Increased Serotonin-1A (5-HT1A) Autoreceptor Expression and Reduced Raphe Serotonin Levels in Deformed Epidermal Autoregulatory Factor-1 (Deaf-1) Gene Knock-out Mice* §

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Background: Dysregulation of 5-HT1A receptors is associated with depression, but the transcriptional mechanisms are unclear.

Results: Deaf-1 binds the 5-HT1A promoter, and loss of Deaf-1 results in altered expression of 5-HT1A receptors and reduced serotonin levels.

Conclusion: Deaf-1 is a key regulator of 5-HT1A expression in vivo that is affected by a promoter polymorphism prevalent in human depression.

Significance: Understanding transcriptional regulators of serotonin could lead to improved antidepressant strategies.

Altered regulation of the serotonin-1A (5-HT1A) receptor gene is implicated in major depression and mood disorders. The functional human 5-HT1A C(−1019)G promoter polymorphism (rs6295), which prevents the binding of Deaf-1/NUDR leading to dysregulation of the receptor, has been associated with major depression. In cell models Deaf-1 displays dual activity, repressing 5-HT1A autoreceptor expression in serotonergic raphe cells while enhancing postsynaptic 5-HT1A heteroreceptor expression in nonserotonergic neurons. A functional Deaf-1 binding site on the mouse 5-HT1A promoter was recognized by Deaf-1 in vitro and in vivo and mediated dual activity of Deaf-1 on 5-HT1A gene transcription. To address regulation by Deaf-1 in vivo, Deaf-1 knock-out mice bred to a C57BL/6 background were compared with wild-type siblings for changes in 5-HT1A RNA and protein by quantitative RT-PCR, in situ hybridization, and immunofluorescence. In the dorsal raphe, Deaf-1 knock-out mice displayed increased 5-HT1A mRNA, protein, and 5-HT1A-positive cell counts but reduced 5-HT levels, whereas other serotonergic markers, such as tryptophan hydroxylase (TPH)- or 5-HT-positive cells and TPH2 RNA levels, were unchanged. By contrast, 5-HT1A mRNA and 5-HT1A-positive cells were reduced in the frontal cortex of Deaf-1-null mice, with no significant change in hippocampal 5-HT1A RNA, protein, or cell counts. The region-specific alterations of brain 5-HT1A gene expression and reduced raphe 5-HT content in Deaf-1−/− mice indicate the importance of Deaf-1 in regulation of 5-HT1A gene expression and provide insight into the role of the 5-HT1A G(−1019) allele in reducing serotonergic neurotransmission by derepression of 5-HT1A autoreceptors.

Reduced activity of the brain serotonin system has been implicated in major depression and suicide (1–3), and antidepressant treatments including serotonin reuptake inhibitors (SSRIs)5 increase serotonergic neurotransmission, either directly or indirectly (4, 5). The serotonin-1A (5-HT1A) somatodendritic autoreceptor inhibits the firing of raphe serotonin neurons to negatively regulate the serotonin system (4). Postsynaptic 5-HT1A receptors are strongly expressed in limbic and cortical brain regions (6, 7) and mediate serotonin action to reduce fear and anxiety. 5-HT1A-null mice display increased anxiety behaviors (8–10) and lack response to SSRI treatment (11), whereas transgenic overexpression of the 5-HT1A receptor decreases anxiety (12). Rescue of 5-HT1A receptor expression in the early postnatal forebrain rescues the anxiety phenotype (13). Hence, the 5-HT1A receptor functions as a critical regulator of the serotonin system and is implicated in mood disorders.

The 5-HT1A receptor gene is regulated by a proximal promoter and an upstream repressor region that determines basal expression in neuronal cells (14, 15). In the human 5-HT1A repressor region, we previously identified a functional C(−1019)G polymorphism associated with major depression and suicide, as well as anxiety-related phenotypes, which has

