nature neuroscience

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

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Transcriptional cascades are required for the specification of serotonin (5-HT) neurons and behaviors modulated by 5-HT. Several cascade factors are expressed throughout the lifespan, which suggests that their control of behavior might not be temporally restricted to programming normal numbers of 5-HT neurons. We used new mouse conditional targeting approaches to investigate the ongoing requirements for *Pet-1* (also called *Fev*), a cascade factor that is required for the initiation of 5-HT synthesis, but whose expression persists into adulthood. We found that *Pet-1* was required after the generation of 5-HT neurons for multiple steps in 5-HT neuron maturation, including axonal innervation of the somatosensory cortex, expression of appropriate firing properties, and the expression of the *Htr1a* and *Htr1b* autoreceptors. *Pet-1* was still required in adult 5-HT neurons to preserve normal anxiety-related behaviors through direct autoregulated control of serotonergic gene expression. These findings indicate that *Pet-1* is required across the lifespan of the mouse and that behavioral pathogenesis can result from both developmental and adult-onset alterations in serotonergic transcription.

The 5-HT transmitter system in the brain is an essential homeostatic modulator of neural circuits that shape emotional behaviors in response to stressors in the environment[1]. A widely discussed theory supported by a rich literature emphasizes the importance of 5-HT function for the maturation of neural circuits and the development of normal adult emotional behaviors[2]. Altered serotonergic signaling and gene expression during embryonic development disrupts cortical dendritic arborization[3] and the patterning of forebrain afferents[4,5]. Other studies show that early postnatal perturbation of the serotonergic system can cause emotional disorders in adult animals[6–8]. These findings, together with correlative studies of serotonergic indices and gene variants in monkeys and humans, support the idea that alterations in serotonergic function are involved in establishing vulnerability for several mood and neurological disorders[1,9].

The likelihood that altered serotonergic function during development contributes to behavioral pathogenesis has stimulated interest in the genetic mechanisms that direct the formation of the 5-HT system[10]. A cascade (**Supplementary Fig. 1**) of transcriptional regulators has been identified that progressively restricts multi-potent neuronal progenitors to a 5-HT neuron fate in the embryonic ventral hindbrain[11]. Genetic targeting of factors in the cascade causes alterations in 5-HT-modulated emotional responses in adults[12,13], and this finding provides a link between the transcriptional regulation of 5-HT neuron birth and adult behavior. Nevertheless, the mechanisms through which transcription factors in the cascade regulate behavior are poorly understood and might not be simply the result of programming normal 5-HT neuron numbers and 5-HT levels. For example, although all of the factors known to compose the cascade are necessary for the initiation of 5-HT synthesis at the cell fate specification stage, transcriptional control of subsequent steps in the maturation of the 5-HT system might also be crucial for programming normal 5-HT-modulated behaviors. However, whether or not factors in the cascade are responsible for additional transcriptional events in the maturation of the system has not been investigated. Furthermore, it is not known whether the critical period for transcription directed by these developmental determinants extends into adulthood to regulate the maintenance of 5-HT signaling and preserve behavioral integrity. The concept of a transcriptional maintenance mechanism could be crucial for understanding the regulation of behavioral and psychiatric pathogenesis as drug, toxin and dietary perturbation studies in adults including humans demonstrate the importance of ongoing presynaptic serotonergic function in emotional and behavioral processing[9].

Expression of the rodent *Pet-1* ETS domain transcription factor (human orthologue, *FEV*) is restricted in the CNS to 5-HT neurons and is induced in postmitotic precursors just before the initiation of 5-HT synthesis in the ventral hindbrain[14]. *Pet-1* has a crucial role in the cascade through its coordinate induction of the enzymatic pathway responsible for 5-HT synthesis in immature postmitotic precursors[12]. Interestingly, *Pet-1* expression seems to continue undiminished in all adult 5-HT neurons[14]. This persistent expression suggests that *Pet-1* might be required for events in 5-HT neuron maturation that occur subsequent to their specification and possibly in adulthood for transcriptional maintenance of the 5-HT system. Here, we used new 5-HT neuron-specific and temporally restricted conditional targeting approaches to investigate requirements for continued *Pet-1*–dependent transcription in the 5-HT system.

RESULTS

Conditional deletion of *Pet-1* after 5-HT neuron generation

To investigate the function of *Pet-1* after its initial role in 5-HT neuron generation, we inserted two *loxP* sites into introns on each side of exon 3, which encodes most of the *Pet-1* protein coding sequences

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Received 10 March; accepted 23 July; published online 5 September 2010; doi:10.1038/nn.2623

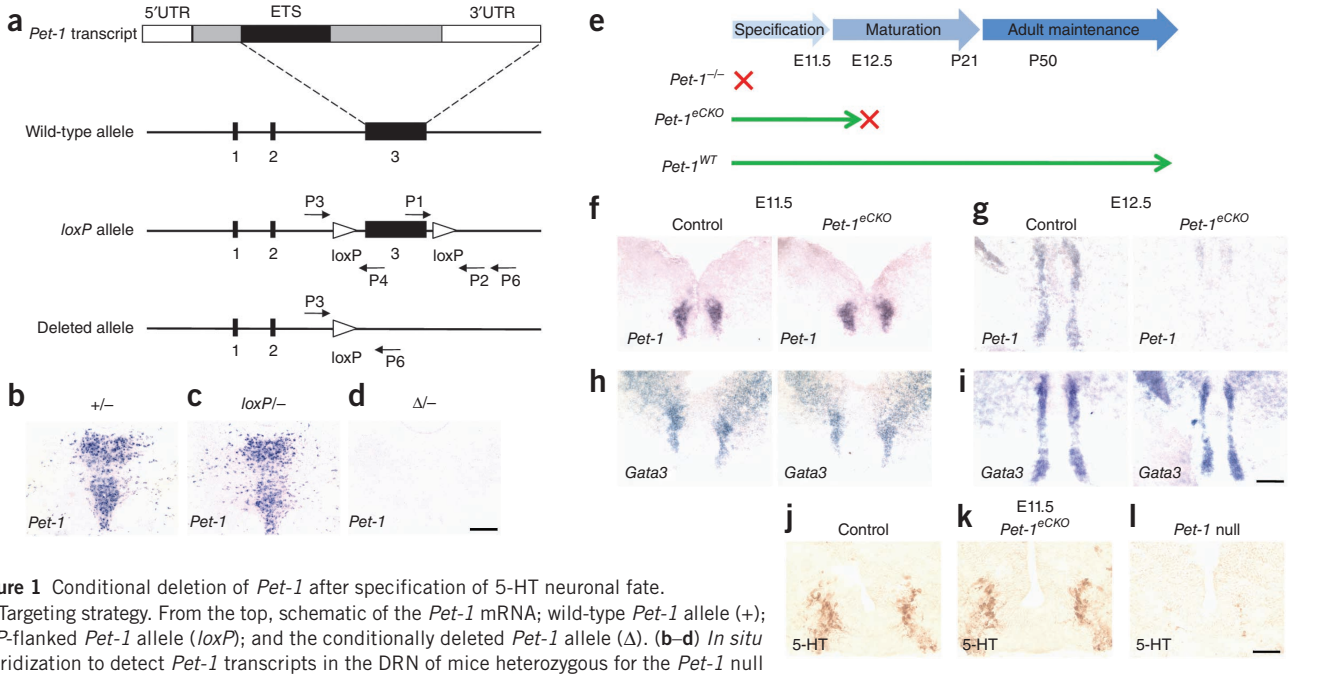

Figure 1 Conditional deletion of *Pet-1* after specification of 5-HT neuronal fate. (**a**) Targeting strategy. From the top, schematic of the *Pet-1* mRNA; wild-type *Pet-1* allele (+); *loxP*-flanked *Pet-1* allele (*loxP*); and the conditionally deleted *Pet-1* allele (Δ). (**b**–**d**) *In situ* hybridization to detect *Pet-1* transcripts in the DRN of mice heterozygous for the *Pet-1* null allele and the wild-type (**b**), *loxP*-flanked (**c**) or conditionally deleted (**d**) *Pet-1* alleles. (**e**) Time frame of *Pet-1* expression in *Pet-1*$^{-/-}$, *Pet-1*^{eCKO} (*Pet-1*$^{loxP/-}$ *ePet::Cre*) and wild-type mice. (**f**–**i**) *In situ* hybridization to detect *Pet-1* and *Gata3* mRNAs in control (*Pet-1*$^{loxP/+}$ *ePet::Cre*) and *Pet-1*^{eCKO} mice at either E11.5 (**f**,**h**) or E12.5 (**g**,**i**). (**j**–**l**) 5-HT immunostaining in control, *Pet-1*^{eCKO} and *Pet-1*$^{-/-}$ mice at E11.5. Scale bars represent 100 μm (**i**,**l**) and 200 μm (**d**).

including the ETS DNA-binding domain (**Fig. 1**). *In situ* hybridization (**Fig. 1b**,**c**) and quantitative reverse transcriptase PCR (RT-qPCR; **Supplementary Fig. 2**) indicated that *Pet-1* expression was indistinguishable in mice carrying either one copy of the wild-type or *loxP*-flanked *Pet-1* allele together with a constitutive null allele. Excision of exon 3 generated a deleted *Pet-1* allele (Δ) in which all protein coding sequences except those encoding 42 amino acids at the N terminus were eliminated (**Fig. 1a**). *In situ* hybridization showed that *Pet-1* was not expressed in mice heterozygous for the deleted and null *Pet-1* alleles (**Fig. 1d**). Furthermore, *Pet-1*$^{loxP/-}$ mice had normal numbers of tryptophan hydroxylase (Tph)-positive neurons but *Pet-1*$^{\Delta/-}$ mice did not (**Supplementary Fig. 2**). Thus, the unrecombined *loxP*-flanked allele is functionally equivalent to the wild-type allele and the conditionally deleted *Pet-1* allele is functionally equivalent to the constitutive null *Pet-1* allele.

We crossed loxP-flanked *Pet-1* mice with *ePet::Cre* transgenic mice[15], which express *Cre* recombinase only in postmitotic 5-HT neurons, to generate *Pet-1* early conditional knockout mice (*Pet-1*$^{loxP/-}$ *ePet::Cre*, designated *Pet-1*^{eCKO}). Nearly all 5-HT neurons derived from progenitors in rhombomeres 1 and 2 are born by embryonic days (E)10 and 11, respectively[16,17]. However, reduced serotonergic gene expression is not seen in mice carrying this *ePet::Cre* transgene until about E12.5 (ref. 18). Thus, *Pet-1* expression should be maintained in *Pet-1*^{eCKO} mice for about 2 d after fulfilling its early role in 5-HT neuron generation (**Fig. 1e**). On the basis of this reasoning, we predicted that normal numbers of 5-HT neurons would be generated in *Pet-1*^{eCKO} mice.

