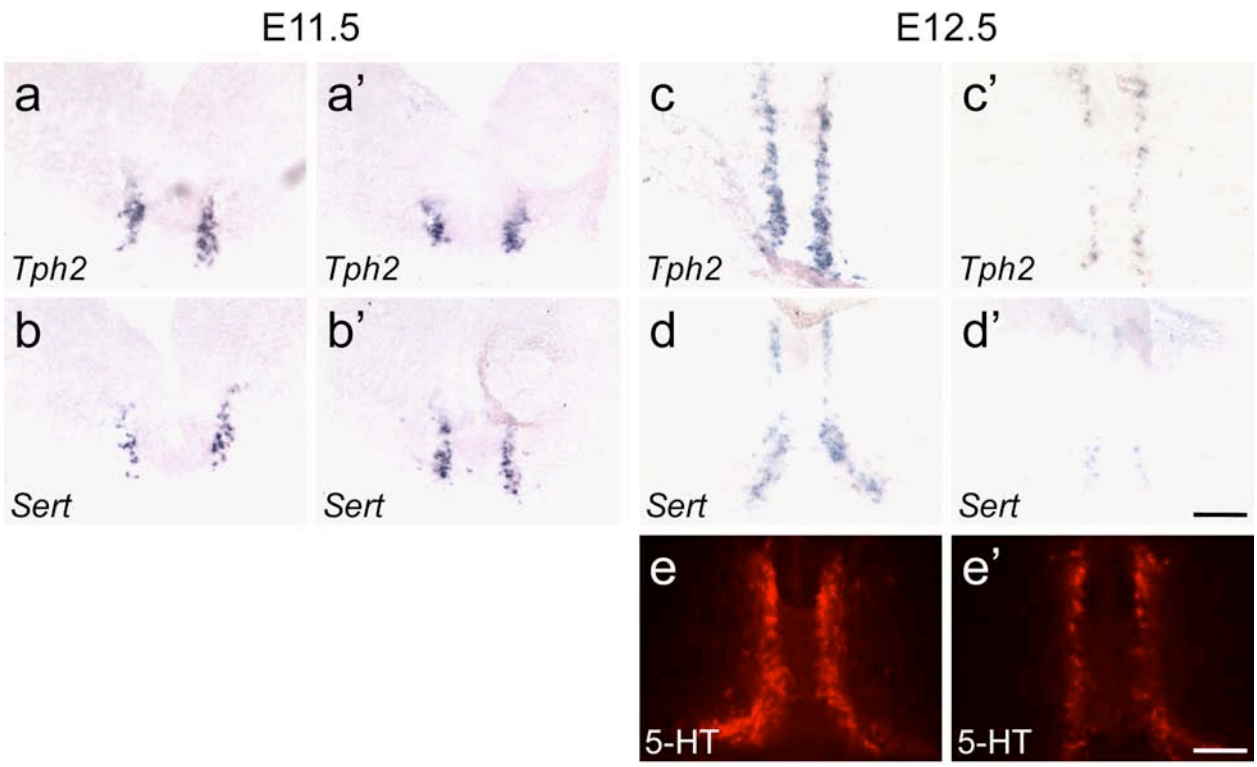
	Pet-1	*Gata3*	*Lmx1b*
5-HT neuron survival	N	N	Y
5-HT synthesis and reuptake	Y	Y	Y
Formation of the dorsal raphe nucleus	Y	N	N
Axonal innervation in somatosensory cortex	Y	?	?
Autoreceptor expression and firing properties	Y	N	?



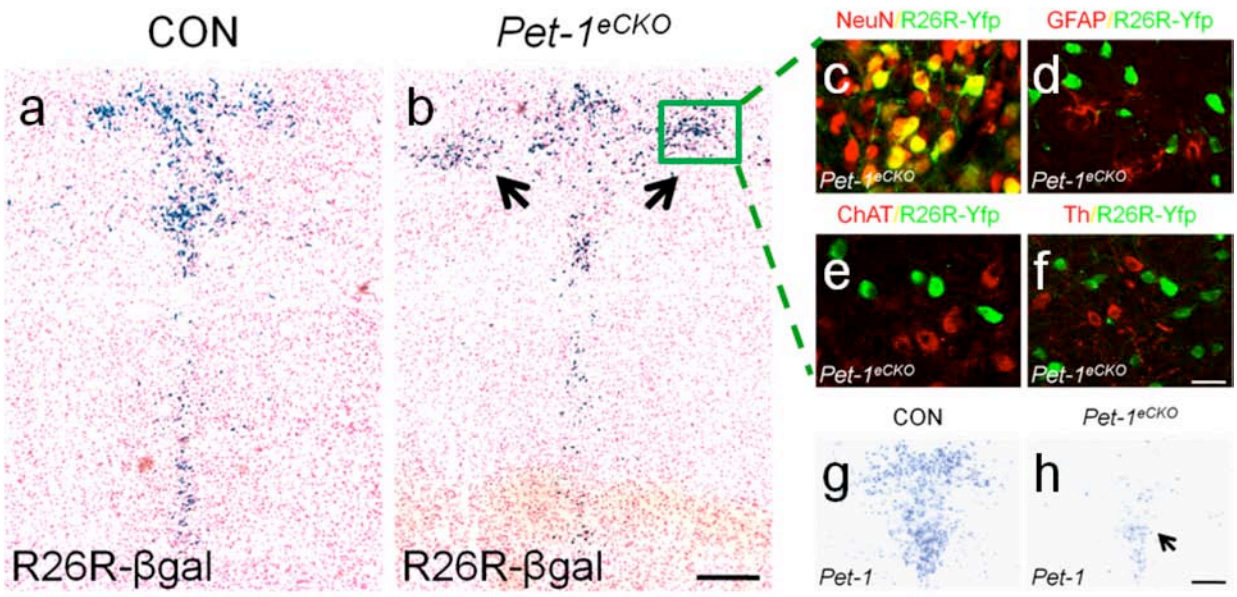
Supplementary Figure 1 Transcriptional control of 5-HT neurons across life span. Top, Transcriptional cascade that directs 5-HT neuron development. Middle, Distinct requirements for continued *Pet-1*, *Gata3* and *Lmx1b* expression during 5-HT neuron maturation. ?, not determined. Bottom, Proposed adult homeostatic transcriptional mechanism for regulation for 5-HT modulated behaviors.