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5 The abbreviations used are: SSRI, serotonin-selective reuptake inhibitor; BES, 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid; Deaf-1, deformed epidermal autoregulatory factor-1; DRN, dorsal raphe nuclei; 5-HIAA, 5-hydroxyindole acetic acid; 5-HT, 5-hydroxytryptamine (serotonin); PEA, polyoma virus enhancer; TPH, tryptophan hydroxylase.
been replicated in independent studies (16, 17). The G-allele prevents the binding of the transcription factor deformed epidermal autoregulatory factor-1 (Defa-1) (18). In serotonergic cells, Defa-1 represses 5-HT1A autoreceptor expression, whereas in nonserotonergic neuronal cells Defa-1 enhances 5-HT1A promoter activity (19). However, the dual activity of Defa-1 on endogenous 5-HT1A expression in vivo has not been demonstrated. Based on promoter studies, the G-allele should increase 5-HT1A autoreceptor levels to reduce 5-HT neuron firing and decrease postsynaptic 5-HT1A receptors to synergistically reduce serotonin neurotransmission. In depressed subjects, the homozygous 5-HT1A G/G(−1019) genotype was associated with increased 5-HT1A autoreceptor binding potential (20–22), consistent with increased 5-HT1A autoreceptors in postmortem studies of depressed suicides (23, 24). Thus, the 5-HT1A G(−1019) allele may alter 5-HT1A receptor expression in vivo because of its inability to bind Defa-1.

The role of Defa-1 in the regulation of 5-HT1A receptor expression in vivo was addressed using the Defa-1−/− mouse (25). In Defa-1-null mice compared with littermate wild-type controls, 5-HT1A mRNA and protein levels were increased in the dorsal raphe region, whereas serotonin cell number and tryptophan hydroxylase 2 (TPH2) mRNA were unchanged. By contrast, postsynaptic 5-HT1A receptors were down-regulated in cell lines, we find a region-specific dysregulation of 5-HT1A receptor expression in Defa-1−/− mice. The Defa-1 knock-out mice indicate the importance of Defa-1 in regulation of the 5-HT1A receptor gene in vivo.

MATERIALS AND METHODS

Electrophoretic Mobility Shift Assay (EMSA)—EMSA was conducted as described previously (19). Briefly, sense and antisense oligonucleotides of the human (26C) and mouse (mouse#1, 5′-GGAGAGTCCTGAGGGTTTCTCCTGTGCCTGATA) Defa-1 elements synthesized with GG overhangs were hybridized and labeled with [32P]dCTP using Klenow fragment DNA polymerase (15). Purified bacterially expressed human Defa-1 protein (5 or 7.5 μg/reaction) was preincubated with or without competitor DNA in a 20-μl reaction containing labeled probe in DNA-binding buffer (20 mM HEPES, 0.2 mM EDTA, 0.2 mM EGTA, 100 mM KCl, 5% glycerol, and 2 mM DTT, pH 7.9). 1 μg of poly(dl-dC), at 22 °C for 20 min and resolved on a nondenaturing 5% acrylamide/Tris-glycine gel at 4 °C. Unlabeled doubled-stranded polyoma virus enhancer fragment DNA polymerase (15). Purified bacterially expressed 5-HT1A promoter (position −1 to −984 or −942 relative to the ATG) was amplified by PCR using the following primers: forward, 5′-CTCGAGGCCCATCTTCTCAGGTCCTGAGGG or 5′-GCCACATGTGAACCTGCTCCT, reverse, 5′-AAGCCTT-GCCTGCTGCACTCCCG) and SN48 cells genomic DNA as a template and verified by sequence analysis. The forward primer included an XhoI site and the reverse primer a HindIII site for cloning into the pGL3B luciferase reporter vector (Promega). The 3×-mDefa-1RE (three copies of mouse Defa-1 response element) construct was generated by cloning complementary 78-bp primers containing three copies of the mouse Defa-1 palindromic 1 (AGTCTCTAGGGTTTCTCGTGCT) in the Nhel/Xhol site of pGL3P. For luciferase assays, HEK293 cells or Ltk− cells were plated at a density of 2 × 105 and 1.5 × 105 cells/ml respectively, in 6-well plates. The following day cells were transfected with 1 μg of reporter plasmid, 1 μg of pCMV-β-galactosidase, and 1 μg of either pcDNA3 or pcDNA3 His-Defa-1 using calcium phosphate (HEK293) or Lipofectamine 2000 (Ltk−) with a lipid:DNA ratio of 2:1. Cells were incubated 24–36 h following transfection and lysed in reporter lysis buffer (Promega). β-Galactosidase activity was used to normalize luciferase activity to correct for transient transfection efficiency.

