

Region-specific regulation of 5-HT_{1A} receptor expression by Pet-1-dependent mechanisms *in vivo*

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Abstract

Serotonin (5-hydroxytryptamine, 5-HT) neurotransmission is negatively regulated by 5-HT_{1A} autoreceptors on raphe neurons, and is implicated in mood disorders. Pet-1/FEV is an ETS transcription factor expressed exclusively in serotonergic neurons and is essential for serotonergic differentiation, although its regulation of 5-HT receptors has not yet been studied. Here, we show by electrophoretic mobility shift assay that recombinant human Pet-1/FEV binds directly to multiple Pet-1 elements of the human 5-HT_{1A} receptor promoter to enhance its transcriptional activity. In luciferase reporter assays, mutational analysis indicated that while several sites contribute, the Pet-1 site at –1406 bp had the greatest effect on 5-HT_{1A} promoter activity. To address the effect of Pet-1 on 5-HT_{1A} receptor regulation *in vivo*, we compared the expression of 5-HT_{1A} receptor RNA and protein in Pet-1 null and wild-type littermate mice. In the raphe nuclei of Pet-1^{–/–} mice tryptophan hydroxylase 2 (TPH2) RNA, and 5-HT and TPH immunostaining were greatly reduced, indicating a deficit in 5-HT production. Raphe 5-HT_{1A} RNA and protein levels were also reduced in Pet-1-deficient mice, consistent with an absence of Pet-1-mediated transcriptional enhancement of 5-HT_{1A} autoreceptors in serotonergic neurons. Interestingly, 5-HT_{1A} receptor expression was up-regulated in the hippocampus, but down-regulated in the striatum and cortex. These data indicate that, in addition to transcriptional regulation by Pet-1 in raphe neurons, 5-HT_{1A} receptor expression is regulated indirectly by alterations in 5-HT neurotransmission in a region-specific manner that together may contribute to the aggressive/anxiety phenotype observed in Pet-1 null mice.

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The authors declare no conflicts of interest.

Supporting information

Additional supporting information may be found in the online version of this article:

Figure S1. Pet-1 mRNA is expressed in multiple cell lines.

Figure S2. 5-HT_{1A} protein expression in prefrontal cortex and hippocampus.

Figure S3. GFAP expression in DRN.

Figure S4. NeuN expression in raphe nuclei.

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Keywords

depression; development; ETS transcription factor; serotonin; transcription; transgenic

The serotonin (5-hydroxytryptamine, 5-HT) system, which originates from neurons of the midbrain raphe nuclei and projects widely throughout the brain (Dahlstrom and Fuxe 1964), has been implicated in behavioural disorders, including major depression, anxiety and aggression (Mann 1999; Albert and Lemonde 2004; Gordon and Hen 2004; Lesch 2005; Akimova *et al.* 2009). Gene knockout mouse models have demonstrated contrasting roles for 5-HT_{1A} and 5-HT_{1B} receptors in anxiety and aggressive behaviours, with 5-HT_{1B} null mice having an aggressive phenotype and 5-HT_{1A} null mice having an anxious phenotype (Zhuang *et al.* 1999; Lesch 2005). Tissue-specific, conditional rescue of the 5-HT_{1A} receptor in the 5-HT_{1A}^{-/-} background and pharmacological studies have identified a critical timeframe of forebrain 5-HT_{1A} receptor expression during embryonic and early postnatal development to establish a normal anxiety phenotype in the adult (Gross *et al.* 2002; Lo Iacono and Gross 2008). In addition, the 5-HT_{1A} receptor is expressed pre-synaptically on serotonergic neurons where it functions as a negative regulator of neuronal activity and differentiation (Sotelo *et al.* 1990; Rumajogee *et al.* 2004). Thus, the developmental regulation of the 5-HT_{1A} receptor is a critical determinant of the activity of the serotonin system, as well as the behavioural phenotype in the adult.

In response to elevation of 5-HT using serotonin-selective reuptake blockers, the pre-synaptic 5-HT_{1A} autoreceptor appears to preferentially desensitize compared with post-synaptic 5-HT_{1A} receptors (Le Poul *et al.* 2000), and this correlates with disinhibition of raphe neurons and clinical improvement of depression in patients (Pineyro and Blier 1999). Similarly, in 5-HT transporter (5-HTT) null mice, a preferential down-regulation of 5-HT_{1A} RNA and protein was observed in the dorsal raphe nucleus compared with post-synaptic sites (Fabre *et al.* 2000; Li *et al.* 2000). These data suggest that adaptive changes in 5-HT_{1A} receptor expression are implicated in depression and response to antidepressants.

Several transcription factors have been implicated in the development of the serotonin system, including Pet-1/FEV [pheochromocytoma 12 ETS (E26 transformation-specific) factor/Fifth Ewing Variant], an ETS-domain factor that is exclusively localized in 5-HT neurons (Maurer *et al.* 2004; Iyo *et al.* 2005). Pet-1 is expressed approximately 1 day before 5-HT (E11 in the mouse) (Hendricks *et al.* 1999). Pet-1 null mice (Hendricks *et al.* 2003) have a severe deficit in the serotonin system, with a 70–80% reduction in 5-HT levels, and display an anxious-aggressive phenotype that is reminiscent of the combined phenotypes of 5-HT_{1A} and 5-HT_{1B} knockout mice (Zhuang *et al.* 1999; Lesch 2005). These mice further display reduced expression of a number of Pet-1 target serotonergic genes that contain consensus Pet-1 elements (Hendricks *et al.* 1999), including the serotonin transporter (5-HTT) and tryptophan hydroxylase (TPH); however, the expression of raphe-specific TPH2 or specific 5-HT receptors in these mice has not been examined.

The presence of a Pet-1 consensus DNA element identified in the 5-HT_{1A} receptor promoter suggests its potential role in regulation of pre-synaptic 5-HT_{1A} receptor expression (Hendricks *et al.* 1999). Pet-1 was shown to enhance transcription of a reporter containing

four copies of the 5-HT1A Pet-1 element, but the role of Pet-1 in the regulation of 5-HT1A gene transcription has not been addressed. In this study, we examined the role of Pet-1 (i) as a direct regulator of the 5-HT1A receptor gene and (ii) in the postnatal expression of 5-HT1A receptors *in vivo*. Our results demonstrate that Pet-1 acts by direct and indirect mechanisms to regulate the expression of pre- and post-synaptic 5-HT1A receptors *in vivo*, which could contribute to the mature affective phenotype of Pet-1-null mice.

Materials and methods

Cell culture

Human SK-N-AS neuroblastoma cells (from Dr Michael Bannon, Wayne State University, Detroit, MI, USA), human SK-N-SH neuroblastoma cells (from Dr Lakshmi Devi, New York University; Fricker *et al.* 2005) and human embryonic kidney cells (HEK293 cells) were cultured on polystyrene plates (Fisher, Ottawa, ON, Canada) in Dulbecco's Modified Eagle's Medium (Wisent, St. Bruno, QC, Canada), supplemented with 8% v/v heat inactivated foetal bovine serum at 37°C in 5% CO₂. Human retinoblastoma Y79 cells were cultured in suspension in RPMI-1640 medium supplemented with 1.5 g/L NaHCO₃, 10 mM HEPES, 1 mM sodium pyruvate, 20% foetal bovine serum and 4.5 g/L glucose (Wisent).