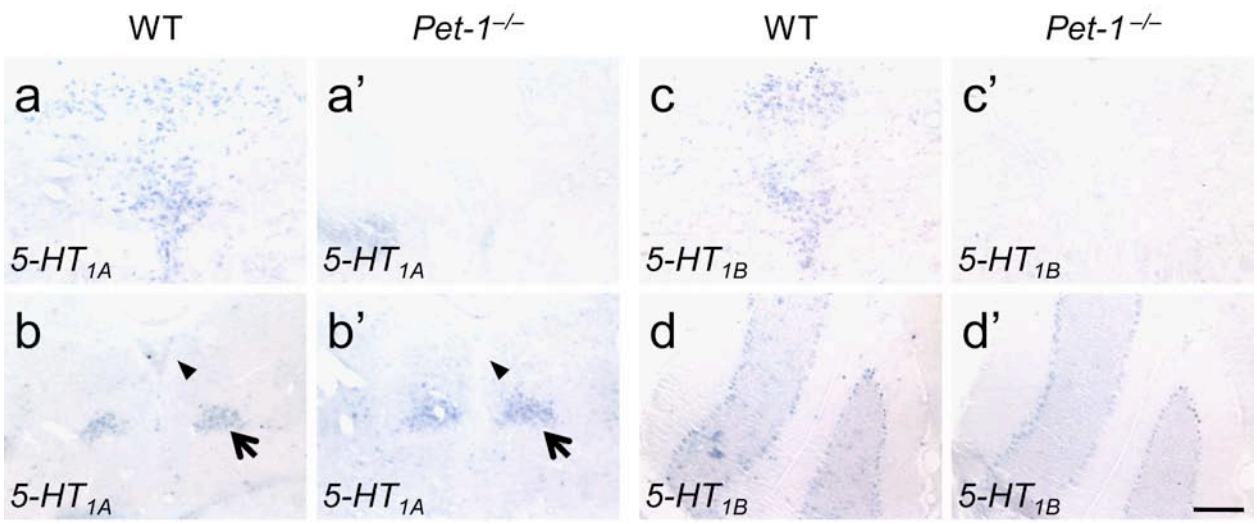
Supplementary Figure 2 Analysis of the floxed and conditional deleted *Pet-1* alleles. (**a**) PCR genotyping using primer sets identified predicted wild-type (+), floxed (*fl*) and conditionally deleted (*Δ*) *Pet-1* alleles. (**b**) RT-qPCR to measure relative *Pet-1* expression levels in the indicated *Pet-1* genotypes (*Pet-1*$^{+/-}$, n=6, normalized to 1; *Pet-1*$^{fl/-}$, n=6; mean + s.e.m; p>0.05, two tailed t test). (**c–f**) anti-Tph immunoreactivity in adult dorsal raphe nucleus (DRN) demonstrated that the number of DRN Tph^{+} neurons in *Pet-1*$^{+/-}$ (**c**) and *Pet-1*$^{fl/-}$ (**d**) mice was similar and the number in *Pet-1*$^{\Delta/-}$ (**e**) and *Pet-1*$^{-/-}$ (**f**) mice was similar. Scale bar is 200 μm.