Chromatin Immunoprecipitation (ChIP)—SN48 cells were fixed with 1% formaldehyde for 10 min at 22 °C. Fixation was stopped following addition of 0.125 M glycine for 5 min, and cells were then washed 3 times with PBS. Cells were scraped in 10 mM Tris, pH 8.0, 85 mM KCl, and 0.5% Nonidet P-40 and harvested by centrifugation. Cells were then lysed in 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100 supplemented with protease inhibitors and sonicated with 16 pulses of 30 s. Extracts were then incubated with preimmune serum or Defa-1 rabbit antibody (1/100 dilution) overnight at 4 °C. Extracts were then incubated for 2 h with protein A-Sepharose beads at 4 °C, washed once with 1 ml of lysis buffer, 4 times with 1 ml of wash buffer (100 mM Tris-HCl, pH 7.5, 500 mM LiCl, 1% Nonidet P-40, and 1% deoxycholate) and once with 1 ml of TE. DNA was eluted in 0.1 M NaHCO3 with 1% SDS and samples reverse cross-linked overnight at 65 °C. DNA was purified following phenol/chloroform extraction and precipitated with 2 volumes of EtOH and 20 μg of linear acrylamide for 2 h at −20 °C. PCR amplifications were performed using 5 μl of each elution with the following primers (forward/reverse): 5-HT1A promoter, ATGAAAAGTCAGCCAGCACC/TCA-CAGTGTGCTGACTATCAGGC; 5-HT1A open reading frame, GCCGAGACGCTTCAAGAGG/GGCGTCGCTCCTCACCCTG; GAPDH promoter, GTCCAAAGAGGAGGGAGGAGGGCGTCAGTCCGTATTTA.

Animals and Genotyping—All animal studies were conducted in accordance with the University of Ottawa Animal Care Committee guidelines using protocol NSI-57. Animals were maintained on a 12-h light/dark cycle with lights on at 6:00 a.m., at 21 °C with food and water ad libitum. Defa-1 knock-out mice were received from Dr. Jane Visvader (25) and rederived by embryo transfer (Taconic, Hudson, NY) on a mixed C57BL/6-BALB/c-129 background. Pilot experiments revealed no significant differences in 5-HT1A mRNA levels between mixed background Defa-1-null (−/−) and wild-type (+/+) mice, but differences were observed compared with pure C57BL/6 mice. Hence, the mice were backcrossed for five generations onto the C57BL/6 background. All experiments were performed on adult (8–10 weeks old) knock-out mice with age-matched littermate wild-type controls from the C56BL/6 background, unless otherwise specified.

For genotyping, tail DNA was isolated using DNA lysis buffer (100 mM Tris, pH 8.0, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 10
µg/ml proteinase K). PCR was done using the primers: forward, 5’-GTGCTCTCATCTCATTGCTC; reverse1, 3’-GAAACA-
GAAACACGGATCGGAATCA; reverse2, 3’-AAAG-
AATAGAACACCACCACCCGTC, with 267-bp wild-type and 421-bp knock-out products. Each mouse used in this study was genotyped at weaning and verified at sacrifice.

Two-dimensional SDS-PAGE—Prefrontal cortex was dis-
sected, rinsed in ice-cold 10 mM BES pH 7.4, 0.3 M sucrose, homogenized in isolectric focusing buffer (7 M urea, 2 M thio-
urea, 4% CHAPS, 1% DTT, 0.001% bromphenol blue) and incu-
bated for 90 min at 22 °C. Samples were centrifuged at 10,000 ×
g for 5 min, the supernatant was precipitated in 90% acetone and centrifuged (2000 × g for 5 min), and the pellet was resus-
pended in isolectric focusing buffer supplemented with biotyes. IPG strips (pH 3–10; Bio-Rad) were rehydrated over-
night with 300 µl of isolectric focusing buffer containing 1 mg of total protein. Isoelectric focusing was done using the follow-
ing conditions: desalting for 20 min at 500 V; 2.5 h ramp to 10,000 V; 40,000 VH focusing at 10,000 V. IPG strips were then treated for 15 min with a reducing solution (375 mM Tris-HCl, pH 8.8, 20% glycerol, 2% SDS, 65 mM DTT) followed by a 15-min incubation with an alkylation solution (375 mM Tris-
HCl, pH 8.8, 2% SDS, 20% glycerol, 215 mM iodoacetamide) and placed on top of a 12% SDS-polyacrylamide gel using a 0.5% agarose overlay. Gels were run for 5 h at 30 mA and transferred to PVDF membranes. Membranes were blocked in 5% milk and incubated overnight at 4 °C with rabbit anti-Deaf-1 antibody (1:1000; custom raised against full-length Deaf-1 peptide; Cedarlane).