Nuclear fractionation

Nuclear extracts from HEK293 cells were obtained as described previously (Czesak *et al.* 2006). Successful nuclear fractionation was validated by Western blotting using monoclonal mouse antibodies raised against Histone H1 (1 : 2500; Upstate, Lake Placid, NY, USA) as a nuclear marker and against c-raf (1 : 1000; BD Biosciences, Mississauga, ON, Canada) as a cytosolic marker.

Plasmid constructs

Human Pet-1 (FEV) was PCR-amplified from human SK-N-AS cells and cloned into the *EcoRV* sites of pcDNA3.1 and the *KpnI* and *EcoRV* sites of pTriEx-4 and verified by DNA sequence analysis. The human 5-HT1A promoter pGL3-Basic luciferase reporter constructs were described previously (Lemondé *et al.* 2004). The -1517 wt 5-HT1A construct was mutated using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, Cedar Creek, TX, USA) at the core GGAA nucleotides of the Pet-1 consensus motif using complementary primers: 111–101: 5'-ggctctctgcatccctAGctccgaaactcccaggagaagg; 136–126: 5'-cgccgaagcagta agaactAGctgcttgggt ctctgc; 449–439: 5'-ggagagagggaaggaagCTaat agggagaggagggtc; 532–522: 5'-ggaagagg gagactgaaTCggaaggcagg tggggag; 931–921: 5'ccattcaggctccctatgctAGcttttctacatctctattgc; 1406–1396: 5'-catatgcaaaatattCGcatccctgaattgactagccacaaagc.

Transfection

HEK293 cells were split into 12-well plates 16 h prior to transfection with luciferase constructs and pCMV-β-gal as control for transfection efficiency. Transfection was done using Lipofectamine Plus (Invitrogen, Burlington, ON, Canada) and 0.5 μg DNA/well at a ratio of 1 : 1 : 1 DNA : lipofectamine : plus for 3 h in serum-free OptiMEM (Invitrogen). After 48 h, plates were rinsed with phosphate-buffered saline (PBS) and lysed with 200 μL

1× reporter lysis buffer at 4°C (Promega, Madison, WI, USA). Luciferase and β-galactosidase activity were assessed using L-MaxII and SpectroMax M2 (Molecular Devices, Woodbridge, ON, Canada), respectively.

Protein expression

Pet-1 : pTriEx-4 was transformed into BL21 (DE3) *Escherichia coli* and grown at 37°C to OD₆₀₀ of 0.6, and induced with addition of 1 mM isopropyl-β-D-thiogalactopyranoside (Wisent) for 3.5 h. Cells were harvested by centrifugation and Pet-1 protein was isolated under denaturing conditions following the QIAexpressionist protocol (Qiagen, Mississauga, ON, Canada) and using Ni-nitrilotriacetic acid beads to select for His-tagged proteins. Fractions with the highest expression were combined and dialysed against DNA binding buffer (20 mM HEPES, 0.2 mM EDTA, 0.2 mM EGTA, 100 mM KCl, 5% glycerol, pH 7.9) containing 2 mM dithiothreitol. Protein was quantified using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

Electrophoretic mobility shift assay (EMSA)

Oligonucleotides corresponding to known 5-HT1A Pet-1 binding elements (5-HT1A –137), were annealed and end-labelled with [α -³²P]deoxy-CTP using 2.5 U Klenow (New England Biolabs, Pickering, ON, Canada) and purified using a Sephadex G-50 column (GE HealthCare, Baie D'Urfe, QC, Canada). Bacterially expressed, purified human Pet-1 was pre-incubated with or without annealed competitor probes: polyomavirus enhancer activator 3 (PEA3) wt (5'-gggatccaggaagtga), PEA3 mut (5'-gggatccatcaagtga), E2F (5'-tatagtgtactctactattctgctc), 5-HT1A –111/101 (5'-ggtgcattcccttccctccgaaac), 5-HT1A –136/126 (ggagtaagaactcctgcttggg), 5-HT1A –450/440 (5'-ggggaaggaaggaatagggaga), 5-HT1A –532/522 (5'-ggaataaaggaagtgaggagg), 5-HT1A –931/921 (5'-ggtccatgatcttctttctca), 5-HT1A –1406/1396 (5'-gggcaaaatattccatccctga), 5-HTT –1154/-1144 (5'-gggaacgataggaagttagaac), 5-HTT –1172/-1162 (5'-gggaccgaaaggaaatagcagtg), some of which have been described previously (Hendricks *et al.* 1999; Rogaeva and Albert 2007). Binding was done in 25-μL reaction containing DNA binding buffer, 250 ng of herring sperm DNA (Roche, Laval, QC, Canada) for 30 min at 22°C, and ³²P-labelled 5-HT1A –137 probe (50 000 cpm/reaction) was added and incubated 20 min, followed by electrophoresis on a 5% native polyacrylamide gel at 4°C, vacuum-drying and exposure to film. Band intensity was quantified in Adobe Photoshop (Adobe Systems, San Jose, CA, USA), normalizing to the specific Pet-1 + ³²P-136/126 band (in %) to combine multiple experiments and to calculate SEM.

Animals

The University of Ottawa Animal Care Committee approved all animal care procedures, in accordance with requirements of the Canadian Council on Animal Care. Pet-1 null (B6.129-Pet1^{Tm Eds/SJL}) mice were generously provided by Dr Evan S. Deneris, Case Western Reserve University, Cleveland, OH, Canada (Hendricks *et al.* 2003) and were genotyped by PCR for Pet-1 using the following primers: (forward, wild-type) 5'-gcgacttgggggtcattatcac, (reverse) 5'-gcctgatgttcaaggaagacctcgg, (forward knockout) 5'-cgggtgatgtggaatgtgtcgg with products of (wild-type) 209 bp or 361 bp (knockout). Equal proportions of male and female adult knockout mice or wild-type littermates (8–10 weeks) were killed using sodium

pentobarbital (0.01 mL/g body wt, intraperitoneal; Somnitol; MTC Pharmaceuticals, Cambridge, ON, Canada), brains removed, dissected and extracted; dorsal raphe region was dissected as described (Czesak *et al.* 2007). For immunofluorescence, mice were perfused transcardially with 25 mL PBS followed by 50 mL 4% paraformaldehyde containing 0.2% picric acid in PBS, pH 6.9. Brains were removed and post-fixed for 90 min and then placed in 10% sucrose containing 0.01% sodium azide for storage at 4°C. Brains were rapidly frozen with dry ice and cryostat sections were cut at 10 µm and thaw mounted onto chrome alum gelatin-coated glass slides. Coronal brain slices were obtained according to the Mouse Brain Atlas (Paxinos and Franklin 2001) using the following coordinates: rostral raphe, which includes dorsal and median areas (Bregma -4.2 to -4.96 mm), hippocampus (Bregma 1.46 to -2.30 mm), and prefrontal cortex (Bregma 2.96-2.80 mm). Sections were allowed to air dry and then rinsed in PBS prior to incubation with antibodies.