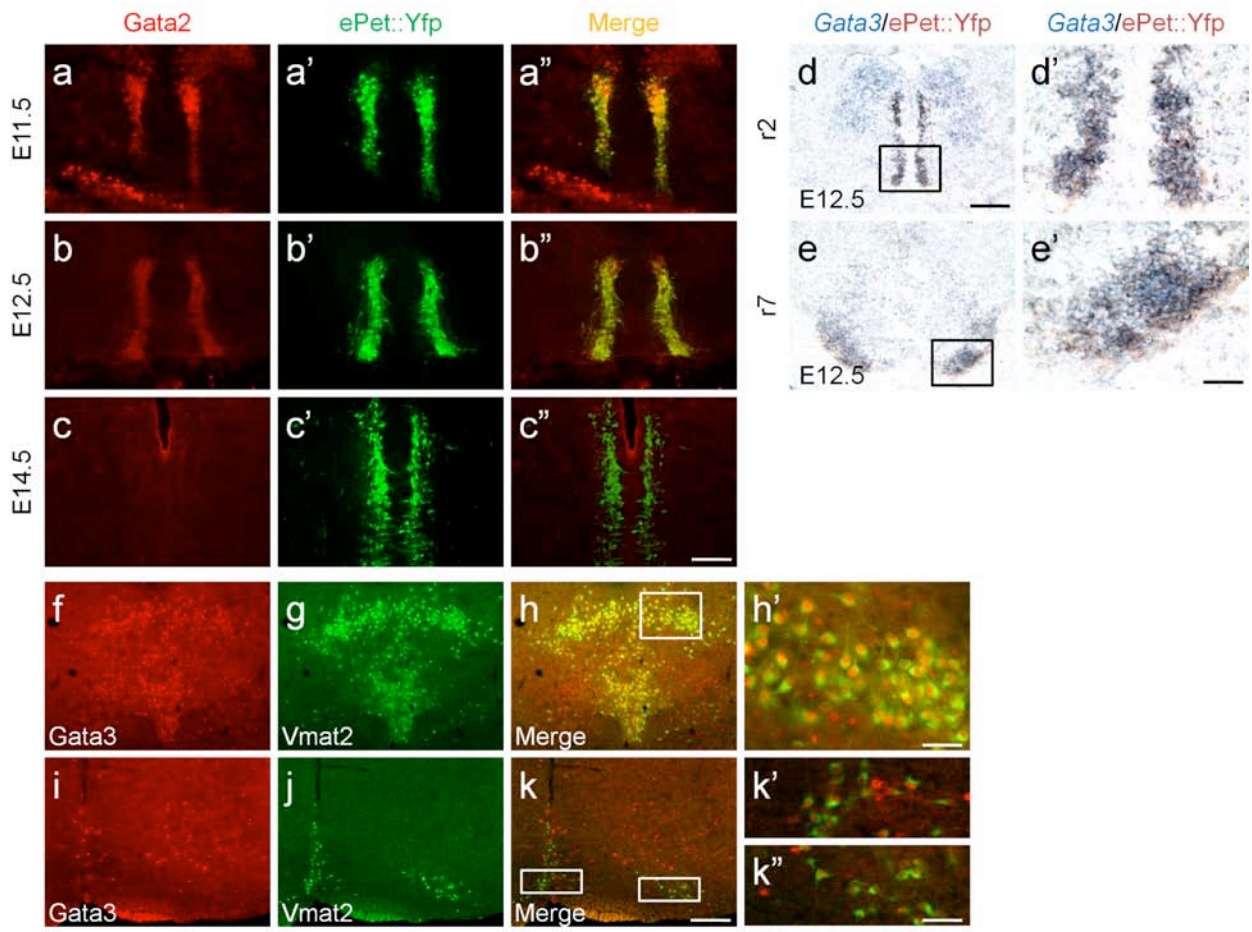
Supplementary Figure 3 Down-regulation of *Tph2*, *Sert* and 5-HT levels at E12.5 following conditional deletion of *Pet-1*in *Pet-1*^{eCKO} embryos. (**a–d'**) In situ hybridization to detect *Tph2* and *Sert* mRNAs in control (*Pet-1*$^{fl/+}$, *ePet::Cre*) and *Pet-1*^{eCKO} embryos. (**e, e'**) 5-HT immunostaining in control (**e**) and *Pet-1*^{eCKO} (**e'**) embryos at E12.5. Scale bars are 100 µm.



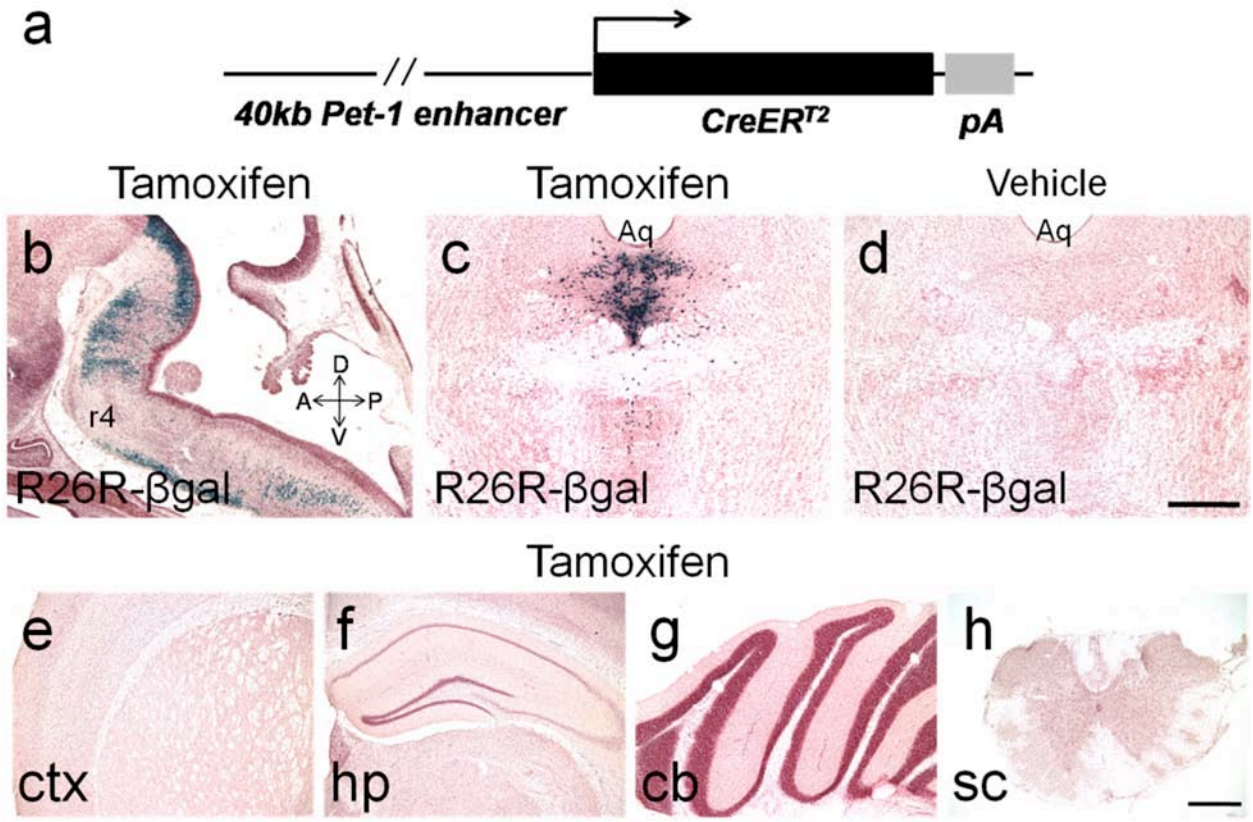
Supplementary Figure 4 Neurochemical fate mapping of *Pet-1* deficient 5-HT neurons in *Pet-1*^{eCKO} mice. (**a, b**) Xgal staining showing disrupted organization of 5-HT neurons in the *Pet-1*^{eCKO} DRN (black arrows in **b**). (**c–f**) Confocal microscopy showing co-immunostaining of R26R-Yfp with NeuN (**c**), GFAP (**d**), ChAT (**e**) or TH (**f**) in the DRN of *Pet-1*^{eCKO} mice. *Pet-1* deficient cells maintained their generic neuronal–type identity as they expressed the neuronal marker NeuN, but not markers for other transmitter identities such as those for motor neurons, dopamine or noradrenergic neurons. (**g, h**) *Pet-1* in situ hybridization showing *Pet-1* deletion was spared in a small number of 5-HT neurons in *Pet-1*^{eCKO} mice. Scale bar is 50 μm in (**f**), 200 μm in (**b**) and (**h**).