Immunofluorescence—Mice were anesthetized by lethal intraperitoneal injection (0.01 ml/g of body weight) of sodium pentobarbital (Somnotol; MTC Pharmaceuticals, Cambridge, ON, Canada) and perfused by cardiac infusion of 20 ml of saline, then 20 ml of 4% paraformaldehyde. Whole brains were isolated, postfixed overnight in 4% paraformaldehyde, washed and centrifuged (2,000 × g) for 5 min, the supernatant was precipitated in 90% acetone and centrifuged (2,000 × g for 5 min), and the pellet was resuspended in isolectric focusing buffer supplemented with biotyes. Samples were treated for 15 min with a reducing solution (375 mM Tris-HCl, pH 8.8, 20% glycerol, 2% SDS, 65 mM DTT) followed by a 15-min incubation with an alkylation solution (375 mM Tris-
HCl, pH 8.8, 2% SDS, 20% glycerol, 215 mM iodoacetamide) and placed on top of a 12% SDS-polyacrylamide gel using a 0.5% agarose overlay. Gels were run for 5 h at 30 mA and transferred to PVDF membranes. Membranes were blocked in 5% milk and incubated overnight at 4 °C with rabbit anti-Deaf-1 antibody (1:1000; custom raised against full-length Deaf-1 peptide; Cedarlane).

RNA Extraction/Reverse Transcription—Adult mice were euthanized by CO2 asphyxiation followed by decapitation and removal and dissection of brains. For preparation of total RNA, brain samples were triturated and extracted in 600 µl of TRIzol (Invitrogen), centrifuged in chloroform, and the supernatant was precipitated and resuspended in 30 µl of diethylpyrocar-
bonate-treated water to 0.5–2 µg/µl. Samples were treated with 1 µl of DNase/5 µg of total RNA for 30 min at 37 °C using the Turbo DNA-free kit (Ambion, Foster City, CA), terminated in DNs
ase inactivation buffer, and quantified at OD260. Reverse transcription was carried out using 0.05 µg/µl DNase-treated RNA, with 1:10 random decamer oligonucleotides (Ambion) and 0.4 mM dNTP mix. Samples were incubated for 3 min at 70 °C and annealed on ice. First-strand buffer (Ambion) was then added along with 25 units of M-MLV reverse transcriptase (Ambion) and 10 units of RNase inhibitor (Promega), and reac-
tions were incubated for 60 min at 42 °C and inactivated for 10 min at 95 °C. Parallel reactions lacking M-MLV enzyme served as no reverse transcription controls. Newly synthesized cDNA was stored at −20 °C.

Quantitative Real-time RT-PCR—Quantitative real-time RT-PCR was performed as described (29) using TaqMan

5-HT1A Receptor Dysregulation in Deaf-1+/− Mice

FEBRUARY 24, 2012•VOLUME 287•NUMBER 9

JOURNAL OF BIOLOGICAL CHEMISTRY 6617
Gene Expression Assay kits (Applied Biosystems). Product numbers were as follows: TPH2, Mm00557717_m1; Deaf-1, Mm00516805_m1; 5-HT1AR, Mm00434106_s1; and GAPDH, 4352339E. TPH2, Deaf-1, and 5-HT1A probes are FAM-labeled probes, whereas GAPDH is a VIC-labeled probe. GAPDH amplification was used as internal control and was included in every multiplex reaction. Each reaction contained 5 μl of TaqMan Universal PCR Mastermix (Applied Biosystems), 0.5 μl each of FAM- and VIC-labeled probes, 3 μl of nuclease-free water, and 1 μl of sample. Standard curves ($r^2 \geq 0.996$) from serial dilution of the most concentrated cDNA sample spanned the range of test sample duplicates and at least one standard was included in every multiplex reaction. Each reaction contained 5 μl of TaqMan Universal PCR Mastermix (Applied Biosystems), 0.5 μl each of FAM- and VIC-labeled probes, 3 μl of nuclease-free water, and 1 μl of sample. Standard curves ($r^2 \geq 0.996$) from serial dilution of the most concentrated cDNA sample spanned the range of test sample duplicates and at least one standard was included in every multiplex reaction. Each reaction contained 5 μl of TaqMan Universal PCR Mastermix (Applied Biosystems), 0.5 μl each of FAM- and VIC-labeled probes, 3 μl of nuclease-free water, and 1 μl of sample. Standard curves ($r^2 \geq 0.996$) from serial dilution of the most concentrated cDNA sample spanned the range of test sample duplicates and at least one standard was included in every multiplex reaction.