Consistent with our expectation, we found that expression of *Pet-1* and other 5-HT neuron markers including tryptophan hydroxylase 2 (*Tph2*), serotonin transporter (*Slc6a4*, also called *Sert*) and another transcription factor, *Gata3*, was indistinguishable between *Pet-1*^{eCKO} mice and wild-type controls in the anterior hindbrain at E11.5 (**Fig. 1f**,**h** and **Supplementary Fig. 3**). Furthermore, immunohistochemistry using antibodies to 5-HT showed that *Pet-1*^{eCKO} mice had the same number of 5-HT^{+} neurons as did controls (**Fig. 1j**,**k**). By contrast, *Pet-1*$^{-/-}$ mice had very few 5-HT^{+} cells at this stage (**Fig. 1l**). We did not observe a decrease in *Pet-1* transcripts in *Pet-1*^{eCKO} mice until E12.5 (**Fig. 1g**). Concomitant with the conditional deletion of *Pet-1* at E12.5, the expression of *Tph2*, *Slc6a4* and 5-HT in *Pet-1*^{eCKO} mice was diminished (**Supplementary Fig. 3**), whereas the expression of *Gata3* was not altered (**Fig. 1i**). *Pet-1* is not required for the survival of 5-HT neurons[19], and all *Pet-1*-deficient cells were present in the brains of *Pet-1*^{eCKO} mice through adulthood (**Supplementary Fig. 4**). These findings indicate that *Pet-1*^{eCKO} mice can be used to investigate *Pet-1* function in 5-HT system maturation after it has fulfilled its initial role in 5-HT neuron generation.

Continued *Pet-1* function controls serotonergic innervation

Immediately after the birth of 5-HT neurons, maturation of the 5-HT system depends on proper cell body migration, axon pathfinding and innervation in terminal fields[20]. To investigate the role of Pet-1 in these maturation events, we used *Cre*-mediated activation of the *R26R*^{Yfp} (ref. 21) reporter allele to permanently mark *Pet-1*-deficient 5-HT neurons in *Pet-1*^{eCKO} mice (**Fig. 2**). *Pet-1* deletion was spared in a small subset (~15%) of 5-HT neurons in *Pet-1*^{eCKO} mice (**Supplementary Fig. 4**) and therefore *Pet-1*-deficient Tph^{-} cells were situated side by side with untargeted Tph^{+} 5-HT neurons (**Fig. 2a**). Examination of R26R-Yfp^{+} cells in *Pet-1*^{eCKO} mice showed that these *Pet-1*-deficient 5-HT neurons extended axons from their cell bodies, similar to intermingled wild-type Tph^{+} cells, and therefore *Pet-1* was not essential for proximal axonal outgrowth (**Fig. 2a**). Immunostaining for yellow fluorescent protein (YFP) in *Pet-1*^{eCKO} mice revealed that axon bundles from *Pet-1*-deficient 5-HT neurons crossed the midbrain-hindbrain boundary and entered the midbrain at E14.5 (**Fig. 2b**).

To determine whether these axons could properly reach their forebrain targets, we performed a retrograde tracing experiment by injecting tracer into the somatosensory cortex, which receives serotonergic afferents mainly from 5-HT neurons in the dorsal raphe

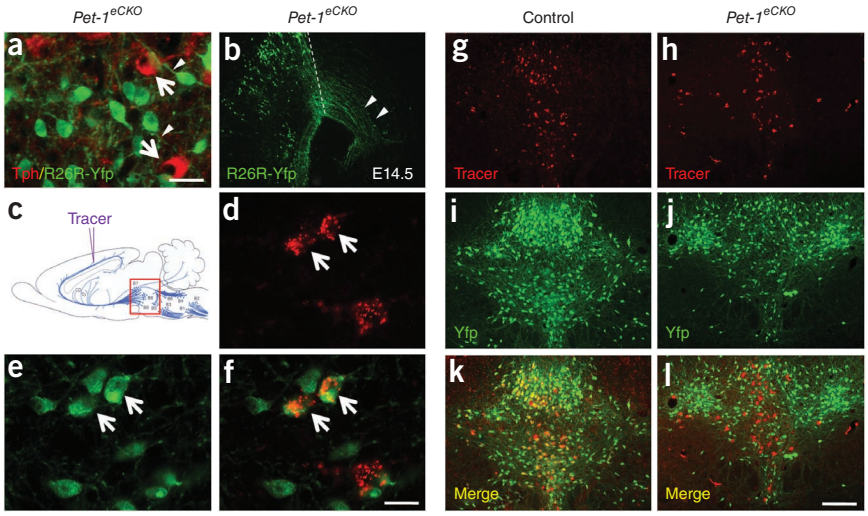

Figure 2 Continued *Pet-1* function is required for maturation of serotonergic axonal innervation patterns. (**a**) Co-immunostaining for YFP and Tph in adult *Pet-1*^{eCKO} mice. White arrows, untargeted 5-HT neurons; arrowheads, proximal axons extending from cell bodies of *Pet-1*–deficient 5-HT neurons. (**b**) YFP immunostaining of *Pet-1*-deficient 5-HT neuron axon bundles at E14.5 in *Pet-1*^{eCKO} mice. Arrowheads mark axons that have crossed the midbrain-hindbrain boundary (dashed lines) and entered the midbrain. (**c**) Schematic of the retrograde tracing experiment. (**d**) Tracer-labeled cells in the DRN. (**e**) YFP immunolabeling of *Pet-1*–deficient 5-HT neurons in the DRN. (**f**) Merge of **d** and **e**. (**g**–**l**) Significantly fewer retrogradely labeled cells were found in the DRN of the *Pet-1*^{eCKO} brain (**g**,**h**; 49.9 ± 3.1%, mean ± s.e.m., relative to control, $n = 6$ for each genotype). Overlay of tracer signal with YFP immunostaining (**i**,**j**) showed that 83.0 ± 1.8% (mean ± s.e.m.) of the retrogradely labeled DRN cells in control mice were *Yfp*$^{+}$ 5-HT neurons (**k**), whereas only 19.3 ± 1.7% YFP^{+} *Pet-1*-deficient 5-HT neurons (**l**) were labeled by the same tracer injection in *Pet-1*^{eCKO} mice. $P < 0.001$, two tailed t test. Scale bars represent 20 μm (**a**,**f**) and 200 μm (**l**).

nucleus[22] (DRN; **Fig. 2c**). In 3-week-old wild-type and *Pet-1*^{eCKO} mice, such injection resulted in the retrograde labeling of both 5-HT and non-5-HT neurons in the DRN (**Fig. 2d**–**f**). The tracer intensity in *Pet-1*-deficient 5-HT neurons was comparable to that in other retrogradely labeled cells, suggesting that *Pet-1*-deficient cells could retrogradely transport tracer (**Fig. 2f**). We found a significant decrease in the total number of labeled cells across the entire DRN in *Pet-1*^{eCKO} mice (**Fig. 2g**,**h**). In wild-type mice, most retrogradely labeled cells were 5-HT neurons (**Fig. 2i**,**k**). In contrast, far fewer *Pet-1*-deficient YFP^{+} 5-HT neurons were labeled by similar tracer injections (**Fig. 2j**,**l**), indicating that the serotonergic innervation of the somatosensory cortex from DRN 5-HT neurons was significantly disrupted in the brains of *Pet-1*^{eCKO} mice.

Continued Pet-1 function controls autoreceptor pathways

Another key event in the maturation of 5-HT neuron function in the brain is the acquisition of 5-HT neuron-specific firing properties[23]. To study whether continued Pet-1 function is required for normal firing of 5-HT neurons, we analyzed *Pet-1*-deficient *R26R-Yfp*$^{+}$ cells in postnatal brain slices from *Pet-1*^{eCKO} mice by using whole-cell recordings under current-clamp conditions. Compared with the aged-matched controls, many *Pet-1*-deficient cells showed increased spontaneous firing of action potentials (**Fig. 3a**–**c**). This increased excitability could result from alterations in Htr1a autoreceptor signaling, which normally inhibits the firing of 5-HT neurons through negative feedback inhibition[24]. As previously described[25], activation of the Htr1a receptor with its specific agonist

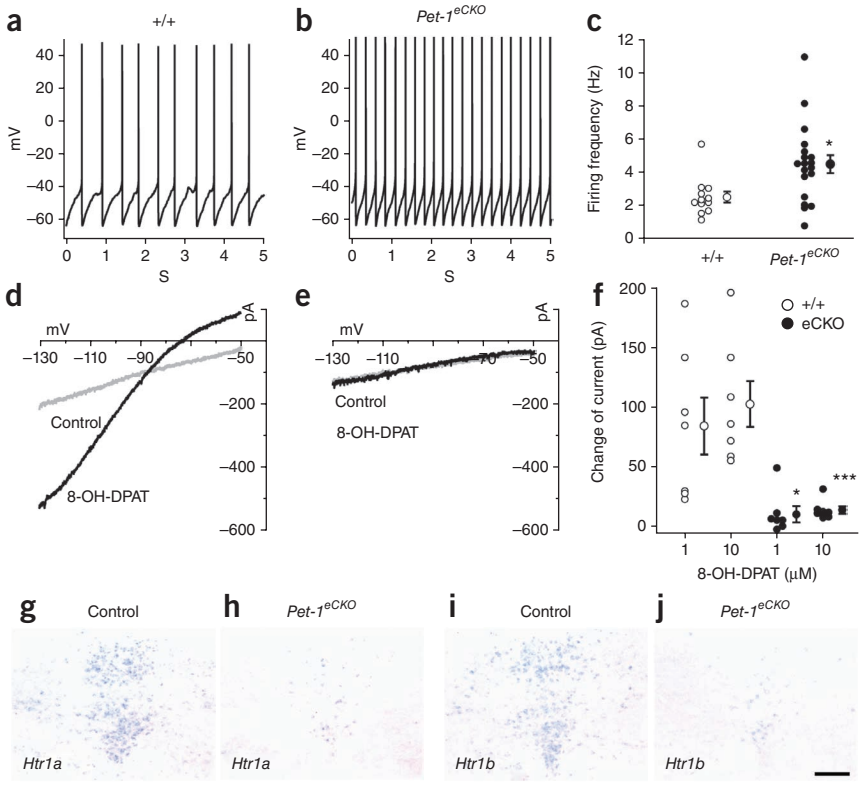

Figure 3 Continued *Pet-1* function is required for 5-HT neuron firing properties and inhibitory autoreceptor function. (**a**,**b**) Whole-cell current-clamp recordings measuring spontaneous firing of YFP^{+} neurons with indicated genotypes. (**c**) Quantification of firing frequencies in **a** and **b** (+/+, $n = 12$; *Pet-1*^{eCKO}, $n = 19$; $^{*}P < 0.05$, two tailed t-test). (**d**,**e**) Whole-cell voltage-clamp recordings measuring current changes in YFP^{+} neurons induced with 1 or 10 μM of the Htr1a receptor agonist 8-OH-DPAT. A ramp voltage was applied at 200 mV s^{-1}. The intersection voltage, –87 mV, of the control and 8-OH-DPAT traces in **d** was close to the estimated K^{+} equilibrium potential (–99 mV), considering that recordings were not corrected for the liquid junction potential of around 10 mV. (**f**) Quantification of current changes at –110 mV in **d** and **e** ($^{*}P < 0.05$, $^{***}P < 0.001$; two-tailed t-test). (**g**–**j**) *In situ* hybridization of *Htr1a* (**g**,**h**) and *Htr1b* (**i**,**j**) in control and *Pet-1*^{eCKO} mice. Scale bar, 200 μm. Error bars show mean ± s.e.m.