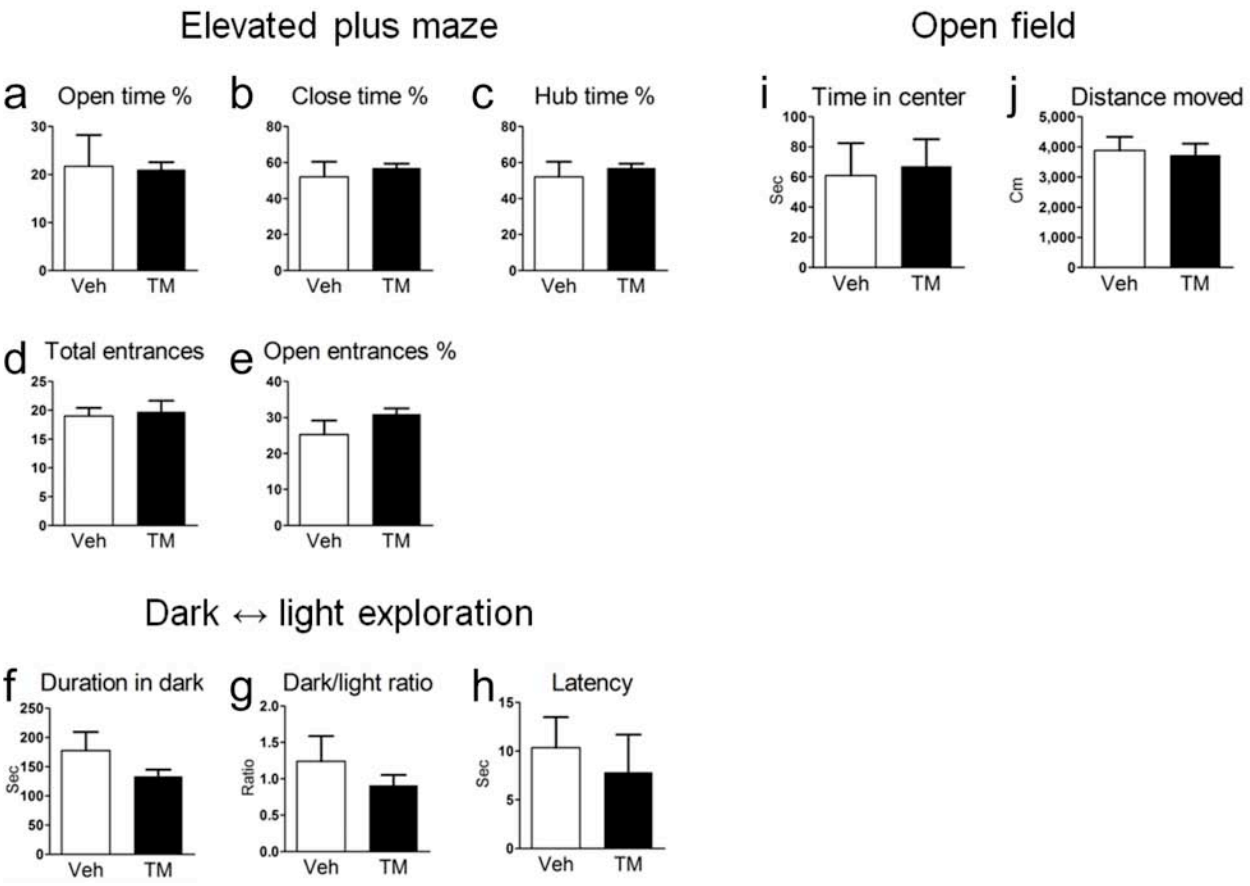
Supplementary Figure 5 Loss of *5-HT$_{1A}$* and *5-HT$_{1B}$* autoreceptor expression in the DRN of *Pet-1*$^{-/-}$ mice. (**a–b'**) *5-HT$_{1A}$* expression analysis in DRN (arrow heads, B6 nucleus) and dorsal tegmental nucleus (arrows) in wild type or *Pet-1*$^{-/-}$ mice. (**c–d'**) *5-HT$_{1B}$* analysis in DRN (**c, c'**) and Purkinje cells (**d, d'**) in wild type or *Pet-1*$^{-/-}$ mice. Loss of *5-HT$_{1A}$* and *5-HT$_{1B}$* receptor expression was observed only in raphe 5-HT neurons as their expression in neighboring structures were not affected (**b, b', d, d'**). Scale bar is 200 μm.