Control samples without template DNA (no template controls) or reverse-transcriptase (no RT) controls were run for each gene analyzed. The cycling program used was: 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 60 s on the Rotor Gene 3000 cycler (Corbett Research, Sydney, Australia). Data were analyzed using Rotor Gene v6.0 software using the ΔΔCt method (30). Briefly, relative amounts of each target gene to GAPDH were obtained as $10^{(Ct_T-Ct_G)}$, where CtT is the threshold cycle for the target gene and CtG is the threshold cycle for GAPDH in the same tube. To verify the deleted exon sequence in Deaf-1 knock-out mice, SYBR Green technology (Molecular Probes) was applied. The following probes were designed to the deleted sequence of the Deaf-1 gene: forward, 5′-CTGTCGGAACATCAGTGGCA; reverse, 5′-CACAGGCAACTCGCTGTCATAC. The amplification cycles were 92 °C for 10 min, 92 °C for 30 s, 58 °C for 15 s, 72 °C for 20 s (40 cycles), terminated at 72 °C for 10 min. Ct values were quantified using SYBR Green incorporation following amplification with Qtaq™ DNA polymerase mix (Clontech). Averages were normalized to GAPDH.

High Performance Liquid Chromatography (HPLC) Analysis—Frozen cryostat sections of the raphe region from mixed background Deaf-1−/− or wild-type littermates were pooled (five/animal), placed in 0.3M monochloroacetic acid containing 10% methanol and internal standards, and stored at −80 °C for HPLC analysis of levels of 5-HT and its metabolite 5-HIAA. Tissue samples were sonicated in 300 μl of homogenizing solution (14.17 g of monochloroacetic acid, 18.6 mg of EDTA, 5.0 ml of methanol, and 500 ml of H2O). Following centrifugation, the supernatants were used for HPLC analysis using an Agilent pump, guard column, radial compression column (5 m, C18 reverse phase, 8 mm × 10 cm), and coulometric electrochemical detector (ESA model 5100A). Supernatant (40 μl/sample) was passed through the system at a flow rate of 1.5 ml/min (1400–1600 p.s.i.). The mobile phase contained 90 mm
RESULTS

Deaf-1 Binding and Dual Activity at Mouse 5-HT1A Promoter—Previous studies have characterized the allele-specific recognition by Deaf-1 at the C-allele of a 26-bp palindromic sequence at −919 bp of the human 5-HT1A promoter sequence (18, 19), but the corresponding site in the mouse 5-HT1A promoter sequence has not been identified. Based on location and palindrome structure of the human 5-HT1A Deaf-1 site, two imperfect palindrome mouse promoter sequences (mouse#1 and #2) were identified containing the minimal Deaf-1 consensus TCG sequence (31) (Fig. 1A). EMSA was done using recombinant purified Deaf-1 protein incubated with labeled 26-bp human 5-HT1A C-allele Deaf-1 site (26C), in the absence or presence of excess unlabeled double-stranded primers (Fig. 1B). Deaf-1 binding was competed with equal potency by the human 26C and mouse#1 primers, but not mouse#2 primers. As a control, the human 26-bp G-allele palindrome (26G) that does not bind Deaf-1, or unrelated DNA elements for PEA3 or E2F, failed to compete Deaf-1 binding. Conversely, Deaf-1 bound to the labeled mouse#1 DNA element and was competed by unlabeled mouse or human Deaf-1 elements but not the PEA3 element (left panel). Thus, the mouse 5-HT1A promoter contains a Deaf-1-binding element located 947 bp upstream of the translation start site.