Immunofluorescence

Slides were incubated overnight using the following antibodies: 1 : 50 rabbit anti-5-HT1A (custom made primary antibody designed to the i2 loop of the 5-HT1A receptor sequence; Cedarlane, Hornby, ON, Canada); 1 : 100 sheep anti-TPH antibody (Chemicon International, Temecula, CA, USA); 1 : 100 rat anti-5-HT antibody (Chemicon) or rabbit anti-5-HT (1 : 1000; Protos Biotech Corp, New York, NY, USA); 1 : 50 mouse anti-NeuN antibody (Chemicon); and 1 : 500 rabbit anti-glial acidic fibrillary protein antibody (DAKO Inc., Mississauga, ON, Canada), in PBS containing 0.3% Triton-X100 and incubated under gentle vibration for 3 h at 22°C (Jacobsen and Staines 2004). For the 5-HT1A primary antibody control, pre-immune rabbit serum 1 : 5000 (Cedarlane). Secondary antibody (goat anti-rabbit Alexa Fluor 488, 1 : 200, Alexa Fluor 488 donkey anti-rabbit IgG; Alexa Fluor 594 goat anti-rat IgG; anti-sheep Alexa Fluor 488, or Alexa Fluor 594 rabbit anti-mouse; Molecular Probes, Eugene, OR, USA) in PBS was incubated at 37°C for 45 min. Sections were examined using an Axiophot II microscope (Carl Zeiss Canada, Toronto, ON). Images were acquired using Northern Eclipse imaging software (EMPIX, Toronto, ON, Canada) and a Retiga Qimage CCD camera (QIMAGING, Burnaby, BC, Canada). Blind counts of positive-labelled cells were performed on images taken at 20× magnification using Adobe Photoshop CS3. A constant area and minimum-threshold of 15% brightness (compared with background) was defined for each image counted.

In situ hybridization

Plasmid containing a 417-bp sequence of the mouse 5-HT1A receptor cDNA from Dr Alexandre Bonnin (Bonnin *et al.* 2006) was linearized and transcribed to incorporate digoxigenin-UTP (Roche Diagnostics) to generate sense or anti-sense riboprobes using SP6 or T7 polymerases, respectively (Jacobsen *et al.* 2008). Tissue sections were incubated overnight with riboprobes (1 : 1000) in hybridization buffer (150 mM NaCl, 15 mM sodium citrate, pH 7.0 (SSC), 50% deionized formamide, 10% dextran sulphate, 1 mg/mL rRNA, 1× Denhardt's solution). Slides were washed three times for 30 min each, followed by two washes, 30 min each with 1× MABT (100 mM maleic acid, 150 mM NaCl, pH 7.5, 0.1% Tween-20). Slides were then blocked for 1 h at 22°C in blocking reagent and followed by an overnight incubation with 1 : 1000 anti-digoxigenin antibody (Roche). The following day slides were washed five times for 20 min with MABT, equilibrated with pre-staining buffer

(100 mM NaCl, 50 mM MgCl₂, 100 mM Tris pH 9.5, 0.1% Tween- 20) three times for 10 min at 22°C, developed using staining solution (100 mM NaCl, 50 mM MgCl₂, 100 mM Tris pH 9.5, 0.1% Tween-20, 10% polyvinyl alcohol) containing 3.5 µL/mL of 5-bromo-4-chloro-3-indolyl-phosphate (Roche) and 4.5 µL/mL of 4-nitro blue tetrazolium chloride (Roche) for 2–24 h at 22°C and terminated by rinsing in PBS. Sections were examined using a Zeiss Axiophot II light microscope at 10× magnification. Image data were acquired using Northern Eclipse imaging software and an Axiovert S100 Zeiss microscope (EMPIX).

RNA isolation

Total RNA was extracted by trituration in 800 µL TRIzol (Invitrogen). For RT-PCR, 800 µL TRIzol (Invitrogen) was added directly to cells pre-rinsed in PBS. Chloroform (160 µL) was added and centrifuged for 25 min at 4°C and RNA precipitated using isopropanol. RNA was treated with 1 µL TURBO DNase (Ambion, Austin, TX, USA) to remove genomic DNA in 50 µL, for 30 min at 37°C, terminated using 5 µL DNase Inactivation Reagent and converted to cDNA using the Cells-to-cDNA™ II kit (Ambion). Reverse Transcriptase was omitted in -RT control.

RT-qPCR

Real-time PCR of cDNA prepared as described above was done using a Rotor-Gene RG-3000 (Corbett research, Sydney, Australia) and with TaqMan Gene Expression Assay using fluorophore labelled probes designed to discriminate between genomic DNA and cDNA (Applied Biosystems, Foster City, CA, USA): GAPDH (Mm99999915_g1), Htr1a (Mm00434106_s1) and TPH2 (Mm00557717_m1). A standard was included for comparison with standard curves, with GAPDH as an internal control; 1.5 µL of cDNA was added to 0.5 µL 20× specific probe, 5 µL 2× TaqMan Universal PCR Master Mix and 3 µL H₂O, to make up a final volume 10 µL. For each probe, no-template controls and -RT controls demonstrated no amplification. The cycling programme was: 95°C, 10 min; 40 cycles of 95°C, 15 s; 60°C, 45 s. Data were analysed using Rotor Gene v6.0 software. Data were analysed by two-tailed, paired Students *t*-test, where **p* < 0.05, ***p* < 0.005, ****p* < 0.0005.

Western blot

Tissue was homogenized on ice in 50 µL of IP Buffer (25 mM Tris pH 7.4, 150 mM NaCl, 1 mM CaCl₂, 1% Triton X-100) containing phenylmethylsulfonyl fluoride (Sigma, Oakville, ON, Canada), Protease Inhibitors (Roche, Basel, Switzerland) and dithiothreitol (Fisher). Western blot analysis was performed (Banihashemi and Albert 2002) using (1 : 4000) anti-5-HT1A (Millipore, Mississauga, ON, Canada), and developed using BM Chemiluminescence Blotting Substrate (Roche). The membranes were re-probed with anti-β-actin (1 : 1000) to control for loading. Band intensity of four independent experiments was quantified using Adobe Photoshop (Adobe Systems).

Statistical analyses

One-way ANOVA was performed for luciferase experiments using the Newman-Keuls Multiple Comparison *post hoc* test. Comparison between +/+ and -/- samples was by two-tailed unpaired *t*-test.