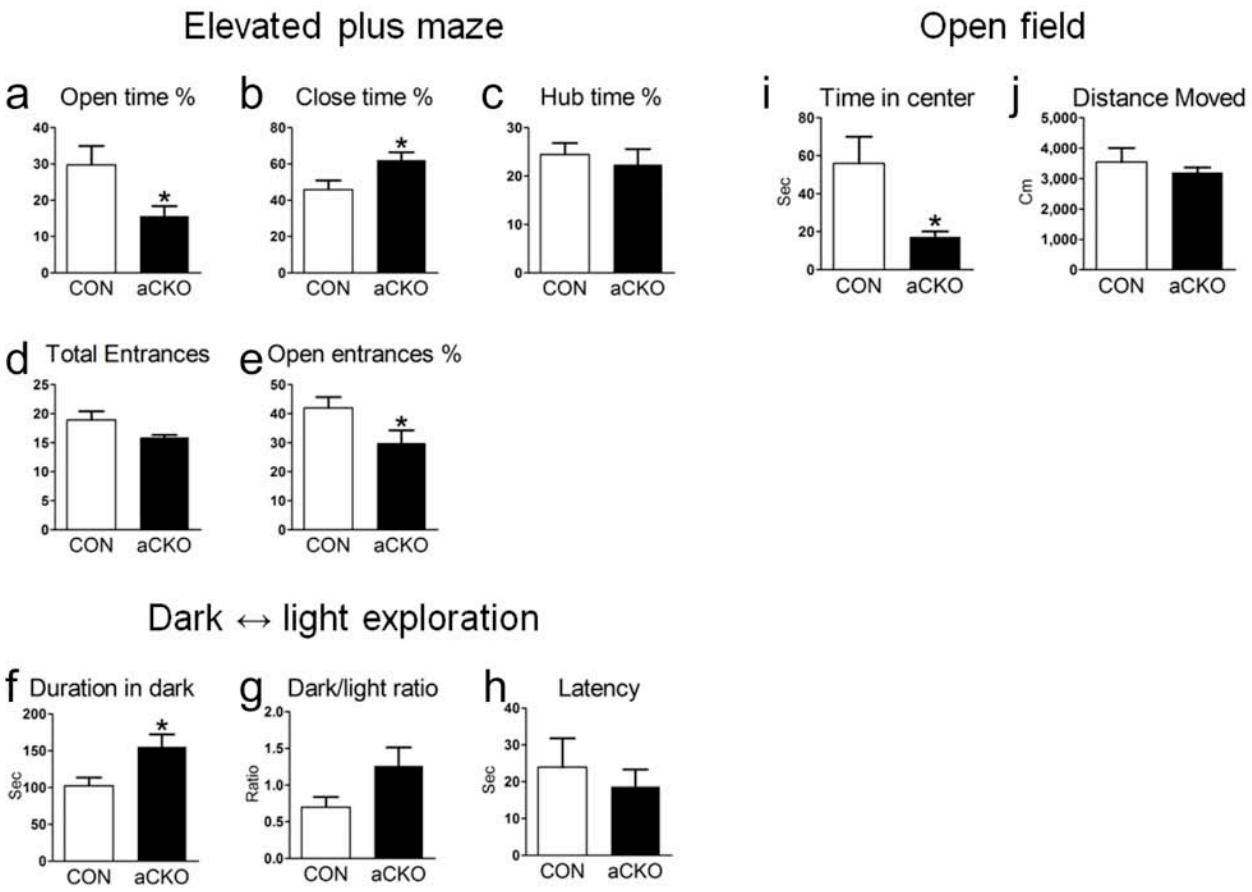
Supplementary Figure 6 Gata3 expression persists while Gata2 expression decreases following 5-HT neuron differentiation. (**a–c''**) Co-immunostaining for Gata2 (**a, b, and c**) and Yfp (**a', b' and c'**) in coronal sections of the rostral hindbrain at E11.5, E12.5, and E14.5. (**a"**), (**b"**) and (**c"**), Merge of (**a**), (**a'**); (**b**), (**b'**); and (**c**), (**c'**) respectively. (**d–e'**) Coronal views of embryonic rostral (r2, **d, d'**) and caudal (r7, **e, e'**) hindbrain sections showing Yfp immunostaining (brown) after *Gata3* in situ hybridization (blue) at E12.5. (**d'**) and (**e'**), Higher magnification of boxed areas in (**d**) and (**e**). (**f–k"**) Co-immunostaining of adult coronal sections for Gata3 (**f, i**) and Vmat2 (**g, j**) in DRN (**f–h'**) and ventral medulla (**i–k"**). (**h**), (**k**), Merge of (**f**), (**g**); and (**i**), (**j**) respectively. (**h**'), (**k**'), and (**k**"), Higher magnification of boxed areas in (**h**) and (**k**). Scale bars in (**c**"), and (**k**) are 200 μm. Scale bars in (**e'**), (**h'**), and (**k"**) are 50 μm.