To determine whether Deaf-1 regulates the transcriptional activity of the mouse HTR1A promoter, luciferase transcriptional reporter assays were done using the mouse 5-HT1A promoter construct that includes (−974) or lacks (−942) the mouse#1 site (Fig. 2A). In the presence of Deaf-1, luciferase activity of the mouse 5-HT1A reporter construct was decreased by 60% in mouse Ltk− cells and by 40% in human HEK293 cells, whereas repression was not observed upon deletion of the mouse#1 element. In SN48 cells, a model of mouse septal neurons that expresses 5-HT1A heteroreceptors, Deaf-1 significantly increased the activity of the −984 5-HT1A promoter fragment by 3.5-fold, but had no significant effect in the −942 fragment lacking the Deaf-1 element (Fig. 2B). Taken together, these results highlight the dual activity of Deaf-1 at the mouse 5-HT1A promoter, consistent with findings previously reported for the human promoter (18). We examined Deaf-1 activity using a reporter construct containing three copies of mouse#1 (3×-Deaf-1RE) to address whether this element is...
sufficient to confer cell specific regulation by Deaf-1 (Fig. 2C). In the presence of Deaf-1 this construct was repressed in HEK293 cells, whereas it was activated in SN48 cells. These results indicate that Deaf-1 acts at its response element to mediate dual regulation of the mouse 5-HT1A promoter, as observed for the human 5-HT1A promoter (18). To specifically demonstrate that Deaf-1 is present at the 5-HT1A promoter in vivo, we performed ChIP assays. In SN48 cells, the mouse 5-HT1A promoter region containing the palindrome was enriched upon immunoprecipitation with Deaf-1 antibody compared with preimmune serum, whereas there was no enrichment of the 5-HT1A coding sequence or the GAPDH promoter (Fig. 2D). These results implicate Deaf-1 in regulating the 5-HT1A promoter in vivo.

Characterization of Deaf-1-deficient Mice—The importance of Deaf-1 in regulation of the 5-HT1A receptor in vivo was addressed using Deaf-1 knock-out mice (25), which were backcrossed from a mixed background onto the C57BL/6 background (see “Materials and Methods”). After genotyping, the presence of the knock-out mutation in genomic DNA and mRNA was verified by DNA sequence analysis. In Deaf-1−/− mice, the loss Deaf-1 coding RNA was shown by RT-PCR analysis (Fig. 3A), and the loss of Deaf-1 protein was resolved by two-dimensional gel electrophoresis/Western blot analysis (Fig. 3B). Morphological analysis of the brains of Deaf-1−/− mice using cresyl violet staining showed no gross alteration (Fig. 3C).

Altered 5-HT1A mRNA Levels in Deaf-1-deficient Mice—To address whether loss of Deaf-1 affects 5-HT1A receptor expression, brain tissues from Deaf-1-null (−/−) and wild-type (+/+) littermates were examined for levels of 5-HT1A receptor RNA using quantitative RT-PCR (29). Interestingly, we observed a significant 38% decrease in 5-HT1A mRNA levels in the frontal cortex when compared with wild-type littermate controls (Fig. 4A) and a trend toward decreased 5-HT1A mRNA levels (23%) in the hippocampus (Fig. 4B). Oppositely, there was a significant 46% increase in 5-HT1A mRNA in midbrain tissue from the rostral raphe region (Fig. 4C). Although the midbrain tissue dissection used was enriched for serotonin neurons (29), in situ hybridization was performed to determine the precise localization of 5-HT1A RNA in the dorsal raphe nuclei (DRN) (Fig. 4D). Compared with wild-type littermates, 5-HT1A RNA was markedly up-regulated in the DRN of Deaf-1-null mice, consistent with the up-regulation of 5-HT1A autoreceptor expression in serotonin neurons of the DRN. By contrast, no change in 5-HT1A mRNA was observed in the median raphe nuclei of these mice (data not shown), implying that up-regulation of 5-HT1A receptor mRNA in Deaf-1−/− mice is specific to the DRN. The DRN is composed primarily of rhombomere-1-de-
Deaf-1 knock-out mice. Sense probe was used as negative control. Representative images are shown at magnification of ×20. Scale bar, 100 μm.

**FIGURE 4. Brain region-specific alterations in 5-HT1A mRNA expression in Deaf-1 knock-out mice.** RNA isolated from prefrontal cortex (A), hippocampus (B), and rostral raphe nuclei (C) was analyzed by Multiplex RT-PCR for 5-HT1A mRNA and GAPDH RNA and quantified as 5-HT1A mRNA normalized to GAPDH RNA using the 2^(-ΔΔCt) formula. Data are presented as mean ± S.E. (error bars), n = 4 mice/group; *, p < 0.05 by two-tailed unpaired t test. In Deaf-1 knock-out (−/−) compared with wild-type mice (+/+), 5-HT1A RNA was reduced by 38% in prefrontal cortex, 23% in hippocampus, and increased by 46% in raphe region. D, increased 5-HT1A mRNA in DRN of Deaf-1−/− mice. *In situ hybridization showing representative sections of the DRN hybridized with 5-HT1A antisense RNA probe in wild-type (+/+ versus Deaf-1 knock-out (−/−) mice. Increased 5-HT1A-positive cell counts were observed in Deaf-1−/− mice. Sense probe was used as negative control. Representative images are shown at magnification of ×20. Scale bar, 100 μm.