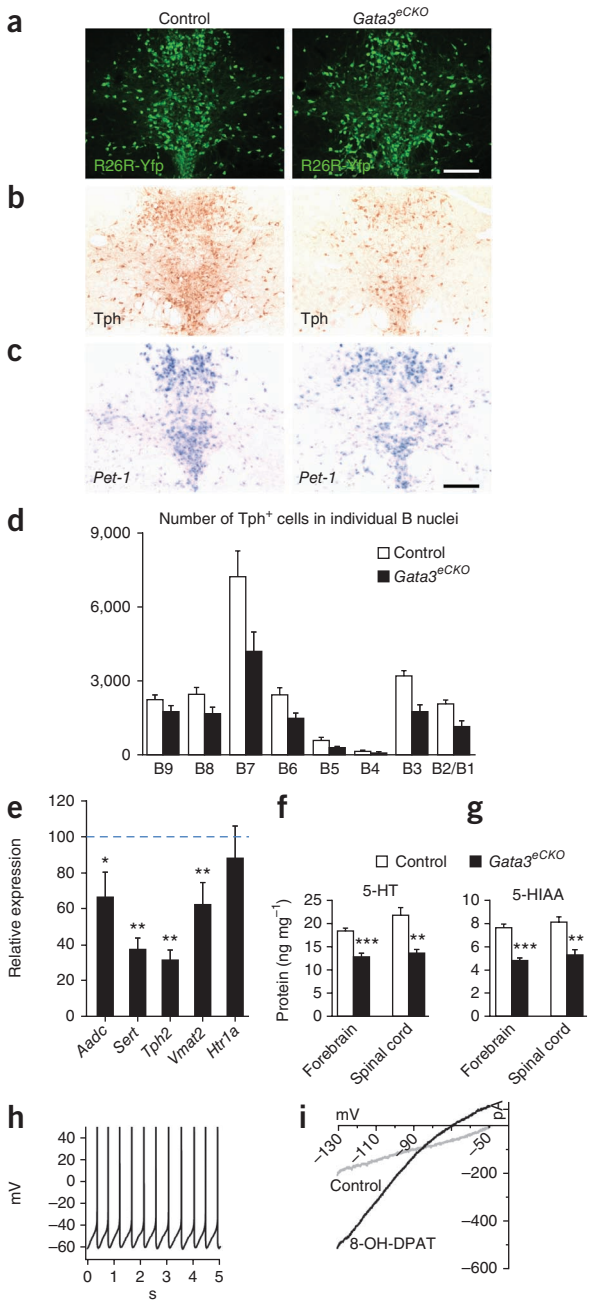

Figure 4 Continued *Gata3* expression is needed to maintain 5-HT gene expression but not autoreceptor function. (**a**) YFP immunostaining in adult DRN. (**b**) Tph immunostaining. (**c**) *In situ* hybridization of *Pet-1* mRNA. (**d**) Counts of Tph$^+$ cell bodies in control versus *Gata3*^{eCKO} mice in individual adult B nuclei ($n = 3$ for each genotype). (**e**) RT-qPCR of *Ddc, Slc6a4, Tph2, Slc18a2* and *Htr1a* mRNAs in control versus *Gata3*^{eCKO} mice (control $n = 7$, normalized to 100%; *Gata3*^{eCKO} $n = 11$; $^*P < 0.05$, $^{**}P < 0.01$, two-tailed *t*-test). (**f**,**g**) HPLC analysis of 5-HT (**f**) and 5-HIAA (**g**) levels in forebrain and spinal cord of control ($n = 7$) and *Gata3*^{eCKO} ($n = 5$) mice ($^{**}P < 0.01$, $^{***}P < 0.001$, two-tailed *t*-test). (**h**) Whole-cell current-clamp recordings of spontaneous firing in R26R-YFP$^+$ *Gata3*-deficient cells. (**i**) Whole-cell voltage-clamp recordings of current changes in response to 8-OH-DPAT in R26R-YFP$^+$ *Gata3*-deficient cells. Scale bars, 200 µm. Error bars show s.e.m. except for s.d. in **d**.

8-OH-DPAT elicited strong inwardly rectifying potassium currents in control YFP$^+$ 5-HT neurons under voltage clamp (**Fig. 3d**). By contrast, neither low (1 µM) nor high (10 µM) concentrations of 8-OH-DPAT elicited a change in baseline currents in *Pet-1*-deficient YFP$^+$ 5-HT neurons (**Fig. 3e**,**f**). To investigate the mechanism that accounts for the loss of 8-OH-DPAT responses, we used *in situ* hybridization to examine the expression of the Htr1a receptor and found greatly decreased levels of *Htr1a* mRNA in the *Pet-1*^{eCKO} DRN (**Fig. 3g**,**h**).

A second prominent serotonergic autoreceptor that regulates 5-HT release in serotonergic presynaptic terminals is the Htr1b receptor[26]. *In situ* hybridization in *Pet-1*^{eCKO} mice revealed that *Pet-1* was also required for expression of the *Htr1b* gene (**Fig. 3i**,**j**). The residual expression of *Htr1a* and *Htr1b* mRNAs is probably from the remaining untargeted 5-HT neurons in *Pet-1*^{eCKO} mice as the expression of the two autoreceptors in the DRN was almost completely abolished in *Pet-1*$^{-/-}$ mice (**Supplementary Fig. 5**). Our *in situ* hybridization studies (data not shown) indicated that the expression of *Htr1a* and *Htr1b* in nearly all 5-HT neurons begins after E14, which is consistent with their onset in the forebrain at E14.5 (ref. 27). Thus, our findings show that ongoing *Pet-1* expression is required, well after it has completed its role in the initiation of 5-HT synthesis in immature precursors, for maturation of essential serotonergic autoreceptor characteristics that control firing patterns and transmitter release.

Gata3 is not required for Htr1a autoreceptor responses

We investigated whether the establishment of normal 5-HT neuron firing properties requires parallel ongoing activity of other serotonergic developmental control genes, or whether *Pet-1* has a unique role in this process. Germ line targeting of the zinc finger transcription factors Gata2 and Gata3 has shown that both are required for 5-HT neuron differentiation[28]. We found that Gata2 protein expression began to decline in differentiated 5-HT neurons at E12.5 and was not detectable at E14.5. By contrast, Gata3 expression persisted in all 5-HT neurons through adulthood (**Supplementary Fig. 6**).

To compare the role of Gata3 in differentiated 5-HT neurons with that of Pet-1, we crossed *Gata3*$^{loxP/loxP}$ mice[29] with *ePet::Cre* mice to generate *Gata3* conditional knockout mice (*Gata3*$^{loxP/loxP}$ *ePet::Cre*, designated as *Gata3*^{eCKO}). Similar to *Pet-1*, *Gata3* was not required for cell survival as normal numbers of *Gata3*-deficient 5-HT neurons survived in the adult brain (**Fig. 4**). However, there was a substantial reduction in the number of Tph immunoreactive cells (**Fig. 4b**,**d**), levels of 5-HT (**Fig. 4f**,**g**) and expression of several other 5-HT genes (**Fig. 4e**) in the DRN of *Gata3*^{eCKO} mice. Persistent expression of *Gata3* and *Pet-1* seems to be maintained by independent regulatory pathways, as neither of them was required for the other's expression (**Figs. 1i** and **4c**). These findings suggest that Gata3 and Pet-1 function in parallel pathways to coordinate the expression of normal levels of serotonergic gene expression and 5-HT in the brain.

Although Gata3 and Pet-1 share several common transcriptional targets, *Gata3*-deficient 5-HT neurons showed normal *Htr1a* expression (**Fig. 4e**), indicating that Pet-1 and Gata3 regulate distinct sets genes in 5-HT neurons. These findings also suggest that *Gata3* might not be required for serotonergic firing characteristics. Indeed, whole-cell recordings of slices from *Gata3*^{eCKO} mice showed firing properties and Htr1a agonist responses typical of wild-type 5-HT neurons (**Fig. 4h**,**i**).

Targeting of *Pet-1* in the adult ascending 5-HT system

Having shown that ongoing Pet-1 function is needed for multiple steps in the maturation of the 5-HT system, we sought to determine whether a Pet-1–dependent transcriptional program still operates in adulthood to support serotonergic function and 5-HT-modulated

behaviors (**Fig. 5a**). Thus, we used *Pet-1* regulatory elements to generate a transgene that directed tamoxifen-inducible $CreER^{T2}$ (Cre recombinase–estrogen receptor ligand binding domain, ref. 30) expression specifically in brain 5-HT neurons. To identify founder lines expressing inducible *Cre* activity, we gave pregnant females a single dose of tamoxifen (150 µg per g, intraperitoneal) at gestational stage E11.5 and scored embryos for *Cre*-activated β-galactosidase expression from the $R26R^{\beta gal}$ allele at E16.5 (**Supplementary Fig. 7**). Eight-week-old *Cre* reporter mice ($R26R^{\beta gal+}ePet::CreER^{T2}$) were then given a single daily dose of tamoxifen or vehicle for five consecutive days. Most 5-HT neurons in the DRN and median raphe nucleus (MRN) contained β-galactosidase 5 or 30 days after the last tamoxifen injection. Recombination was strictly dependent on tamoxifen treatment as we found no *Cre* activity in the absence of tamoxifen. We did not detect *Cre* activity in other regions of the CNS after tamoxifen injections (**Supplementary Fig. 7**). In one of the transgenic lines, designated $ePet::CreER^{T2ascend}$, we found differential targeting efficacies after treatment of adult mice with tamoxifen between 5-HT neuron raphe nuclei that give rise to ascending and descending 5-HT systems (**Fig. 5b**,**c**). Double-labeling to detect β-galactosidase and Tph revealed tamoxifen-activated *Cre* activity in up to 80% of 5-HT neurons in the DRN (B6, B7), MRN (B5, B8) and B9 nucleus. By contrast, far fewer 5-HT neurons contained β-galactosidase in the medullary nuclei (B1–B3) after tamoxifen treatment (**Fig. 5d**,**e**).