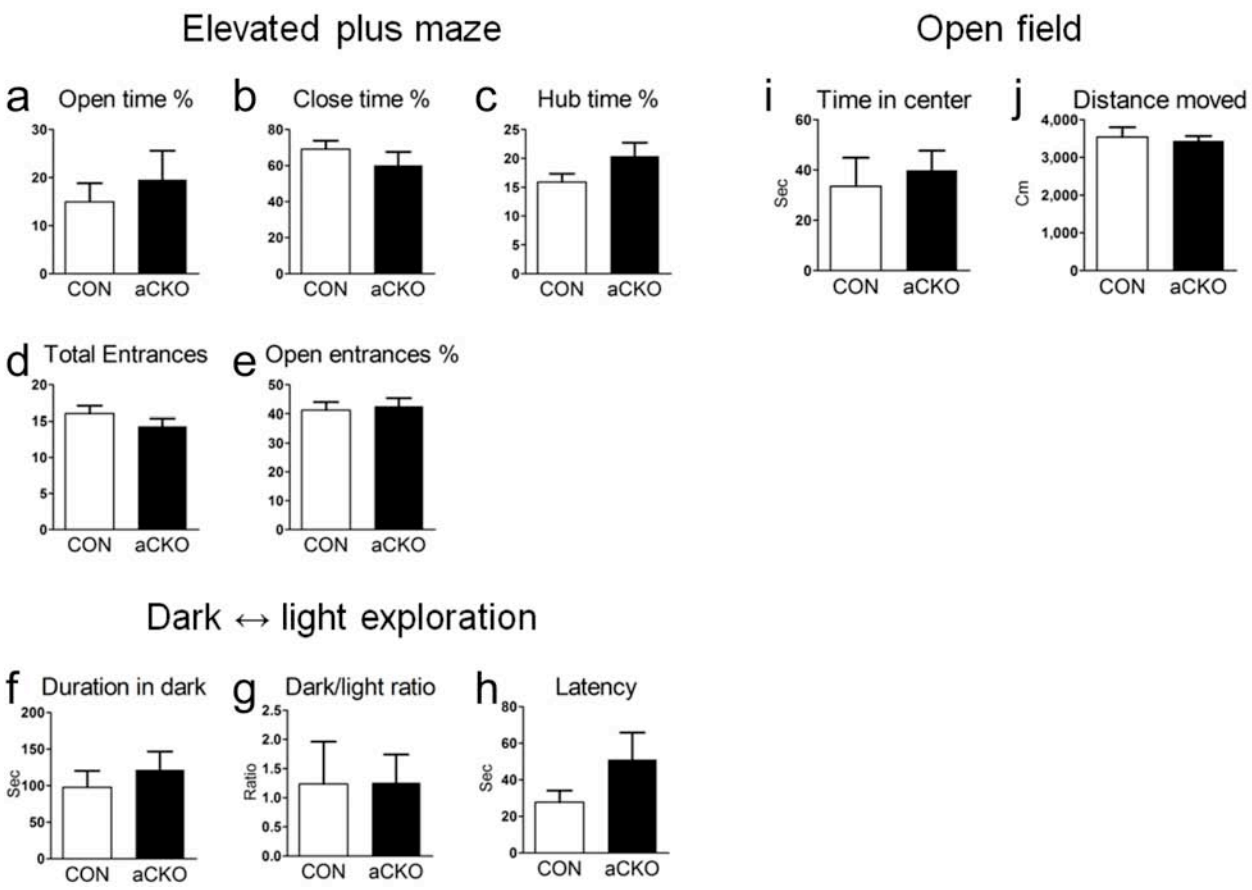
Supplementary Figure 7 Generation and analysis of 5-HT neuron specific tamoxifen inducible *ePet::CreER*^{T2} mice. (**a**) Schematic of the *ePet::CreER*^{T2} transgene. One line, *ePet::CreER*^{T2ascend}, was selected for subsequent experiments as it showed selective targeting efficacies in ascending versus descending 5-HT neurons (**Fig. 5**). (**b**) Xgal staining of an E16.5 sagittal section. r4, rhombomere 4. (**c, d**) Xgal staining of adult coronal sections. (**e–h**) X-gal staining of adult coronal sections from TM treated *ePet::CreER*^{T2}; *R26R*$^{\beta gal+}$ mice. (**e**) ctx, cortex; (**f**) hp, hippocampus; (**g**) cb, cerebellum; (**h**) sc, spinal cord. Scale bars are 200 μm.