Drived 5-HT neurons that are distinct from MRN 5-HT neurons and are implicated in anxiety behavior (32, 33).

5-HT1A Immunostaining in Deaf-1 Mutant Mice—To determine whether altered 5-HT1A mRNA corresponded with changes in 5-HT1A protein levels, immunofluorescent staining for 5-HT1A receptors was performed in the DRN, prefrontal cortex, and dentate gyrus region of the hippocampus (Fig. 5A). No staining was observed in sections not incubated with primary antibody (control) or in sections of cerebellum, which lacks 5-HT1A receptors, whereas cells in the adjacent caudal raphe nuclei were stained in the same section. To demonstrate antibody specificity further, HEK293 cells that were transfected with the mouse 5-HT1A receptor cDNA displayed strong staining compared with cells transfected with empty vector (supplemental Fig. 1).

In Deaf-1-null mice, a 3-fold increase in 5-HT1A receptor-labeled cells was observed in the DRN (Fig. 5B), which correlated with the observed up-regulation of 5-HT1A mRNA (Fig. 3). The increase in 5-HT1A-labeled cells in Deaf-1−/− DRN was greater than the change in 5-HT1A mRNA levels (38%) and may reflect the limit of detection using the 5-HT1A antibody for cell counting (see below). The number of cells labeled with anti-glial fibrillary acidic protein antibody in the DRN region was comparable between knock-out and wild-type mice (data not shown), implying no alteration in activated astroglia. Oppositely, in the prefrontal cortex, a significant 25% decrease in 5-HT1A-labeled cells was observed (Fig. 5C), consistent with reduced levels of 5-HT1A mRNA in this region. No significant change in the number of 5-HT1A-labeled cells was observed in the hippocampal dentate gyrus (data not shown). Thus, Defa-1 deficiency leads to brain region-specific changes in 5-HT1A receptor protein levels that mirrored changes in 5-HT1A mRNA.

**5-HT1A Receptor Dysregulation in Deaf-1−/− Mice**

**FIGURE 6. Dual immunofluorescence labeling for 5-HT1A receptors and TPH in Deaf-1−/− mice.** Sections of DRN stained with primary antibody to 5-HT1A receptors (A) or TPH (B) and secondary antibody (FITC) were double labeled with primary antibody to TPH (red) or 5-HT1A (green), respectively. C, merged image of panels A and B. D, negative control staining showing no significant cross-reactivity between primary antibody to 5-HT1A receptors and TPH. 5-HT1A receptors and TPH were also largely co-localized in DRN (supplemental Fig. 2). 5-HT1A receptor and TPH, the rate-limiting enzyme for serotonin metabolism, are implicated in anxiety behavior (32, 33). The decrease in 5-HT1A receptor-positive cells compared with wild-type mice is consistent with altered 5-HT1A receptor autoreceptors and reduced raphe 5-HT levels, consistent with the DRN 5-HT1A receptor antisense RNA probe in wild-type (A) and Deaf-1 knock-out (B) mice. Increased 5-HT1A-positive cell counts were observed in Deaf-1−/− mice. A similar reduction (40%) was observed upon measurement of the raphe 5-HT content by HPLC, which validates the quantification of 5-HT by immunofluorescence, whereas there was no change in 5-HIAA content (Fig. 7E). These data indicate that Defa-1 deficiency is associated with up-regulation of 5-HT1A autoreceptors and reduced raphe 5-HT levels, consistent with increased inhibition of serotoninergic tone by 5-HT1A autoreceptors. The lack of change in 5-HIAA levels suggests that release and metabolism of 5-HT were compensated in these mice under basal conditions, despite lower 5-HT levels.