Results

Pet-1 regulates 5-HT1A receptor transcription through multiple sites

Based on the consensus Pet-1 binding element (Hendricks *et al.* 1999), we identified six putative Pet-1 elements within the first 1.5 Kb of the human 5-HT1A promoter (Table 1), several of which were highly conserved between human, rat and mouse 5-HT1A promoters (Table 2). To address Pet-1 binding to these elements, EMSA was done using recombinant human Pet-1 incubated with radio-labelled human 5-HT1A element 1A-136/126 and competing with excess unlabelled primers (Fig. 1). A major Pet-1/DNA complex (arrow) was identified that was competed by primers designed against the related PEA3 element, 5-HTT Pet-1 elements or unlabelled 1A –136/126 primers, but not by non-specific competitors (PEA3 mut or E2F). The other putative 5-HT1A Pet-1 elements all competed, but more weakly than the 1A –136/126 site. In the presence of excess competitor primers, a low mobility species (Fig. 1, asterisk) was observed that may represent a low affinity dimeric Pet-1 complex, as observed for other ETS proteins (Lee *et al.* 2005; Green *et al.* 2010). We next isolated nuclear extract from HEK293 cells, which express endogenous Pet-1 (Fig. S1), for use in EMSA experiments (Fig. 2). In the presence of labelled 1A-136/126 and HEK293 nuclear extract, a single band was observed (arrowhead). This experiment revealed a strong competition by all five putative Pet-1 elements, with 1A-449/439 and 1A-533/522 competing better than the 1A-136/126 element. Again, a low mobility complex was observed with excess competitors (Fig. 2, asterisk, and data not shown). Together, these data indicate that like recombinant Pet-1, a Pet-1-like nuclear component binds to multiple Pet-1 response elements in the 5-HT1A promoter, suggesting a role for Pet-1 in the regulation of 5-HT1A receptor transcription.

We screened for a Pet-1-deficient cell line for use in expression studies, but Pet-1 RNA was detected in several human cell lines, including HEK293 cells, retinoblastoma Y79, neuroblastoma SK-N-AS and SK-N-SH cells (Fig. S1, data not shown). The presence of Pet-1 mRNA suggests that endogenous Pet-1 protein is expressed in these cells and may contribute to basal transcription of 5-HT1A reporter constructs. To determine the role of Pet-1 sites of the 5-HT1A receptor gene, HEK293 cells were co-transfected with human Pet-1 expression plasmid or vector, and a series of human 5-HT1A promoter-luciferase reporter constructs (Fig. 3a) and luciferase activity was measured (Fig. 3b). All constructs except the –139 construct displayed significantly increased luciferase activity relative to the pGL3-Basic vector. The shorter constructs (–139, –224 and –391), containing Pet-1 binding elements 1A-111/101 and 1A-136/126, displayed modest but significant activation by Pet-1. In contrast to the –1128 construct which showed negligible activation, the greatest activation was obtained with the longest 5-HT1A promoter construct (–1517), which contains an additional Pet-1 element at –1406 bp. The low level of activation by transfected Pet-1 may reflect elevated basal activity of these constructs because of the presence of endogenous Pet-1 expression in these cells (Fig. S1). These data suggest a role for several Pet-1 response elements in Pet-1-mediated activation of the 5-HT1A promoter.

To address the role of specific Pet-1 elements to enhance 5-HT1A transcription, the GGAA core sequence of each putative Pet-1 element (Table 2) was mutated in the –1517 5-HT1A

promoter construct and co-transfected with the Pet-1 construct in HEK293 cells (Fig. 3c). Each mutant construct displayed substantially reduced reporter activity relative to the non-mutated 5-HT1A promoter construct, indicating that each Pet-1 element contributes to Pet-1 mediated 5-HT1A activation. Strikingly, mutation of the -1406 bp Pet-1 site almost completely abolished transcriptional activity of the -1517 5-HT1A promoter construct, suggesting its importance for both endogenous and transfected Pet-1 action in 5-HT1A transcription.

Pet-1 is critical for 5-HT1A receptor and TPH2 expression *in vivo*

We addressed whether Pet-1 regulates the expression of 5-HT1A receptors *in vivo*, using Pet-1 null mice as a model system. Pet-1 null mice display a 70–80% reduction in serotonin levels as well as a disruption in serotonergic gene expression (Hendricks *et al.* 2003). The level of 5-HT1A receptor RNA in the raphe region was assessed by *in situ* hybridization and was greatly reduced in the dorsal raphe nucleus of Pet-1 knockout compared with wild-type mice (Fig. 4a), consistent with the loss Pet-1 enhancer activity. The level of TPH and 5-HT1A receptor protein in these mice was examined by immunofluorescent staining (Fig. 4b). Compared with wild-type mice, Pet-1 null mice displayed strongly reduced levels of TPH and 5-HT1A immunoreactivity in the dorsal raphe region. Pet-1 knockout mice also displayed significant reductions in 5-HT staining (data not shown) and 5-HT1A-immunoreactive cells in the dorsal and median raphe nuclei, although this reduction was greater in the rostral than caudal portion (Fig. 4c). The smaller reduction in 5-HT1A-positive cells in the dorsal raphe nucleus (Fig. 4c) may represent the presence of 5-HT1A receptors in non-serotonergic cells that become up-regulated because of low 5-HT levels. In contrast, in the hippocampal dentate gyrus and prefrontal cortex, 5-HT1A receptor immunostaining and cell counts were similar in wild-type and Pet-1 knockout brains (Fig. S2). In contrast, there was no change in a glial marker (glial fibrillary acidic protein, Fig. S3), but a 25% reduction in NeuN-positive cells was observed in Pet-1^{-/-} dorsal raphe nucleus (Fig. S4), which may reflect a partial loss of 5-HT neurons. Thus, the loss of Pet-1 leads to a reduction in 5-HT1A receptor expression in the raphe nuclei.

To quantify changes in 5-HT1A receptor expression, brain tissue was analysed using RT-qPCR (Fig. 5a and b) or Western blot analysis (Fig. 5c). In Pet-1^{-/-} cortex, 5-HT1A RNA levels were reduced compared with wild-type controls; while a reduction in 5-HT1A protein was detected in striatum. In contrast, in Pet-1^{-/-} hippocampus, 5-HT1A protein but not mRNA was elevated (Fig. 5c). The discrepancy between the changes observed in cortex and hippocampus by Western blot compared with 5-HT1A immunofluorescence (Fig. 4) could be owing to analysis of whole tissues using RT-qPCR and Western blot, or the small magnitude of changes and the non-quantitative nature of staining. In the dorsal raphe region (Fig. 4c), TPH2 RNA levels were nearly abolished, consistent with the observed loss of TPH protein in Pet-1^{-/-} raphe (Fig. 4b; Hendricks *et al.* 2003). Similarly, raphe 5-HT1A RNA levels were reduced by 40%, consistent with but less striking than the reduction observed by *in situ* hybridization (Fig. 4a). Up-regulation of 5-HT1A receptors in non-serotonergic neurons in the midbrain could account for the smaller effects observed when whole midbrain tissue was analysed by Western blot or RT-qPCR. Taken together, these data suggest that while Pet-1 may act as a transcriptional enhancer of 5-HT1A receptor activity in the raphe

serotonergic neurons, additional compensatory mechanisms that could involve the serotonergic deficit in Pet-1^{-/-} mice and lead to region-specific alterations in post-synaptic 5-HT_{1A} receptor expression.