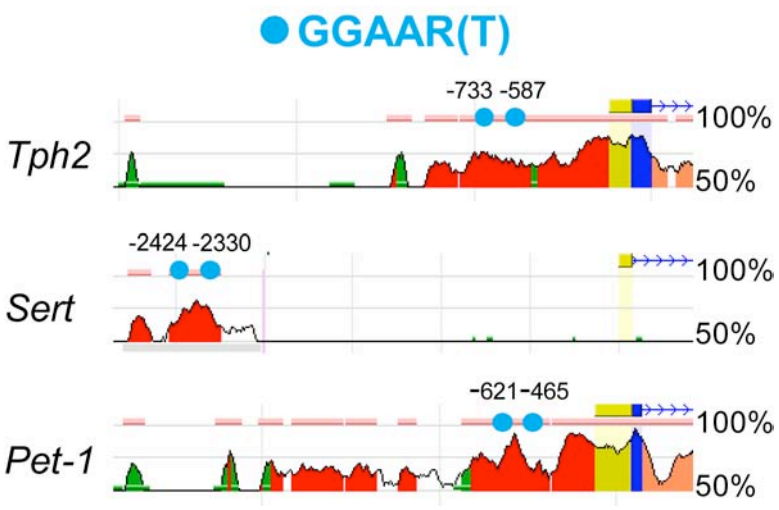
Supplementary Figure 8 Adult TM treatments do not alter behavioral performance in anxiety-related tests. Six to eight-week-old male wildtype mice were treated with either vehicle (n=7) or TM (n=7) for 5 consecutive days and then acclimated for another 4 weeks before behavioral testing. (**a–e**) Elevated plus maze. (**f–h**) Dark ↔ light exploration. (**i, j**) Open field. Error bars represent s.e.m.



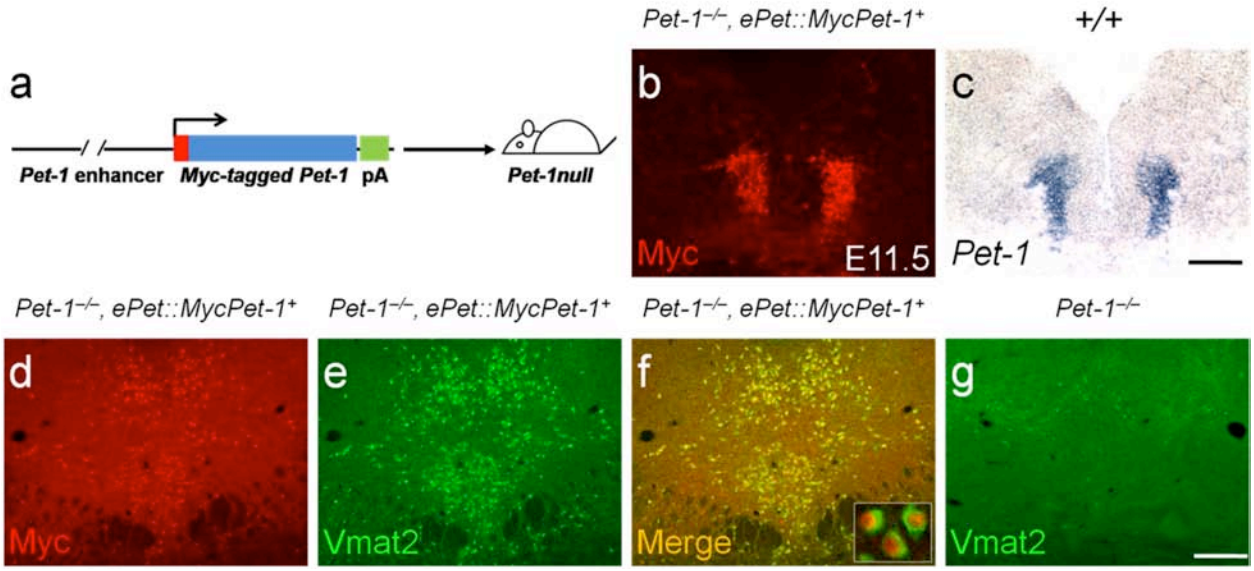
Supplementary Figure 9 Adult deletion of *Pet-1* causes elevated anxiety-like behavior in a second independent cohort. Six to eight-week-old male *Pet-1*^{aCKO} mice (*ePet::CreER*^{T2}; *Pet-1*$^{fl/–}$, n=10) and their littermate controls (*Pet-1*$^{fl/–}$, n=10) were treated with TM for 5 consecutive days and then acclimated for another 4 weeks before behavioral testing. (**a–e**) Elevated plus maze. * p<0.05, two tailed t test. (**f–h**) Dark ↔ light exploration. * p<0.05, p=0.056 for (**g**), two tailed t test. (**i, j**) Open field. * p<0.05, two tailed t test. Error bars represent s.e.m.



Supplementary Figure 10 Anxiety-like behavior is not observed in vehicle treated *Pet-1*^{aCKO} mice. Six to eight-week-old male *Pet-1*^{aCKO} mice (*ePet::CreER*^{T2}; *Pet-1*$^{fl/–}$, n=7) and their littermate controls (*Pet-1*$^{fl/–}$, n=7) were treated with vehicle for 5 consecutive days and then acclimated for another 4 weeks before behavioral testing. (**a–e**) Elevated plus maze. (**f–h**) Dark ↔ light exploration. (**i, j**) Open field. Error bars represent s.e.m.



Supplementary Figure 11 Phylogenetically conserved consensus Pet-1 binding sites. Blue dots identify positions of predicted Pet-1 binding sites (GGAAR*T*) upstream of the *Tph2*, *Sert* and *Pet-1* genes. Colored peaks indicate significant sequence conservation. Red, intergenic sequences; yellow, 5' untranslated exons; green, repetitive sequences; blue, protein coding sequences; orange, introns. Right axis shows percent identity between human and mouse sequences.