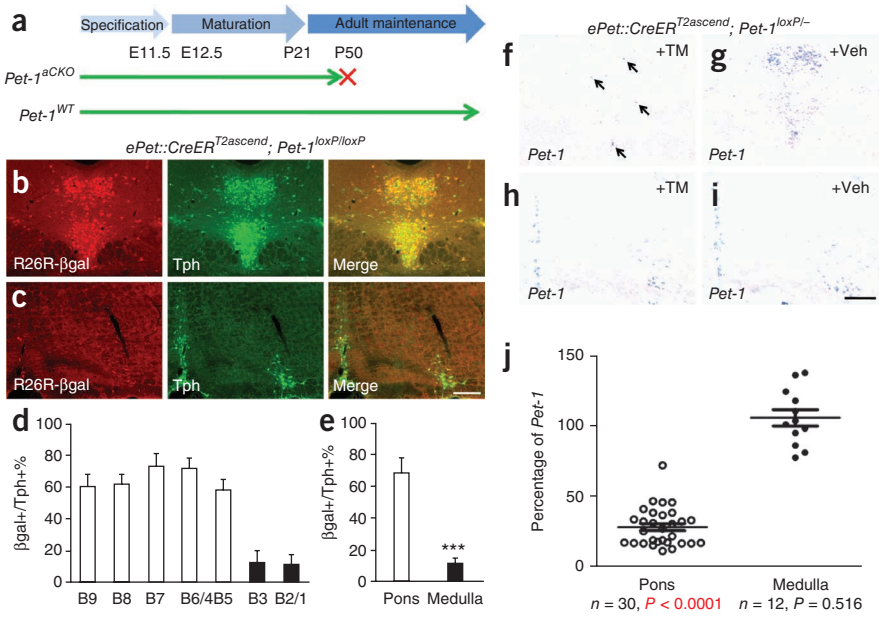

Figure 5 Stage-specific disruption of *Pet-1* in the adult ascending 5-HT system. (**a**) Adult stage-specific deletion of *Pet-1* in $Pet\text{-}1^{aCKO}$ mice. (**b**,**c**) Co-immunostaining for β-galactosidase and Tph in adult DRN (**b**) and medullary raphe (**c**). (**d**,**e**) Percentage of TPH^+ cells expressing *CreER*-activated β-galactosidase in individual adult B nuclei (**d**) and in 5-HT neurons of ascending versus descending pathways (**e**; 68.5 ± 9.5% in the pons (B4–B9) and 12.1 ± 6.8% in medullary nuclei (B1–B3); $n = 7$, mean ± s.d., $^{***}P < 0.001$, two-tailed *t*-test). (**f**–**i**) *In situ* hybridization of *Pet-1* mRNA in coronal sections from adult $Pet\text{-}1^{aCKO}$ mice treated with tamoxifen (TM) or vehicle (Veh). (**j**) RT-qPCR of *Pet-1* mRNA in tamoxifen-treated adult $Pet\text{-}1^{aCKO}$ mice (pons, $n = 30$, 28.0 ± 2.5% relative to control, $n = 35$; medulla, $n = 12$, 105.9 ± 5.8% relative to control, $n = 15$). Each dot represents a sample from the indicated group; data shown are mean ± s.e.m., two-tailed *t*-test. Scale bar, 200 µm.

To determine the efficacy of the $ePet::CreER^{T2ascend}$ line for excision of *Pet-1* in $Pet\text{-}1^{loxP/-}$ mice, we crossed $ePet::CreER^{T2ascend}$, $Pet\text{-}1^{-/-}$ and $Pet\text{-}1^{loxP/loxP}$ mice to generate $Pet\text{-}1^{loxP/-}$ $ePet::CreER^{T2ascend}$ mice, designated as $Pet\text{-}1^{aCKO}$. Six-to-eight-week-old $Pet\text{-}1^{aCKO}$ mice were given single daily tamoxifen treatments for 5 d and killed 5 or 30 d after tamoxifen treatments for evaluation of *Pet-1* expression. *In situ* hybridization showed that tamoxifen treatment abolished the majority of *Pet-1* expression in the adult DRN (B6 and B7 nuclei), MRN (B5 and B8 nuclei) and the B9 group of 5-HT neurons (**Fig. 5f**,**g**). By contrast, *Pet-1* mRNA was not decreased in the B1–B3 groups of 5-HT neurons in the ventral medulla (**Fig. 5h**,**i**). The reduction of *Pet-1* in $Pet\text{-}1^{aCKO}$ mice treated with tamoxifen was further quantified by RT-qPCR, which revealed a >70% loss of *Pet-1* mRNA in pontine tissue containing the B5–B9 serotonergic nuclei, but no significant change in tissue containing the medullary B1–B3 nuclei (**Fig. 5j**). The loss of *Pet-1* mRNA in the B5–B9 nuclei showed that $ePet::CreER^{T2ascend}$ could be used for highly reproducible and stage-specific disruption of *Pet-1* expression in adult ascending 5-HT neurons.

Adult Pet-1 is required for normal anxiety-like behaviors

Germ line targeting of *Pet-1* results in increased anxiety-like behaviors in the adult. However, it remains unclear whether Pet-1–dependent transcription is needed only during development or also in adulthood to modulate normal anxiety responses. To address this question, we treated 6–8-week-old $Pet\text{-}1^{aCKO}$ mice with tamoxifen to delete *Pet-1* in the ascending 5-HT system. We then investigated the effect of adult *Pet-1* deletion on anxiety-related behaviors 4 weeks after the last tamoxifen treatment.

It was recently shown, using an administration protocol similar to that used here, that tamoxifen does not alter anxiety-related behaviors in mice[31]. We verified this finding on a separate cohort of wild-type mice treated with either vehicle or tamoxifen (**Supplementary Fig. 8**). We then tested tamoxifen-treated control and $Pet\text{-}1^{aCKO}$ mice on the elevated plus maze test and found that tamoxifen-treated $Pet\text{-}1^{aCKO}$ mice spent significantly less time in and initiated fewer entries into the open unprotected arms of the maze than did tamoxifen-treated control mice (**Fig. 6a**,**b**). In addition, these mice spent less time in the hub area but significantly more time in the closed arm (**Fig. 6c**,**d**). We found no differences between genotypes in overall explorative activities determined as the number of total open/closed arm entrances (**Fig. 6e**). Their increased avoidance of the aversive properties of height and openness suggests that tamoxifen-treated $Pet\text{-}1^{aCKO}$ mice show augmented anxiety-like behavior. To further study this behavior, we tested the same mice in the light↔dark exploration paradigm, which presents the mice with a similar conflict between the desire to explore a novel environment and the aversive features of a brightly illuminated open field. As compared to tamoxifen-treated littermate controls, tamoxifen-treated $Pet\text{-}1^{aCKO}$ mice spent significantly more time in the dark chamber (**Fig. 6f**,**g**) with a trend towards reduced latency to enter the dark area from the beginning of the test (**Fig. 6h**). The increased time in the dark area and avoidance of the bright open areas supported the idea that tamoxifen-treated $Pet\text{-}1^{aCKO}$ mice show increased anxiety-like behavior. Finally, tamoxifen-treated $Pet\text{-}1^{aCKO}$ mice spent significantly less time than controls in the center of an open field (**Fig. 6i**), whereas overall locomotor activity

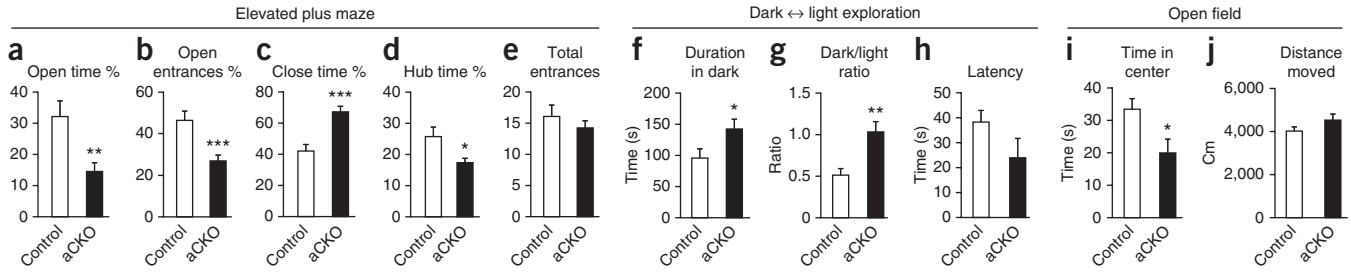

Figure 6 Disruption of *Pet-1*-dependent transcription in the adult ascending 5-HT system causes elevated anxiety-like behavior. Six-to-eight-week-old male *Pet-1*^{aCKO} mice ($n = 12$) and their littermate controls (*Pet-1*$^{loxP/-}$, $n = 12$) were treated with tamoxifen for 5 consecutive days and then acclimated for another 4 weeks before behavioral testing. (**a**–**e**) Elevated plus maze. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; two-tailed *t*-test. (**f**–**h**) Dark ↔ light exploration. $^{*}P < 0.05$, $^{**}P < 0.01$; two-tailed *t*-test. (**i**,**j**) Open field. $^{*}P < 0.05$; two-tailed *t*-test. Error bars represent s.e.m.

was not different (**Fig. 6j**). A second, independent cohort mice showed similar significant increases in all three tests of anxiety-like behaviors in tamoxifen-treated *Pet-1*^{aCKO} mice (**Supplementary Fig. 9**). Furthermore, the increased anxiety-like behavior seen in all three tests depended on reduced *Pet-1* levels, as we found no differences in a separate cohort of *Pet-1*^{aCKO} mice treated with vehicle (**Supplementary Fig. 10**). Overall growth measured by body weight was similar in control and *Pet-1*^{aCKO} mice after tamoxifen treatments (data not shown).

Adult Pet-1 is required for serotonergic gene expression

The altered anxiety-like behaviors in tamoxifen-treated *Pet-1*^{aCKO} mice show that Pet-1 is required in adulthood to maintain serotonergic function. To explore the mechanisms that underlie the alterations in serotonergic function, we first measured levels of brain 5-HT and its metabolite 5-HIAA in *Pet-1*^{aCKO} and control mice killed 5 days after tamoxifen treatments. High-performance liquid chromatography (HPLC) analysis showed that both 5-HT and 5-HIAA were significantly decreased in the forebrain of tamoxifen-treated *Pet-1*^{aCKO} mice (**Fig. 7a**,**b**), but, as predicted, 5-HT levels were not altered in the spinal cord (data not shown), which is innervated by the descending 5-HT system. Western blotting using a monoclonal antibody to both Tph1 and Tph2 indicated that levels of Tph were reduced by about 50% in tamoxifen-treated *Pet-1*^{aCKO} mice relative to controls (**Fig. 7c**,**d**). Consistent with these findings, we found a comparable decrease in *Tph2* mRNA levels in tamoxifen-treated *Pet-1*^{aCKO} mice as early as 5 d after the last tamoxifen treatment (**Fig. 7e**,**f**). Furthermore, *Tph2* mRNA was still decreased 30 d after the last treatment and we did not find a compensatory increase in *Tph1* expression with the loss of *Tph2* (**Fig. 7e**). Together, these findings indicate that Pet-1 is required in adult 5-HT neurons to regulate 5-HT synthesis by maintaining *Tph2* expression.