Discussion

Transcriptional regulation of the 5-HT_{1A} receptor gene by Pet-1

Our data illustrate a role for Pet-1 as an enhancer of 5-HT_{1A} transcription that binds to multiple Pet-1 elements in the 5-HT_{1A} promoter to enhance its transcription (Figs 1–3). In EMSA experiments performed with recombinant human Pet-1, the 5-HT_{1A} –136/126 element displayed the greatest binding efficacy (Fig. 1); however, in HEK293 nuclear extracts, all of the Pet-1 elements displayed similar binding efficacy (Fig. 2). While HEK293 cells express Pet-1, other ETS transcription factors or co-regulators may be present in this cell line to influence the binding efficiency. The enhancement provided by co-transfection of Pet-1 with 5-HT_{1A} promoter constructs (Fig. 3) suggests a direct role for Pet-1 in transcriptional regulation of the 5-HT_{1A} receptor. The reduced promoter activity upon mutation of any one of the Pet-1 elements suggests that they all contribute to transcriptional regulation of the 5-HT_{1A} receptor. The nearly complete loss of transcriptional activity upon mutation of the –1406 Pet-1 site indicates its predominant role in basal and Pet-1 mediated transcription of the 5-HT_{1A} promoter. Since Pet-1 is expressed in the brain exclusively in 5-HT neurons (Hendricks *et al.* 1999), these data suggest that Pet-1 is crucial for the basal expression of the 5-HT_{1A} autoreceptor expression. These findings demonstrate for the first time the activity of human Pet-1 to enhance transcription of a human gene target and to validate the role of Pet-1 in transcriptional regulation of the human 5-HT_{1A} receptor promoter. The human Pet-1 homologue (FEV) is exclusively expressed in serotonergic neurons of the raphe nuclei (Maurer *et al.* 2004; Iyo *et al.* 2005), hence Pet-1 is likely to regulate pre-synaptic 5-HT_{1A} receptor expression in humans.

Regulation of TPH2 and 5-HT_{1A} receptor expression by Pet-1 *in vivo*

To address the impact of Pet-1 on 5-HT_{1A} receptor expression *in vivo*, we compared the 5-HT_{1A} expression in Pet-1 knockout and wild-type brains. The Pet-1 null mice displayed a marked reduction in 5-HT immunoreactive cell bodies in all raphe nuclei examined (Fig., data not shown), reduced dorsal raphe 5-HT levels, and almost undetectable levels of TPH2 RNA (Fig. 5b). This result provides evidence suggesting that the TPH2 gene specifically is regulated by Pet-1, although the presence and identification of functional Pet-1 sites on the TPH2 gene remains to be elucidated. Previous studies have shown that the proximal TPH2 promoter is strongly repressed by RE-1 silencer of transcription/neural restrictive silencing factor, and activated by a cell-specific and calcium-dependent mechanism (Lenicov *et al.* 2007; Patel *et al.* 2007). Additionally, Lmx1b appears to coordinate with Pet-1 to regulate TPH2 expression (Zhao *et al.* 2006). The presence of detectable 5-HT levels in Pet-1^{-/-} mice, despite the loss of TPH2 RNA, suggests that sufficient serotonin re-uptake activity is present to capture serotonin derived from pineal gland or serotonin entry via circumventricular organs.

The loss of Pet-1 reduced 5-HT_{1A} RNA and protein levels in the dorsal and median raphe nuclei (Fig. 4), and this was confirmed by quantification of 5-HT_{1A} mRNA from the dorsal raphe region (Fig. 5b), which contains the majority of the serotonergic raphe nuclei (Dahlstrom and Fuxe 1964). Since 5-HT-positive cells express Pet-1, the reduction in detectable 5-HT- and 5-HT_{1A}-positive cells raphe nuclei indicates that 5-HT_{1A} autoreceptor expression *in vivo* is strongly regulated by Pet-1. The remaining 5-HT_{1A} receptor expression in the Pet-1-deficient raphe region may be because of compensatory up-regulation in response to low 5-HT levels. In contrast, in 5-HTT knockout mice, which display a persistent increase in synaptic 5-HT (Schmitt *et al.* 2007), 5-HT_{1A} receptor binding sites and mRNA levels were significantly decreased in the dorsal raphe (Fabre *et al.* 2000; Li *et al.* 2000). Consistent with this, the 5-HTT Long Polymorphic Repeat short allele, which reduces 5-HTT expression, has been associated with a reduction in 5-HT_{1A} receptor levels in humans (David *et al.* 2005). Despite the complete loss of Pet-1, a fraction of 5-HT neurons are still able to differentiate (Hendricks *et al.* 2003). Thus, while Pet-1 appears required for 5-HT_{1A} autoreceptor expression, compensatory mechanisms that permit serotonergic differentiation observed in a fraction of cells may account for low levels of 5-HT_{1A} receptors observed in the Pet-1^{-/-} mice.

Consistent with our findings, a substantial reduction in 5-HT_{1A} receptor mRNA levels was recently reported in the dorsal raphe nuclei of mice in which Pet-1 gene was disrupted at the onset of 5-HT differentiation (E12.5) (Liu *et al.* 2010). Importantly, whole cell recordings of Pet-1 promoter-driven yellow fluorescent protein-labelled raphe neurons demonstrate that 5-HT_{1A} autoreceptor function is almost completely impaired in these conditional Pet-1-null mice. These results clearly indicate a major desensitization of functional 5-HT_{1A} autoreceptors, consistent with the strong reduction in 5-HT_{1A} RNA that we observe. Hence, the residual 5-HT_{1A} protein in Pet-1^{-/-} midbrain is likely because of up-regulation of 5-HT_{1A} expression in non-serotonergic cells.

In target regions for 5-HT neurons, which receive a substantially reduced input of functional 5-HT projections in Pet-1 null mice (Hendricks *et al.* 2003), several alterations in 5-HT_{1A} RNA and protein were observed. These changes were not obvious in immunofluorescence, where 5-HT_{1A}-positive cell counts in dentate gyrus were not altered. Western blot measured an increase in whole hippocampal 5-HT_{1A} protein, suggesting that 5-HT_{1A} protein/cell could be increased, which would not be detectable by immunofluorescence. Lesion of 5-HT projections to the hippocampus using the 5-HT neurotoxin, 5,7-dihydroxytryptamine, results in increased levels of 5-HT_{1A} protein in the hippocampus (Patel *et al.* 1996), and thus the lack of 5-HT in the Pet-1 knockout mice may also result in up-regulation of these post-synaptic 5-HT_{1A} receptors. In agreement with this, acute treatment with 5-HT_{1A} antagonist WAY100,635 was also shown to up-regulate 5-HT_{1A} receptor number in cortex and hippocampus (Abbas *et al.* 2007). The reduction in 5-HT_{1A} RNA levels in the cortex agrees with pharmacological studies demonstrating that reduced 5-HT signalling delays expression of cortical 5-HT type-1 receptors (Whitaker-Azmitia *et al.* 1996; Lauder *et al.* 2000). The increase of 5-HT_{1A} receptor expression in the hippocampus versus a decrease in striatum of Pet-1 knockout mice, suggests that reduced serotonergic signalling may alter receptor expression in a site-specific manner.