Supplementary Figure 12 *ePet::mycPet-1* expression recapitulates endogenous *Pet-1* expression and rescues 5-HT neuron number in *Pet-1*$^{-/-}$ mice. (**a**) Schematic of the *ePet::mycPet-1* transgene injected into pronuclei derived from *Pet-1*$^{-/-}$ female fertilized eggs. (**b**) Anti-myc immunohistochemistry in E11.5 coronal hindbrain sections. (**c**) In situ hybridization of *Pet-1* mRNA in E11.5 wildtype embryos. (**d–f**) Co-staining of adult coronal sections for myc (**d**) and Vmat2 (**e**) in *Pet-1*$^{-/-}$; *ePet::mycPet-1*$^{+}$ mice demonstrating that expression of *ePet::mycPet-1* transgene (hemizygous) rescued the number of Vmat2^{+} 5-HT neurons in the *Pet-1*$^{-/-}$ background (**g**). (**f**), Merge of (**d**) and (**e**). Inset: higher magnification showing co-localization of mycPet-1 and Vmat2 proteins within 5-HT neurons. Scale bar in (**c**) is 50 μm. Scale bar in (**g**) is 200 μm.

Gene	Forward Primer (contains T7 promoter sequence)	Reverse Primer (contains T3 promoter sequence)	Size
5HT 1A	GTAATACGACTCACTATAGGGCGAAGAGCCTGAATGGTCAGC	GAATTAACCCTCACTAAAGGGCCTTCTCCATCACCACCACT	564
5HT 1B	GTAATACGACTCACTATAGGGCGATGCTGGACTGCTTTGTGA	GAATTAACCCTCACTAAAGGGTGTGGAACGCTTGTTTGAAG	560
Aadc	GTAATACGACTCACTATAGGGCGAGCTGGGTTAATTGGTGGA	GAATTAACCCTCACTAAAGGGCTGGCGTACCAGTGACTCAA	593
Cpne7	GTAATACGACTCACTATAGGGCCACGGTGATAGCAGAGGACA	GAATTAACCCTCACTAAAGGGGGTACTCGTTGGGCTGGTAA	590
Cre	GTAATACGACTCACTATAGGGCAAAATTTGCCTGCATTACCG	GAATTAACCCTCACTAAAGGGATTCTCCCACCGTCAGTACG	554
Gata3	GTAATACGACTCACTATAGGGCCTTATCAAGCCCAAGCGAAG	GAATTAACCCTCACTAAAGGGGTAGAAGGGGTCGGAGGAAC	506
Lmx1b	GTAATACGACTCACTATAGGGCCTGATGCGAGTCAACGAGTC	GAATTAACCCTCACTAAAGGGGGCCAGCTTCTTCATCTTTG	633
Maob	GTAATACGACTCACTATAGGGCGTGTGCCTTTGGGTTCAGTT	GAATTAACCCTCACTAAAGGGGAAGGCAGGTGTCTCTCCAG	603
Pet-1	GTAATACGACTCACTATAGGGCCAGTGACCAATCCCATCCTC	GAATTAACCCTCACTAAAGGGGTAATGGGGCTGAAAGGGATA	511
Sert	GTAATACGACTCACTATAGGGCGTCAAAACGTCTGGCAAGGT	GAATTAACCCTCACTAAAGGGATGACCACGATGAGCACAAA	593
Tph2	GTAATACGACTCACTATAGGGCGCTACACGCAGAGCATTGAA	GAATTAACCCTCACTAAAGGGATCCATCCCAACTGCTGTGT	659
Vmat2	GTAATACGACTCACTATAGGGCAGACCATGTGTTCCCGAAAG	GAATTAACCCTCACTAAAGGGACATTGGGCAACGTTAGAGG	607

Supplementary Table 1 Primer pairs for synthesis of antisense riboprobes.

Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')
5HT1A	GGAGCAAAAGGGCTCCTAAG	AAAATTGCCCCTTTAGGTCAC
Aadc	CTAAGGCCAACCGTGAAAAG	ACCAGAGGCATACAGGGACA
Cpne7	GGCTGCCAGATCCACTGTA	CAGCTGTTCCTCGGGTCA
Gch1	GCCTCACCAAACAGATTGC	CACGCCTCGCATTACCAT
Gata3	CTATGTGCCCGAGTACAGCTC	ACACACTCCCTGCCTTCTGT
Lmx1b	AAAGAGCAAAGATGAAGAAGCTG	CGGCTTGACAGAACCTCTTG
Maob	GCCATTGGGAAGATTCCAG	ACTGGTAATGGGTCGTGCAG
Pet-1	GTCGGAGATGGTCTTTTTAAGG	TGCCACAACTGGATCTGC
Sert	CAAGTTCAACAACAACTGTTACCAA	TAGCCAAGCACCGTGAAGAT
Tph1	AAGAAATTGGCCTGGCTTC	GTTTGCACAGCCCAAACTC
Tph2	GAGCTTGATGCCGACCAT	TGGCCACATCCACAAAATAC
Vglut3	TTTGTCCCCTCATTGTTGGT	GCGCTGCTATGAGGAACAC
Vmat2	TGCTGAAGGACCCATACATTC	CACATGGTCTCCATCATCCA

Supplementary Table 2 Primer Pairs for RT-qPCR.