To determine whether disruption of the Htr1a autoreceptor pathway might have contributed to the abnormal anxiety-like behavior, we performed whole-cell recordings in slices from tamoxifen-treated *Pet-1*^{aCKO} mice but found that Pet-1 was no longer required in the adult brain for spontaneous firing, autoreceptor agonist responses

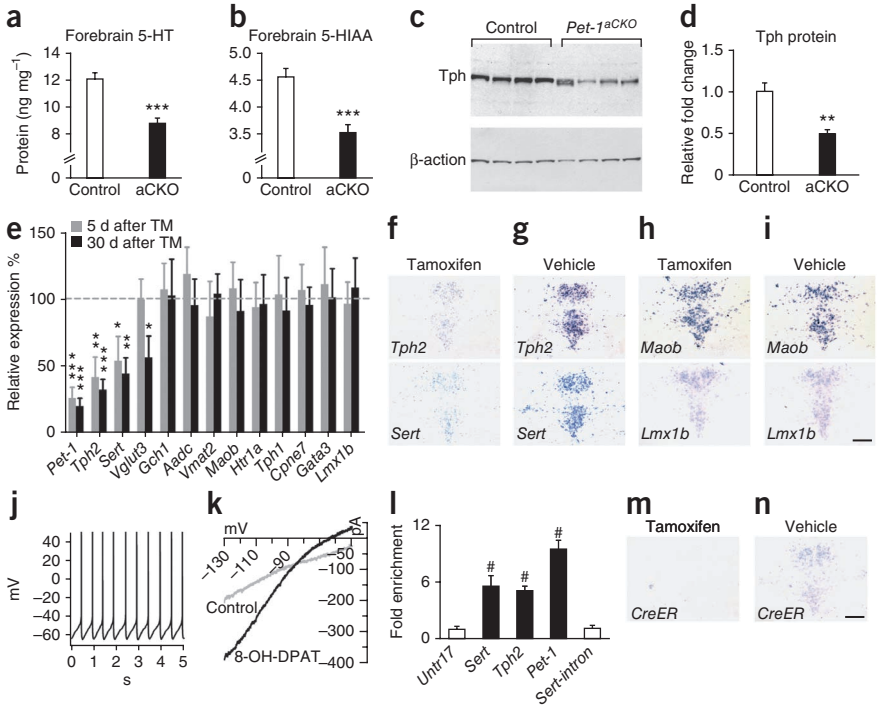

Figure 7 5-HT synthesis and *Slc6a4* expression are maintained in the adult ascending 5-HT system through positively autoregulated direct Pet-1 transactivation. (**a**,**b**) HPLC analysis of 5-HT and 5-HIAA levels in the forebrain of tamoxifen-treated control ($n = 7$) and *Pet-1*^{aCKO} ($n = 7$) mice ($^{***}P < 0.001$, two-tailed *t*-test). (**c**,**d**) Western blotting analysis of Tph protein in DRN of tamoxifen-treated control ($n = 8$) and *Pet-1*^{aCKO} mice ($n = 7$; 50.3 ± 4.0% relative to the control; $^{**}P < 0.01$, two-tailed *t*-test). (**e**) RT-qPCR analysis of serotonergic gene expression in *Pet-1*^{aCKO} or control mice either 5 days (control, $n = 11$; *Pet-1*^{aCKO}, $n = 11$) or 30 days (control $n = 14$; *Pet-1*^{aCKO}, $n = 16$) after treatment with tamoxifen or vehicle ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). (**f**–**i**) *In situ* hybridization to detect *Tph2*, *Slc6a4*, *Maob* and *Lmx1b* mRNAs in coronal sections from *Pet-1*^{aCKO} mice treated with tamoxifen or vehicle. (**j**) Whole-cell current-clamp recordings of spontaneous firing in YFP^{+} *Pet-1*-deficient cells in tamoxifen-treated *Pet-1*^{aCKO} mice. (**k**) Whole-cell voltage-clamp recordings of current changes in response to 8-OH-DPAT in YFP^{+} *Pet-1*-deficient cells. (**l**) RT-qPCR analysis of chromatin immunoprecipitations. Values represent fold enrichment in binding to the indicated regions as compared to negative control region (*Untr17*). Untr, untranscribed genomic region; #, $P < 0.0001$ for *Tph2*, *Slc6a4*, *Pet-1* versus *Untr17* or *Slc6a4* intron, one-way ANOVA with Bonferroni's Multiple Comparison Test. (**m**,**n**) *In situ* hybridization to detect *CreER*^{T2} mRNA in adult *Pet-1*^{aCKO} mice treated with tamoxifen or vehicle. Scale bars, 200 μm. Error bars represent s.e.m except for s.d. in (**l**).

(**Fig. 7j**,**k**), or *Htr1a* gene expression (**Fig. 7e**). To explore other potential deficits beyond the loss of 5-HT, we analyzed the set of additional genes that are known to depend on *Pet-1* in the embryonic hindbrain as well as other genes that are important for 5-HT synthesis and metabolism (**Fig. 7e**,**f**–**i**). The expression of *Slc6a4* and vesicular glutamate transporter 3 (*Slc17a8*, also called *Vglut3*) were also significantly reduced (**Fig. 7e**,**h**), but for *Slc17a8*, decreased expression was not observed until 30 d after tamoxifen treatments (**Fig. 7e**). In contrast to the marked decreases in *Tph2*, *Slc6a4* and *Slc17a8* expression, Pet-1 was no longer required in adulthood to maintain the expression of *Ddc* (also called *Aadc* and *Maob*) and *Slc18a2* (also called *Vmat2*) mRNA (**Fig. 7e**), even though their embryonic expression depends on Pet-1 function (**Supplementary Figure 3**).

Direct autoregulation of serotonergic gene expression

The studies presented so far do not distinguish direct from indirect transcriptional control of target genes by Pet-1. We previously identified a consensus Pet-1-binding sequence, GGAAR(T), upstream of *Slc6a4* and showed that Pet-1 protein interacted with this site *in vitro*[14]. Further analyses identified conserved putative *Pet-1* ETS binding sites in highly conserved upstream regulatory regions of *Tph2* and *Slc6a4* genes (**Supplementary Fig. 11**). To probe the mechanism through which *Pet-1* regulates *Tph2* and *Slc6a4* in 5-HT neurons, we investigated the possibility that *Pet-1* directly regulates their transcription by interacting with conserved upstream regulatory elements.

Because we have been unable to prepare a suitable Pet-1 antibody for chromatin immunoprecipitation (ChIP), we generated a new transgenic mouse line that expressed a myc-epitope-tagged Pet-1 protein in the brains of *Pet-1*$^{-/-}$ mice with *Pet-1* promoter/enhancer sequences[15]. Expression of the *ePet::mycPet-1* transgene recapitulated endogenous *Pet-1* expression in both developing and adult hindbrain, resulting in the rescue of normal numbers of 5-HT neurons in *Pet-1*$^{-/-}$ mice (**Supplementary Fig. 12**). We used this rescue line for ChIP to determine whether Pet-1 directly interacted with *Tph2* and *Slc6a4* promoter sequences *in vivo*. Chromatin was harvested from E12.5 mouse hindbrain before extensive 5-HT neuron dispersion began to scatter these cells, although the dissected tissue was still largely composed of non-serotonergic cells. Sheared chromatin was immunoprecipitated with an anti-myc antibody and analyzed by RT-qPCR for anti-myc enrichment of genomic fragments that included predicted Pet-1–binding sites upstream of *Tph2* and *Slc6a4* as well as in the intron of *Slc6a4* (+11390). Compared to the control, we found no enrichment near the *Slc6a4* intron sequence. By contrast, upstream *Tph2* and *Slc6a4* sequences showed several-fold enrichment compared to both the negative control region *untr17* and the *Slc6a4* intron sequence in two independent immunoprecipitation assays (**Fig. 7l** and data not shown).

Finally, we used the *ePet::CreER*^{T2ascend} transgene as a reporter for Pet-1–dependent regulation of its own enhancer in *Pet-1*^{aCKO} mice. Expression of *CreER*^{T2} in the adult DRN was significantly reduced in *Pet-1*^{aCKO} mice treated with tamoxifen, but not in *Pet-1*^{aCKO} mice treated with vehicle, showing that adult expression of *Pet-1* depends on positive autoregulation (**Fig. 7m**,**n**). Inspection of the upstream *Pet-1* promoter/enhancer sequences[32] revealed conserved *Pet-1* consensus binding sites at −465 and −621 relative to the predicted transcription start site (**Supplementary Fig. 11**). ChIP for genomic fragments with these binding sites revealed a tenfold enrichment relative to control immunoprecipitations (**Fig. 7l** and data not shown). These findings suggest that transcriptional regulation of 5-HT synthesis and serotonergic gene expression in adulthood depends on direct positive autoregulatory maintenance of *Pet-1* expression.

DISCUSSION

In this study, we tested the idea that Pet-1, a key component of an embryonic transcriptional cascade that generates 5-HT neurons in the ventral hindbrain, continues to regulate subsequent milestones in 5-HT system maturation and 5-HT function in adulthood. We have shown that Pet-1 function is not restricted to the induction of serotonergic characteristics in embryonic 5-HT neuron precursors. Instead, ongoing Pet-1–directed transcription is required across the lifespan for multiple regulatory events that shape and maintain the serotonergic neurotransmitter system. Our findings also support the idea that the etiology of behavioral pathogenesis is not limited to dysfunction of the serotonergic system during development but may also result from adult-onset alterations in serotonergic transcription.

These and earlier findings[12] define three general but distinct stages of Pet-1 function. The initial stage occurs during serotonergic neurogenesis, when Pet-1 regulates a late phase of 5-HT neuron generation by coordinating the induction of key serotonergic genes that are required for transmitter synthesis, reuptake and vesicular transport in immature postmitotic precursors[12]. Here, we uncovered a second stage of *Pet-1* function using a conditional targeting approach that did not interfere with *Pet-1* expression until about 2 d after the completion of serotonergic neurogenesis. This transcriptional stage coincides with the prolonged period of 5-HT neuron maturation, during which these cells must negotiate complex axonal growth and pathfinding decisions and acquire their characteristic firing properties. We identified multiple requirements for Pet-1 at this second stage, which showed that Pet-1 is essential for proper 5-HT system maturation. For example, retrograde tracing of R26R-YFP-marked *Pet-1*–deficient 5-HT neurons revealed a substantial deficit in the number of serotonergic projections to the somatosensory cortex. However, initial serotonergic axon-like outgrowth did not appear to be compromised in *Pet-1*^{eCKO} mice, which suggests that Pet-1–dependent transcription regulates subsequent pathfinding decisions that help to build the ascending serotonergic system. The innervation defects in *Pet-1*^{eCKO} mice were probably not contributed to by the reduction in brain 5-HT. Although pharmacological disruption of embryonic 5-HT signaling alters neuronal organization in the presubicular cortex[33] and 5-HT regulates thalamocortical axon pathfinding by modulating axonal responsiveness to guidance cues[5], recent studies of *Tph2*-targeted mice, which lack 5-HT synthesis in the brain, did not find defects in serotonergic innervation patterns[34].