Adaptive regulation of 5-HT_{1A} receptor expression and behaviour in Pet-1 null mice

Pet-1 null mice display a heightened anxiety/aggressivity phenotype (Hendricks *et al.* 2003). Interestingly, 5-HT_{1A} receptor knockout studies have demonstrated that early developmental expression of the forebrain 5-HT_{1A} receptor is critical for establishing normal anxiety-like behaviour (Gross *et al.* 2002; Lo Iacono and Gross 2008). The alterations in hippocampal and cortical 5-HT_{1A} receptors observed in Pet-1^{-/-} mice may contribute to the anxiety phenotype of these mice. On the other hand, 5-HT_{1B} receptor knockout mice display an aggressive phenotype with reduced anxiety (Saudou *et al.* 1994; Zhuang *et al.* 1999). In addition to 5-HT_{1A} receptors, several other 5-HT receptors (5-HT_{1B}, 5-HT_{2A}, 5-HT_{2C}, 5-HT₄ and others) have been implicated in affective disorders and in the therapeutic actions of antidepressants (Schechter *et al.* 2005; Weisstaub *et al.* 2006; Lucas *et al.* 2007), and may be altered in Pet-1^{-/-} mice. In stress-sensitive monkeys, FEV (Pet-1) is down-regulated and can be restored by antidepressant treatment, suggesting a role for Pet-1 expression in depression-related phenotypes in primates (Lima *et al.* 2009). It is as of yet unclear whether there is compensation by any other neurotransmitter system in Pet-1 null mice. Our preliminary data indicate an increase in tyrosine hydroxylase-positive projections (largely noradrenergic) in certain brainstem regions of Pet-1 knockout mice, such as the dorsal raphe (data not shown). The implications of this finding have yet to be elucidated, but suggest that additional adaptive changes may contribute to the behavioural phenotype of the Pet-1 null mice.

Taken together, the above data demonstrate that Pet-1 can activate 5-HT_{1A} receptor expression through multiple Pet-1 response elements in the 5-HT_{1A} promoter, and indicate that Pet-1 is critical for 5-HT_{1A} autoreceptor expression in the raphe nuclei. The Pet-1 null mice illustrate the critical role of Pet-1 in the development of serotonin neurons, and their subsequent role as a differentiation signal for target cells. The Pet-1-dependent cascade that regulates 5-HT neuron differentiation and 5-HT_{1A} autoreceptor expression, also indirectly affects 5-HT_{1A} heteroreceptor expression in 5-HT-innervated target regions, such as cortex or hippocampus. The combination of altered pre- and post-synaptic receptor expression has inextricable implications for the role of this gene in the development of affective behaviours.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

5-HT

5-hydroxytryptamine

| | |
|---------------------|--|
| 5-HTT | 5-hydroxy-tryptamine transporter |
| EMSA | electrophoretic mobility shift assay |
| HEK293 cells | human embryonic kidney cells |
| PBS | phosphate-buffered saline |
| PEA3 | polyomavirus enhancer activator 3 |
| Pet-1/FEV | pheochromocytoma 12 ETS (E26 transformation-specific) factor/Fifth Ewing Variant |
| TPH2 | tryptophan hydroxylase 2 |

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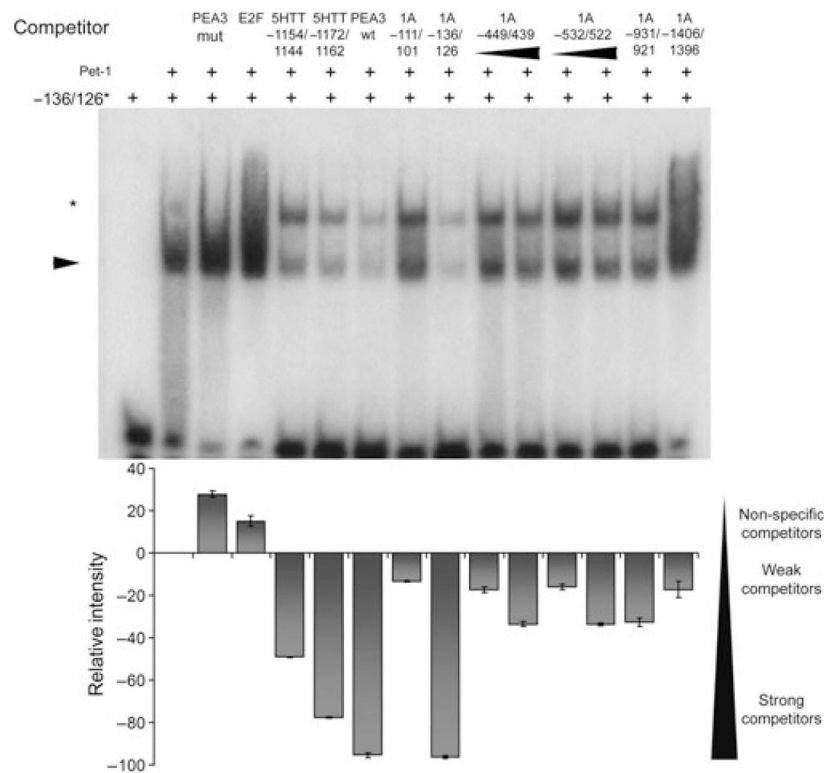


Fig. 1. Human Pet-1 directly binds multiple Pet-1 elements in the human 5-HT1A promoter. Electrophoretic mobility shift assay (EMSA) experiment showing Pet-1 binding to the previously identified Pet-1 element in the 5-HT1A promoter (-136/126, arrow) and subsequent competition by known specific probes (5-HTT -1154/1144, 5-HTT -1172/1162, PEA3 wt, 1A -136/126) but not of known non-specific probes (PEA3 mut, E2F). Putative 5-HT1A Pet-1 elements (1A -111/101, -450/440, -532/522, -931/921 and -1406/1396) were also able to compete for Pet-1 binding, although to a smaller extent than the 5-HT1A -136/126 itself. In all cases, 200× molar excess competitor was used, and where a concentration range was tested, the lower concentration of competitor was 100× molar excess. Intensity of the specific Pet-1 band (arrow) is quantified below each lane and expressed as intensity relative to the baseline Pet-1 interaction with the radiolabelled 1A -136/126 element. (*) A low mobility complex was observed in the presence of excess specific competitors. Error bars represent SEM for band intensity quantified for four independent experiments.

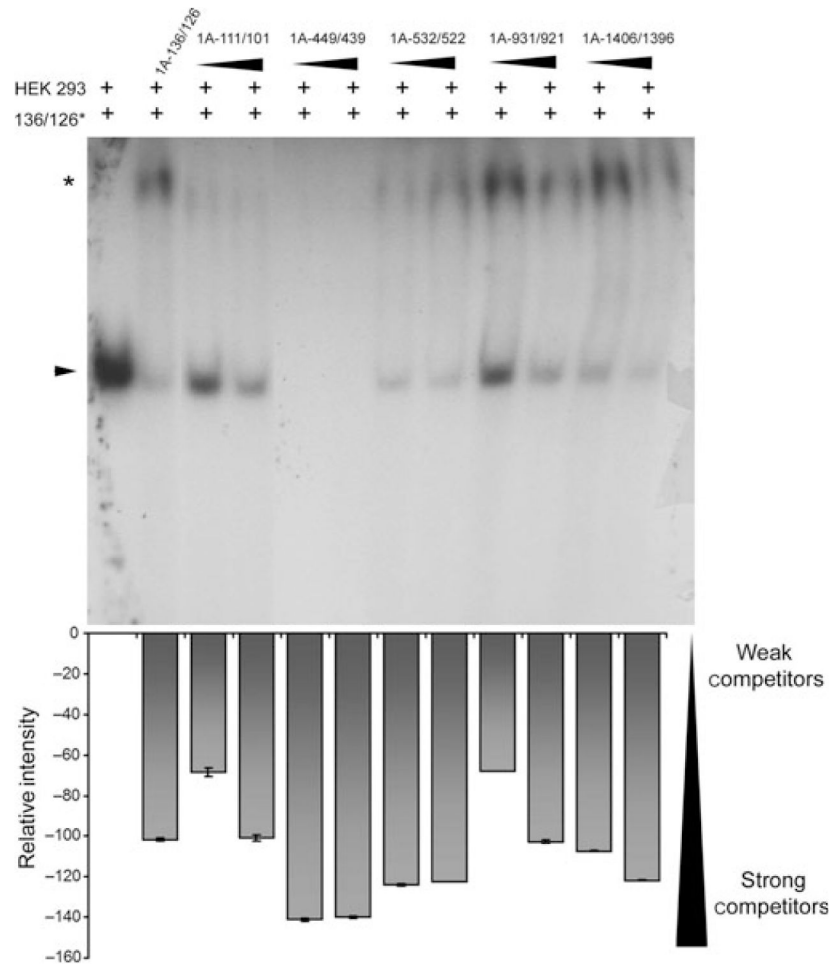


Fig. 2. Specific binding of human embryonic kidney cells (HEK293) nuclear protein and 5-HT1A promoter Pet-1 elements. Electrophoretic mobility shift assay (EMSA) experiment showing complex formed between nuclear protein isolated from HEK293 cells and previously identified Pet-1 element in the 5-HT1A promoter (–136/126, arrow) and competition by putative 5-HT1A promoter Pet-1 elements (1A –111/101, –450/440, –532/522, –931/921 and –1406/1396). In all cases, a concentration range of 100× to 200× molar excess competitor was used, with 200× excess unlabelled 1A-136/126. Intensity of the specific Pet-1 band (arrow) is quantified below each lane and expressed as intensity relative to the baseline Pet-1 interaction with the radiolabelled 1A –136/126 element. (*) A weak low mobility complex was observed in the presence of excess specific, but not non-specific, competitors. Error bars represent SEM of band intensity quantified for four independent experiments.

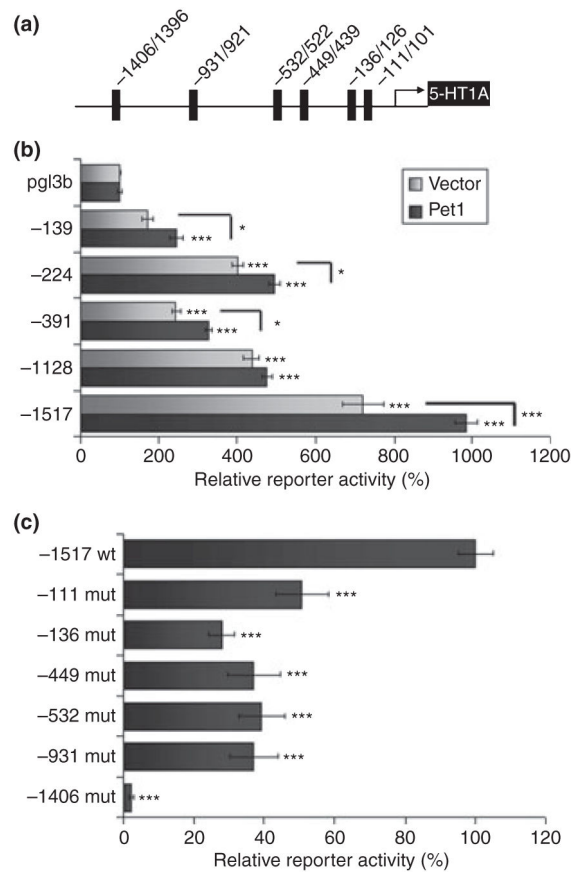


Fig. 3. Human Pet-1 activates transcription through multiple Pet-1 elements in the human 5-HT1A promoter. (a) Map showing position of Pet-1 elements in the 5-HT1A promoter. (b) Transcriptional activity of Pet-1 on 5-HT1A promoter constructs containing increasing lengths of promoter sequence in human embryonic kidney cells (HEK293 cells). Pet-1 activation is strongest for the longest construct that contains all of the putative Pet-1 elements (-1517). Luciferase activity was normalized to β -galactosidase activity and expressed as percent of pGL3B vector (100%). (c) Co-transfection of Pet-1 with wild-type and mutated -1517 bp 5-HT1A promoter reporter constructs. Pet-1-mediated transcriptional activation of the 5-HT1A promoter construct is significantly reduced when any of the putative Pet-1 binding elements is mutated. Luciferase/ β -galactosidase activity ratio is expressed as percent of activity of the non-mutated -1517 construct. Error bars represent SEM. *** $p < 0.0005$, ** $p < 0.005$, * $p < 0.05$; $N = 4$.