We also identified a special role for continued Pet-1–directed transcription, not shared by Gata3, in regulating the maturation of 5-HT neuron firing frequency through the control of 5-HT autoreceptor–mediated inhibitory responses. Similar to the innervation defects, the defects in firing frequencies and autoreceptor-mediated inhibitory responses were probably not caused simply by reduced 5-HT, as 5-HT is also reduced in the brains of *Gata3*^{eCKO} mice. Instead, our findings showed that Pet-1 transcriptionally controls spontaneous firing frequency and inhibitory responses by regulating expression of the *Htr1a* autoreceptor gene. In addition, *Pet-1* was required for expression of the presynaptic Htr1b autoreceptor. Because *Htr1a* and *Htr1b* are not normally expressed until several days after 5-HT neuron generation, our findings support the hypothesis that persistent Pet-1-directed transcription is essential for maturation steps during which 5-HT neurons acquire key functional characteristics.

We identified a third stage of Pet-1 function using a tamoxifen-inducible targeting approach that resulted in a severe and selective reduction in Pet-1 in the adult ascending 5-HT system. Significantly, the targeting of Pet-1 in adult 5-HT neurons revealed that this late stage of Pet-1 function is required in the adult ascending 5-HT system

to maintain emotional behaviors. Our finding that tamoxifen-treated *Pet-1*^{aCKO} mice showed altered emotional behavior was supported by three tests of rodent anxiety-related behavior performed on two independent cohorts of mice that were tested several months apart. The simplest interpretation of our findings is that the accompanying reduction in 5-HT levels in the ascending serotonergic system accounted for the elevated anxiety in tamoxifen-treated *Pet-1*^{aCKO} mice. However, the literature regarding the effect of neurotoxin-mediated depletion of 5-HT on anxiety in adult rats is conflicting, with both anxiogenic and anxiolytic effects being reported depending on experimental design[35–37]. Furthermore, as Pet-1 is likely to control a network of downstream transcriptional targets, the increased anxiety-like behavior in tamoxifen-treated *Pet-1*^{aCKO} mice may have resulted from multiple alterations in adult serotonergic function. We found a reduction in *Slc6a4* and *Slc17a8* gene expression in these mice, which suggests that there were complex changes in the 5-HT neuron genetic network. A recent study showed that at least part of the ascending 5-HT system engages in dual serotonergic/glutamatergic fast synaptic transmission[38]. Reduced expression of *Slc17a8* in tamoxifen-treated *Pet-1*^{aCKO} mice was not observed until 30 d after the tamoxifen treatments, indicating that adult loss of *Pet-1* expression could have elicited gradual changes in glutamatergic transmission that contributed to the behavioral phenotype of tamoxifen-treated *Pet-1*^{aCKO} mice.

The normal expression of the Htr1a autoreceptor and Htr1a-mediated inhibitory responses in tamoxifen-treated *Pet-1*^{aCKO} mice indicates that the increased anxiety-like behaviors that followed adult deletion of *Pet-1* were not due to deficiencies in Htr1a signaling. This result is consistent with the findings that although germline targeting of *Htr1a* leads to increased anxiety-related behaviors[39,40], reduced Htr1a signaling in adulthood does not[41]. These findings suggest that anxiety-like behavior in tamoxifen-treated *Pet-1*^{aCKO} mice may be caused by a different process than that responsible for increased anxiety in *Pet-1*$^{-/-}$ mice. Nevertheless, our findings provide the first direct evidence in support of the concept that adult 5-HT–modulated behaviors are not hardwired during development but are transcriptionally regulated in the adult brain. Moreover, they highlight the potential importance of perturbations in serotonergic transcription at any stage of life in emotional pathogenesis.

Several characteristics of the expression and function of Pet-1 suggest that it is a terminal selector gene analogous to the *Caenorhabditis elegans* ETS terminal selector gene, *ast-1*, which coordinates the induction and maintenance of dopamine synthesis and transport in postmitotic neurons through a common conserved terminal selector motif[42]. Consistent with the fundamental properties of a terminal selector gene, *Pet-1* is expressed throughout the life of postmitotic 5-HT neurons and is required not only to determine serotonergic identity but also to maintain it. However, like a terminal selector gene, it is not required for generic neuronal identity. Further key features of *Pet-1* that fit with its classification as a terminal selector gene are that it directly regulates and maintains expression of terminal differentiation genes that define serotonergic-type identity and positively autoregulates its own expression, all through conserved ETS binding motifs[43]. It remains to be determined whether Pet-1 induces other transcription factors that then function cooperatively in a feedforward loop to control serotonergic identity.

Our findings raise the question of why *Pet-1* is still needed in adult 5-HT neurons for regulation of a subset of its known embryonic targets but not for others such as *Ddc*, *Slc18a2* and *Htr1a*. Expression of *Tph2* and *Slc6a4* in the adult is restricted to Pet-1–expressing 5-HT neurons and is rate-limiting for the essential serotonergic functions of 5-HT synthesis and reuptake. The expression of *Tph2* and *Slc6a4* in the adult DRN are regulated by external stimuli such as selective serotonin reuptake inhibitors and different stress paradigms[44,45]. In addition, the expression of *Tph2* and *Slc6a4 in vivo* are sensitive to the levels of *Pet-1* expression[46]. We hypothesize (**Supplementary Fig. 1**) that environmentally induced alterations in the expression of *Tph2* and *Slc6a4* might be mediated through direct transcriptional activation by Pet-1, which itself is subject to extrinsic regulation[47], thereby providing an efficient homeostatic transcriptional mechanism that acts throughout life to alter serotonergic function in response to environmental challenges.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/.

Note: Supplementary information is available on the Nature Neuroscience website.

ACKNOWLEDGMENTS

We thank L. Landmesser for suggestions on retrograde tracing; S. Dymecki and P. Chambon for *CreER*^{T2} plasmids; Q. Ma for the *mycPet-1* vector; J. Zhu for the *Gata3*$^{loxP/loxP}$ mice; K. Lobur for assistance with genotyping of mice; J. Reeves for behavioral testing in the Case Rodent Behavior Core; and L. Landmesser, S. Maricich and J. Silver for comments on the manuscript. This work was supported by grants MH062723 and MH078028 to E.S.D. (US National Institutes of Health).

AUTHOR CONTRIBUTIONS

E.S.D. conceived the project. C.L. made the transgenic and targeting constructs, characterized all new mouse lines and generated all histological, RT-PCR and retrograde tracing data and images. C.L. and S.C.W. performed western blot analyses. C.L. and G.C. performed behavioral analyses. T.M. and S.H. generated the electrophysiology data. C.L., S.H., T.M., G.C., S.C.W. and E.S.D. analyzed the data. E.S.D. and C.L. designed the experiments and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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1. Holmes, A. Genetic variation in cortico-amygdala serotonin function and risk for stress-related disease. *Neurosci. Biobehav. Rev.* **32**, 1293–1314 (2008).
2. Ansorge, M.S., Hen, R. & Gingrich, J.A. Neurodevelopmental origins of depressive disorders. *Curr. Opin. Pharmacol.* **7**, 8–17 (2007).
3. Vitalis, T., Cases, O., Passemard, S., Callebert, J. & Parnavelas, J.G. Embryonic depletion of serotonin affects cortical development. *Eur. J. Neurosci.* **26**, 331–344 (2007).
4. Salichon, N. *et al.* Excessive activation of serotonin (5-HT) 1B receptors disrupts the formation of sensory maps in monoamine oxidase a and 5-ht transporter knock-out mice. *J. Neurosci.* **21**, 884–896 (2001).
5. Bonnin, A., Torii, M., Wang, L., Rakic, P. & Levitt, P. Serotonin modulates the response of embryonic thalamocortical axons to netrin-1. *Nat. Neurosci.* **10**, 588–597 (2007).
6. Ansorge, M.S., Zhou, M., Lira, A., Hen, R. & Gingrich, J.A. Early-life blockade of the 5-HT transporter alters emotional behavior in adult mice. *Science* **306**, 879–881 (2004).
7. Maciag, D. *et al.* Neonatal antidepressant exposure has lasting effects on behavior and serotonin circuitry. *Neuropsychopharmacology* **31**, 47–57 (2006).
8. Gross, C. *et al.* Serotonin1A receptor acts during development to establish normal anxiety-like behaviour in the adult. *Nature* **416**, 396–400 (2002).
9. Jans, L.A., Riedel, W.J., Markus, C.R. & Blokland, A. Serotonergic vulnerability and depression: assumptions, experimental evidence and implications. *Mol. Psychiatry* **12**, 522–543 (2007).
10. Scott, M.M. & Deneris, E.S. Making and breaking serotonin neurons and autism. *Int. J. Dev. Neurosci.* **23**, 277–285 (2005).
11. Cordes, S.P. Molecular genetics of the early development of hindbrain serotonergic neurons. *Clin. Genet.* **68**, 487–494 (2005).
12. Hendricks, T.J. *et al.* Pet-1 ETS gene plays a critical role in 5-HT neuron development and is required for normal anxiety-like and aggressive behavior. *Neuron* **37**, 233–247 (2003).
13. Dai, J.X. *et al.* Enhanced contextual fear memory in central serotonin-deficient mice. *Proc. Natl. Acad. Sci. USA* **105**, 11981–11986 (2008).
14. Hendricks, T., Francis, N., Fyodorov, D. & Deneris, E. The ETS domain factor Pet-1 is an early and precise marker of central 5-HT neurons and interacts with a conserved element in serotonergic genes. *J. Neurosci.* **19**, 10348–10356 (1999).

15. Scott, M.M. *et al.* A genetic approach to access serotonin neurons for *in vivo* and *in vitro* studies. *Proc. Natl. Acad. Sci. USA* **102**, 16472–16477 (2005).
16. Jacob, J. *et al.* Transcriptional repression coordinates the temporal switch from motor to serotonergic neurogenesis. *Nat. Neurosci.* **10**, 1433–1439 (2007).
17. Pattyn, A. *et al.* Coordinated temporal and spatial control of motor neuron and serotonergic neuron generation from a common pool of CNS progenitors. *Genes Dev.* **17**, 729–737 (2003).
18. Zhao, Z.Q. *et al.* Lmx1b is required for maintenance of central serotonergic neurons and mice lacking central serotonergic system exhibit normal locomotor activity. *J. Neurosci.* **26**, 12781–12788 (2006).
19. Krueger, K.C. & Deneris, E.S. Serotonergic transcription of human FEV reveals direct GATA factor interactions and fate of Pet-1-deficient serotonin neuron precursors. *J. Neurosci.* **28**, 12748–12758 (2008).
20. Lidov, H.G. & Molliver, M.E. An immunohistochemical study of serotonin neuron development in the rat: ascending pathways and terminal fields. *Brain Res. Bull.* **8**, 389–430 (1982).
21. Srinivas, S. *et al.* Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Dev. Biol.* **1**, 4 (2001).
22. Vertes, R.P., Fortin, W.J. & Crane, A.M. Projections of the median raphe nucleus in the rat. *J. Comp. Neurol.* **407**, 555–582 (1999).
23. Le Francois, B., Czesak, M., Steubl, D. & Albert, P.R. Transcriptional regulation at a HTR1A polymorphism associated with mental illness. *Neuropharmacology* **55**, 977–985 (2008).
24. Fornal, C.A. *et al.* Single-unit responses of serotonergic dorsal raphe neurons to 5–HT1A agonist and antagonist drug administration in behaving cats. *J. Pharmacol. Exp. Ther.* **270**, 1345–1358 (1994).
25. Bayliss, D.A., Li, Y.W. & Talley, E.M. Effects of serotonin on caudal raphe neurons: activation of an inwardly rectifying potassium conductance. *J. Neurophysiol.* **77**, 1349–1361 (1997).
26. Sari, Y. Serotonin1B receptors: from protein to physiological function and behavior. *Neurosci. Biobehav. Rev.* **28**, 565–582 (2004).
27. Bonnin, A., Peng, W., Hewlett, W. & Levitt, P. Expression mapping of 5-HT1 serotonin receptor subtypes during fetal and early postnatal mouse forebrain development. *Neuroscience* **141**, 781–794 (2006).
28. Craven, S.E. *et al.* Gata2 specifies serotonergic neurons downstream of sonic hedgehog. *Development* **131**, 1165–1173 (2004).
29. Zhu, J. *et al.* Conditional deletion of Gata3 shows its essential function in T(H)1-T(H)2 responses. *Nat. Immunol.* **5**, 1157–1165 (2004).
30. Feil, R., Wagner, J., Metzger, D. & Chambon, P. Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains. *Biochem. Biophys. Res. Commun.* **237**, 752–757 (1997).
31. Vogt, M.A. *et al.* Suitability of tamoxifen-induced mutagenesis for behavioral phenotyping. *Exp. Neurol.* **211**, 25–33 (2008).
32. Scott, M.M., Krueger, K.C. & Deneris, E.S. A differentially autoregulated Pet-1 enhancer region is a critical target of the transcriptional cascade that governs serotonin neuron development. *J. Neurosci.* **25**, 2628–2636 (2005).
33. Janusonis, S., Gluncic, V. & Rakic, P. Early serotonergic projections to Cajal-Retzius cells: relevance for cortical development. *J. Neurosci.* **24**, 1652–1659 (2004).
34. Gutknecht, L. *et al.* Deficiency of brain 5-HT synthesis but serotonergic neuron formation in Tph2 knockout mice. *J. Neural Transm.* **115**, 1127–1132 (2008).
35. Ludwig, V. & Schwarting, R.K. Neurochemical and behavioral consequences of striatal injection of 5,7-dihydroxytryptamine. *J. Neurosci. Methods* **162**, 108–118 (2007).
36. Pum, M.E., Huston, J.P. & Muller, C.P. The role of cortical serotonin in anxiety and locomotor activity in Wistar rats. *Behav. Neurosci.* **123**, 449–454 (2009).
37. Sommer, W. *et al.* Local 5,7-dihydroxytryptamine lesions of rat amygdala: release of punished drinking, unaffected plus-maze behavior and ethanol consumption. *Neuropsychopharmacology* **24**, 430–440 (2001).
38. Varga, V. *et al.* Fast synaptic subcortical control of hippocampal circuits. *Science* **326**, 449–453 (2009).
39. Heisler, L.K. *et al.* Elevated anxiety and antidepressant-like responses in serotonin 5–HT1A receptor mutant mice. *Proc. Natl. Acad. Sci. USA* **95**, 15049–15054 (1998).
40. Ramboz, S. *et al.* Serotonin receptor 1A knockout: an animal model of anxiety-related disorder. *Proc. Natl. Acad. Sci. USA* **95**, 14476–14481 (1998).
41. Richardson-Jones, J.W. *et al.* 5-HT1A autoreceptor levels determine vulnerability to stress and response to antidepressants. *Neuron* **65**, 40–52 (2010).
42. Flames, N. & Hobert, O. Gene regulatory logic of dopamine neuron differentiation. *Nature* **458**, 885–889 (2009).
43. Hobert, O. Regulatory logic of neuronal diversity: terminal selector genes and selector motifs. *Proc. Natl. Acad. Sci. USA* **105**, 20067–20071 (2008).
44. Gardner, K.L., Hale, M.W., Lightman, S.L., Plotsky, P.M. & Lowry, C.A. Adverse early life experience and social stress during adulthood interact to increase serotonin transporter mRNA expression. *Brain Res.* **1305**, 47–63 (2009).
45. Shishkina, G.T., Kalinina, T.S. & Dygalo, N.N. Up-regulation of tryptophan hydroxylase-2 mRNA in the rat brain by chronic fluoxetine treatment correlates with its antidepressant effect. *Neuroscience* **150**, 404–412 (2007).
46. Lerch-Haner, J.K., Frierson, D., Crawford, L.K., Beck, S.G. & Deneris, E.S. Serotonergic transcriptional programming determines maternal behavior and offspring survival. *Nat. Neurosci.* **11**, 1001–1003 (2008).
47. Rivera, H.M., Oberbeck, D.R., Kwon, B., Houpt, T.A. & Eckel, L.A. Estradiol increases Pet-1 and serotonin transporter mRNA in the midbrain raphe nuclei of ovariectomized rats. *Brain Res.* **1259**, 51–58 (2009).

ONLINE METHODS

Mice. Animal procedures used in this study were approved by the CWRU School of Medicine Institutional Animal Care in compliance with the National Institutes of Health guide for the care and use of laboratory animals.

***loxP*-flanked *Pet-1* mice.** An 11-kb genomic fragment that included *Pet-1* was subcloned into a targeting construct designed to insert *loxP* sites around exon 3. Several rounds of electroporation and G418 selection were performed on genetically modified R1 ES cells containing a protamine *Cre* transgene[48]. A total of 176 colonies were isolated and screened by Southern blot analysis using an NcoI digestion and a 5′ external probe. Nine positive clones were identified and rescreened using a KpnI digestion and 3′ external probe. The 5′ probe hybridized to an 8.4-kb fragment in wild-type DNA and an 11.2-kb fragment in targeted DNA. The 3′ probe hybridized to an 8.0-kb fragment in wild-type DNA and a 10.6-kb fragment in targeted DNA. Two clones, i5h and i7c, were chosen for blastocyst injection. All resulting chimeras showed germline transmission and were bred to mice of mixed 129Sv and C57BL/6 backgrounds. The F1 pups from male chimeras were screened for mice carrying either a *loxP*-flanked *Pet-1* allele or a conditionally deleted *Pet-1* allele using PCR genotyping with following primers. p1: 5′-ACTCTGGCTTCCCTTTCTCC-3′; p2: 5′-ACTTGGAGGCCTTTTGCTCT-3′; p3: 5′-TAGGAGGGTCTGGTGTCTGG-3′; p4: 5′-GCGTCCTTGTGTGTAGCAGA-3′; p6: 5′-ATGCAAGAAGTTTCGGATGG-3′ (**Supplementary Fig. 2**).

***ePet::CreER^{T2}*.** DNA sequences encoding a fusion protein of Cre recombinase with a mutated estrogen receptor (*CreER^{T2}*, a gift from P. Chambon via S. Dymecki) were first subcloned into the pSG5 vector (Stratagene) between the *β-globin* intron and the simian virus 40 polyadenylation sequences. The *β-globin-intron/CreER^{T2}/poly(A)* cassette was then released from the pSG5 vector and subcloned downstream of the *β-globin* minimal promoter in a modified BGZA vector in which *LacZ* was removed. The *β-globin promoter/β-globin-intron/CreER^{T2}/poly(A)* region was subcloned downstream of the 40-kb *ePet* genomic fragment in the modified pBACe3.6 vector[15]. The transgene was released from vector with an AscI digestion and purified for pronuclear injections into hybrid c57B6/129 zygotes. Founders were identified by PCR with 5′-AAAATTTGCCTGCATTACCG-3′ and 5′-ATTCTCCCACCGTCAGTACG-3′ primers.

***ePet::mycPet-1*.** DNA sequences encoding a myc-tagged Pet-1 protein (gift from Q. Ma) as well as a simian virus 40 polyadenylation region were first subcloned downstream of the *β-globin* minimal promoter in a modified BGZA vector. The *β-globin/Pet-1/poly(A)* cassette was then released and subcloned downstream of the *ePet* enhancer sequence in pBACe3.6. The transgene was released from vector with an AscI digestion and purified for pronuclear injections into *Pet-1$^{-/-}$* fertilized eggs in a mixed C57BL/6 and 129 background. Founders were identified by PCR with 5′-GGGCCTATCCAAACTCAACTT-3′ and 5′-GGGAGGTGTGGGAGGTTTT-3′primers.

Histology. Fluorescent and diaminobenzidine (DAB) immunohistochemistry were performed as described[46]. The following primary antibodies were used: rabbit anti-5-HT (1:10,000, ImmunoStar), mouse anti-TPH (1:200, Sigma), rabbit anti-GFP (1:1,000, Invitrogen), rabbit anti-Slc18a2 (1:200, Millipore), goat anti-CHAT (1:200, Millipore), chicken anti-TH (1:100, Aves), mouse anti-NeuN (1:500, Millipore), mouse anti-GFAP (1:200, Imgenex), rabbit anti-β-galactosidase (1:5,000, MP Biomedicals), rabbit anti-Cre (1:500, Covance), mouse anti-myc (9E10, Sigma). Secondary antibodies including FITC, TexRed and Cyanine3 (1:200) were from Jackson ImmunoResearch. Fluorescent and bright field images were collected using a SPOT RT color digital camera (Diagnostic Instruments) attached to an Olympus Optical BX51 microscope. Confocal images were taken on a Zeiss LSM 510 confocal laser microscope.

Retrograde tracing. Six wild-type (*ePet::Yfp+*) and six *Pet-1^{eCKO}* mice (~P22) were deeply anesthetized by 1.5% isoflurane in the air flow. Mice were placed into a stereotaxic frame and a small opening was made in the skull directly over the injection site (−0.5 mm, 3 mm, 0.5 mm from bregma). Coordinates for stereotaxic injections were obtained from the Paxinos mouse brain atlas. About 1 µl of Texas Red–conjugated dextran (5% diluted in 0.5 × PBS, 3,000 Mw, Invitrogen) was pressure injected into the somatosensory barrel cortex using a Hamilton syringe. After injection, animals were allowed to survive for another 3 d before being killed for histology.

***In situ* hybridization.** Gene-specific DNA oligonucleotide primers (**Supplementary Table 1**) were designed to amplify ~600-bp fragments using cDNA synthesized from adult DRN mRNA. Forward and reverse primers contained bacteriophage T7 or T3 promoter sequences at their 5′ ends so that PCR products could be directly used as templates to synthesize digoxigenin (Roche)-labeled sense and antisense riboprobes. *In situ* hybridization was performed as described[14].