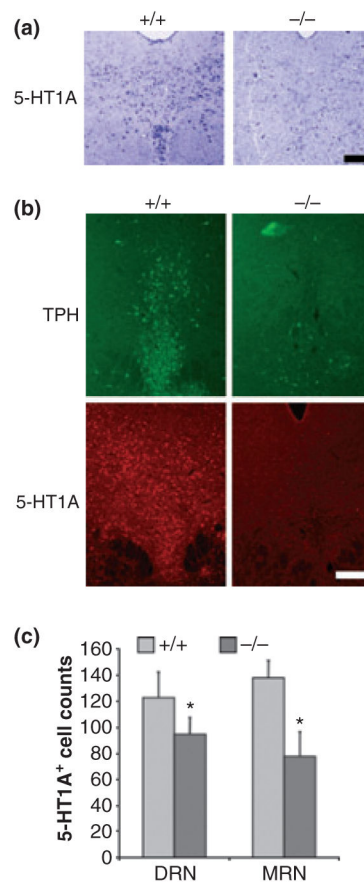
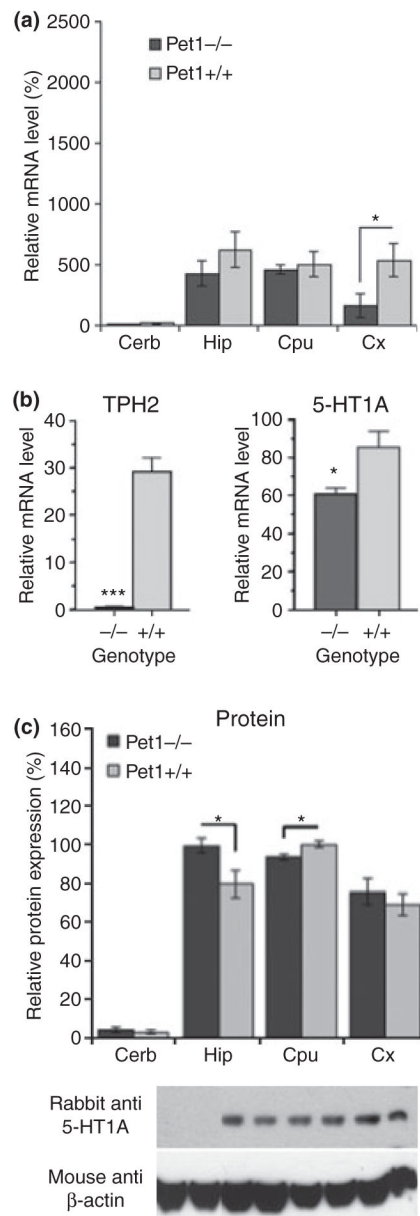


Fig. 4. Reduced 5-HT1A mRNA and protein expression in Pet-1^{-/-} raphe nuclei. (a) *In situ* hybridization showing 5-HT1A mRNA anti-sense probe staining in the dorsal raphe of wild-type (+/+) and Pet-1 knockout (-/-) mice; scale bar = 50 μ m. (b) Immunofluorescent labelling for tryptophan hydroxylase (TPH) or 5-HT1A receptor proteins in sections of DRN from the same wild-type or knockout mice. Representative images are shown at 10 \times magnification with a scale bar = 100 μ m; $n = 3$ mice/group. (c) Quantification of 5-HT1A-immunopositive cells. Cell counts are presented from the dorsal (DR) and median raphe (MRN) of wild-type (+/+) and Pet-1 knockout (-/-) mice; $n = 3$ mice/group. Statistical significance was measured using two-tailed unpaired *t*-test, where * $p < 0.05$.

**Fig. 5.**

Pet-1 knockout mice display altered 5-HT1A receptor expression. Levels of 5-HT1A mRNA (a) or both 5-HT1A and tryptophan hydroxylase 2 (TPH2) RNA (b), and 5-HT1A protein (c) were measured in indicated brain regions by Q-RT-PCR and Western blot, respectively. Regions examined included: 5-HT1A-negative cerebellum (Cerb) as a negative control; hippocampus (Hip), striatum (CPu, including caudate, putamen and globus pallidus), and cortex (Cx); and the dorsal raphe region (DR). (a) 5-HT1A mRNA was reduced in the cortex of Pet-1 null mice. GAPDH was used as an internal control; $N=6$. (b) TPH2 and 5-HT1A RNA levels were quantified in dorsal raphe tissue. GAPDH was used as an internal control; $N=4$. (c) 5-HT1A protein is elevated in hippocampus (HIP), and reduced in the striatum.

Band intensity of four independent experiments was quantified. β -Actin was used as a loading control. Error bars represent SEM. *** $p < 0.001$, * $p < 0.05$.

Table 1

Multiple Pet-1 elements in serotonergic genes

| Gene | Site | Sequence |
|-----------|--------------|-------------|
| h5-HTT | -1154/1144 | GATAGGAAGTA |
| h5-HTT | -1172/1162 | GAAAGGAAATA |
| m5-HTT | -2424/2414 | CCCAGGAAATG |
| m5-HTT | -2024/2014 | GGGAGGAAATG |
| hTPH1 | -1154/1144 | ATACGGAAATT |
| mTPH1 | -790/780 | TACAGGATATA |
| h-AADC | Intron | TTCAGGAAATT |
| h5-HT1A | -136/126 R | AGCAGGAAGTT |
| h5-HT1A | -111/101 R | CGGAGGAAGGG |
| h5-HT1A | -449/439 F | GGAAGGAAATA |
| h5-HT1A | -532/522 F | GAAGGGAAAGT |
| h5-HT1A | -931/921 R | AAAAGGAAGCA |
| h5-HT1A | -1406/1396 R | GGATGGAAATA |
| PEA3 | | TCCAGGAAGTG |
| Consensus | | RRMAGGAARTR |

Alignment of consensus Pet-1 elements previously identified (Hendricks *et al.* 1999), as well as putative additional elements in the 5-HT1A promoter identified using the Transcription Element Search Software (TESS) (Schug 2008), including their respective positions with respect to the start ATG and presence on the coding (F) or non-coding (R) strand. 5-HTT, serotonin transporter; AADC, amino acid decarboxylase; TPH1, tryptophan hydroxylase-1; PEA3, polyomavirus enhancer activator 3. R=G or A, M=A or C. Highlighted is the position of the minimal GGAA Pet-1 binding core.

Table 2

Multiple conserved Pet-1 elements in the 5-HT1A promoter

| Species | Site (human) | Sequence |
|---------|--------------|-------------|
| Human | -111/101 R | CGGAGGAAGGG |
| Rat | | CAGAGGGAGGG |
| Mouse | | CGAAGGGAGGG |
| Human | -136/126 R | AGCAGGAAGTT |
| Rat | | AGCGGGAAGTT |
| Mouse | | AGCGGGAAGTT |
| Human | -449/439 F | GGAAGGAAATA |
| Rat | | AGAAGCAACCA |
| Mouse | | CGAAGGAACTA |
| Human | -532/522 F | GAAGGGAAGT |
| Rat | | GAAGGGAAGGT |
| Mouse | | GGAAGGAAGGT |
| Human | -931/921 R | AAAAGGAAGCA |
| Rat | | TAAACAGAGCC |
| Mouse | | TAAATAGAGCC |
| Human | -1406/1396 R | GGATGGAATA |
| Rat | | GGAAAGAAATC |
| Mouse | | GGAAAGAAATT |

Comparison of putative Pet-1 elements in the promoter of the 5-HT1A receptor. Position of the human element with respect to the start ATG is indicated as well as presence on the coding (F) or non-coding (R) strand.