Western blot analysis. Mice were killed 30 d after the last tamoxifen treatment. DRN tissue was dissected and homogenized in RIPA buffer containing 1× proteinase inhibitor (Sigma). Proteins were quantified using the BCA Protein Assay Kit (Pierce). Seven micrograms of each protein extract was separated by 10% SDS-PAGE (BioRad) and then transferred to a 0.45-µm nitrocellulose membrane (BioRad). The antibodies used were a monoclonal anti-TPH antibody (1:2,000, Sigma), an HRP-conjugated anti-mouse secondary antibody (1:2,000, Cell Signaling Technology), and an anti-β-actin antibody (1:3,000, Millipore). The film was developed and then scanned on a HP Scanjet 8200. The mean band density was measured using ImageJ (http://rsb.info.nih.gov/ij).

Tamoxifen preparation and treatment. Tamoxifen (Sigma) was dissolved in corn oil at 20 mg ml^{-1} according to the Joyner laboratory's protocol (http://www.mskcc.org/mskcc/html/77387.cfm). For tamoxifen treatment in the embryo, one single dose of tamoxifen (150 µg per g body weight) was given to the mother by intraperitoneal injection or oral gavage at E11.5. For treatment in adults, 5 single daily doses of TM (150 µg per g body weight) were given to adult mice by intraperitoneal injection.

Electrophysiology. Coronal slices including the the DRN (250 µm thick) were cut from brainstem of untreated *Pet-1^{eCKO}*, tamoxifen-treated *Pet-1$^{aCKO)}$* and control mice aged 3–5 weeks. Mice were anesthetized with isoflurane and decapitated. The brainstem was cooled and sliced in ice cold solution containing (in mM) 87, NaCl; 75, sucrose; 2.5, KCl; 0.5, $CaCl_2$; 7, $MgCl_2$; 1.25, NaH_2PO4; 25, $NaHCO_3$; and 20, glucose bubbled with 95% O_2 and 5% CO_2 using a vibratome (VT1000S, Leica). Slices were stored for at least 1 h at room temperature in recording artificial cerebrospinal fluid containing (in mM) 124, NaCl; 3, KCl; 2.5, $CaCl_2$; 1.2, $MgSO_4$; 1.23, NaH_2PO4; 26, $NaHCO_3$, and 10, glucose bubbled with 95% O_2 and 5% CO_2. YFP^+ cells were visually identified under an upright microscope (DMLFSA, Leica) equipped with a monochromator system (Polychrome IV, TILL Photonics). Whole-cell recordings were made from the cells in the dorsomedial subregion of the B7 DRN. During recordings, slices were continuously perfused with the external solution containing 10 µM 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX), 20 µM D-(−)-2-amino-5-phosphonopentanoic acid (D-AP5) and 20 µM picrotoxin at room temperature. Patch pipettes (2–4 MΩ) were filled with an internal solution with the following composition (in mM) 140, K-methylsulfate; 4, NaCl; 10, HEPES; 0.2, EGTA; 4, Mg-ATP; 0.3, Na-GTP and 10, Tris-phosphocreatine (pH 7.3, adjusted with KOH). Membrane currents or voltages were recorded with an EPC10/2 amplifier (HEKA). The signals were filtered at 3 kHz and digitized at 50 kHz. PatchMaster software (HEKA) was used for control of voltage and data acquisition. Off-line analysis was performed with Igor Pro software (Wavemetrics). The Htr1a agonist (±)-8-Hydroxy-2-dipropylaminotetralin hydrobromide (8-OH-DPAT) was purchased from Tocris and bath-applied to slices.

Quantitative real-time PCR. Mice were anesthetized with Avertin (0.5 g tribromoethanol per 39.5 ml H_20, 0.02 ml per g body weight) and killed by rapid decapitation. Brains were dissected and placed in RNase-free tissue culture plates. A sterile razor blade was used to cut a transverse section at bregma area −2.92 mm and then again at bregma area −5.46 mm to isolate the area containing the DRN and MRN. The tissue was placed immediately in Trizol (Invitrogen) and RNA was extracted according to the manufacturer's manual. Genomic DNA was removed by DNase I treatment (Roche) and 1 µg RNA was used for first-strand cDNA synthesis (Invitrogen). For real-time RT-qPCR, a SYBR green detection system (Molecular Probes), fluorescein calibration dye (Bio-Rad), Platinum Taq (Invitrogen), specific primers (**Supplementary Table 2**) and 2 µl of undiluted cDNA were used in 20-µl PCR reactions. Each reaction was performed in

doi:10.1038/nn.2623

triplicate. All real time RT-PCR reactions were performed in 40 cycles on the iCycler (Bio-Rad). Relative gene expression and statistics analysis were determined using the Relative Expression Software Tool (http://www.gene-quantification.de/rest-paper.html).

HPLC analysis. Tissues were collected as described[46]. HPLC analysis was performed by the Neurochemistry Core Lab at Vanderbilt University, Center for Molecular Neuroscience.

Sequence analysis. Three kilobases upstream of the predicted human and mouse *Tph2*, *Slc6a4* and *Pet-1* transcription start sites were compared using ECR browser tool (http://ecrbrowser.dcode.org/) as described[19]. The minimum criterion for significant sequence conservation was 70% identity over 100 bp. Gene annotation information was derived from NCBI (*Pet-1*, GeneID 260298; *Tph2*, GeneID 216343; *Slc6a4*, GeneID 15567). Predicted conserved Pet-1 consensus binding sites (GGAAR(T)) were identified using rVista 2.0 (http://rvista.dcode.org/).

ChIP assays. Hindbrain tissue from the mesencephalic flexure to the cervical flexure was removed from 56 E12.5 *ePet::mycPet-1* transgenic embryos and quickly frozen on dry ice. MycPet-1 occupancy of genomic regions was tested by GenPathway, Inc. using goat anti-Myc antibody (Abcam ab9132) and quantitative PCR (qPCR) according to their protocols. Binding was tested in triplicate for the negative control region (untranscribed genomic region *Untr17*) and regions in or near predicted Pet-1–binding sites. Data are expressed as fold enrichment for each sample relative to binding at *Untr17*. Differences in binding among regions were calculated using one-way ANOVA with Bonferroni's Multiple Comparison Test (Prism 5.0, GraphPad Software). Replication of the entire assay gave similar results. Sequences of primers used for qPCR and their positions relative to the predicted conserved Pet-1–binding sites for each of the test genes are shown below. Primers sequences are underlined. Pet-1-binding sites are shown in bold.

Tph2: ***TTTCC***TGTGGCTTTCTAAAGTTGGAAAAGTACAAATATAATC TTGTCTATGCCTGTCAAATTGCTGGGTCTGATCAGGTCATAGATGGA GAGCAATAAAATTGTATCAGAAGAGTATCAAAGGAATGATGGGCCTA TGGGCATTTC**ATTTCC**

Slc6a4: CCCCTTCTTTCCGCTCTATCTTGATTAGCTAGGTCAGCCTCAG GTGGTTGCTGGGGAGATTCCAGGCCTACTGTGGTGGACATCCGAAAC AAGAGATTCCCTGAGAGGGAGGGGTGTGGTAGCC**ATTTCC**TGGGCCT AAGAAGAAGCCCACAAGGAAGGGAGAGCTTCCTCTTCTGTCACGGTG TAAACAGAACACAGGCAGACAGACAGATGGCACCGAGAG**CTTCC**

Slc6a4 intron: CATCCTCAGTCCAGAAGAGAGAGCCCAGCTC**CTTCC**C TGTGCCCCGTCCCGGCAGTGAAATGAAGGTACAGCCT

Pet-1: ***GGAAA***CCAGGAAATCGAGGAGGGGATGGGTCTCTAGGGACC TAAAGAGAGTAGGAAAAAAGGAGGGAGAAGGCACGGGGGTGGGCAA AGATAAAGGGAGCCACGGCAGCGCGGTAGCGCGGCTGGGAGCGCAG CGACAGGCGAGAGGGAG**GGAAGCGGAAAT**

Behavioral tests. All tests were carried out in the Case Western Reserve University Rodent Behavior Core. Six-to-eight-week-old *Pet-1*^{aCKO} (*ePet::CreER*^{T2} *Pet-1*$^{loxP/-}$) and littermate controls (*Pet-1*$^{loxP/-}$) were treated with tamoxifen for 5 consecutive days. After the last tamoxifen injection, mice were rested for 4 weeks before testing with access to food and water *ad libitum*. All tests were performed during the light cycle between 10:30 a.m. and 6:00 p.m. Equipment was cleaned thoroughly with 70% ethanol between each test to remove odor cues. The elevated plus maze test was conducted first because of its sensitivity to prior experience. Individual tests were performed at least 48 h apart. The tester was blinded to group identification. Cohort 1 was tested in autumn and Cohort 2 in spring.

The elevated plus maze, equipped with infrared grid and video tracking system (Med Associates Inc.), was ~1 m high and consisted of two open and two closed arms forming a cross. Mice were placed in the center of the maze facing the open arm and their activity was recorded for 5 min. The total time spent in the open arms, closed arms and hub and the number of entries into each arm were measured. We did not observe differences in frequency of defecation, urination and head dips between control and tamoxifen-treated *Pet-1*^{aCKO} mice.

The light/dark box consists of two square dark gray chambers. The lit open chamber (20 × 20 cm) was illuminated with a 100-W light 40 cm above the chamber floor and the dark chamber (15 × 15 cm) was entirely enclosed with a solid black plastic top. Mice were placed in the open chamber, facing away from the dark side, and their exploration pattern was tracked for 5 min. Latency to cross over into the dark chamber and total duration in light were scored. We did not detect differences in the number of re-entries into the illuminated chamber between control and tamoxifen-treated *Pet-1*^{aCKO} mice.

The open field consisted of a 40 cm × 40 cm box in a dimly lit room. Using EthoVision XT 5.0 (Noldus), the area was digitally subdivided into a 20 cm × 20 cm center area and a peripheral area. The peripheral area was also divided into a middle (inner 10 cm) and an outer area (outer 10 cm) to determine thigmotaxic behavior. Animals were placed in the open field and allowed to explore the enclosure freely for 15 min. During this period locomotor parameters such as total distance moved, velocity, angular velocity and heading degrees were measured to determine basic locomotor activity and presence of stereotypies. Frequency and duration in the center, periphery and outer quadrants were collected to determine anxiety-like behavior. In addition, data were nested into 5-min bins and distance moved during each of these 3 periods was recorded to evaluate habituation differences across groups.

Statistics. All statistical measures on normally distributed data were done using either a two-tailed *t*-test between the control and mutant mice or one-way ANOVA with Bonferroni's multiple comparison test to compare means between all combinations of groups. Statistical analysis in the RT-qPCR experiment was carried out by using the pair-wise fixed reallocation randomization test (http://www.gene-quantification.de/rest-paper.html).

48. O'Gorman, S., Dagenais, N.A., Qian, M. & Marchuk, Y. Protamine-Cre recombinase transgenes efficiently recombine target sequences in the male germ line of mice, but not in embryonic stem cells. *Proc. Natl. Acad. Sci. USA* **94**, 14602–14607 (1997).