To examine whether Defa-1 deficiency has a global action on serotoninergic neurons, we examined the level of TPH2 mRNA in raphe tissue (Fig. 7D). TPH2 is rate-limiting for brain 5-HT synthesis and is not expected to be regulated by Defa-1. Levels of TPH2 mRNA were unchanged between Defa-1 mutant and wild-type mice. Taken together, these results indicate that the absence Defa-1 did not affect the number of serotonin neurons...
or the expression of TPH2 despite reduced levels of 5-HT. This suggests that Deaf-1 deficiency did not affect serotonergic differentiation but specifically increased 5-HT1A autoreceptor expression, which was associated with reduced serotonin levels at the cell body of neurons in the DRN.

**DISCUSSION**

**Altered 5-HT1A Receptor Expression in Deaf-1 Knock-out Mice**—The identification of Deaf-1 as an allele-specific regulator of the human 5-HT1A receptor gene and the association of the 5-HT1A G(−1019) allele with depression and suicide suggest that Deaf-1 may have an important role in the regulation of 5-HT1A receptor expression in vivo. Our previous results demonstrated that Deaf-1 has differential activity depending on the cell model, with repressor activity in serotonergic raphe RN46A cells but enhancer activity in several nonserotonergic neuronal cell lines (18, 19), implying that Deaf-1 regulates 5-HT1A autoreceptors differently than 5-HT1A heteroreceptors expressed on nonserotonergic neurons. By analogy with the human 5-HT1A promoter, we identified a palindromic site located at −947 bp in the mouse 5-HT1A promoter that contains the minimal TCG Deaf-1 consensus sequence (31) and is specifically recognized by Deaf-1 protein. Although the level of nucleotide identity between human and mouse genes in this region is limited (Fig. 1A), the presence of a Deaf-1 element in the same region suggests its importance in regulating 5-HT1A
gene transcription. Similar to the human 5-HT1A promoter Deaf-1 exhibited dual activity at the mouse 5-HT1A promoter that is mediated directly by the Deaf-1 element (Fig. 2). Furthermore, we show for the first time using the ChIP assay that Deaf-1 binds directly to the 5-HT1A promoter in vivo, indicating that Deaf-1 regulates 5-HT1A expression directly via this element in neuronal cells. The evidence from these in vitro and in vivo studies make a strong case for a direct effect of Deaf-1 on 5-HT1A expression in vivo.

To address the effect of Deaf-1 on 5-HT1A receptor gene regulation in vivo, we have examined the levels of 5-HT1A mRNA and protein in Deaf-1 knock-out mice on the C57BL/6 genetic background. The C57BL/6 strain displays higher relative levels of forebrain 5-HT1A receptor RNA (data not shown) and responds to chronic SSRI treatment with increased hippocampal neurogenesis and reduced anxiety-like behavior. These responses are abolished in 5-HT1A knock-out mice (11) but are not observed in the BALB/c strain (34). The C57BL/6 strain also has the higher activity Arg477 variant of TPH2 (35) and the low activity GK-5-HTT variant (36), suggesting a more active serotonin system. Thus, the Deaf-1−/− mouse provides a useful model to study 5-HT1A receptor regulation in vivo.

Deaf-1 binds directly to the mouse 5-HT1A promoter, and mediates dual activity through the Deaf-1 element on the promoter (Figs. 1 and 2). The cell specificity of this activity corresponds to that observed in Deaf-1-null mice, where 5-HT1A mRNA was increased in 5-HT neurons, consistent with the role of Deaf-1 as a repressor of 5-HT1A autoreceptor transcription in serotonergic neurons. Oppositely, in nonserotonergic neurons such as SN48 cells, Deaf-1 activated 5-HT1A autoreceptor transcription, and deficiency of Deaf-1 in vivo reduced 5-HT1A receptor RNA levels in the cortex, a nonserotonergic brain region. These results indicate that altered 5-HT1A regulation in Deaf-1−/− mice is primarily due to loss of Deaf-1-dependent regulation of the 5-HT1A gene, although loss of Deaf-1 may also indirectly regulate 5-HT1A receptor expression in Deaf-1−/− mice. Importantly, the expression of other genes (e.g. TPH2) in 5-HT neurons was not affected in Deaf-1 knock-outs, arguing against a nonspecific global effect, which would be expected to produce a general inhibition or induction of 5-HT1A and other genes. For example, a developmental abnormality that leads to impaired serotonergic differentiation such as Pet-1 knock-out, globally reduces 5-HT1A and other serotonin markers like TPH2 (37, 38), which we did not observe in the Deaf-1−/− mice. Thus, the effects of Deaf-1 deficiency that we observed are both cell- and gene-specific and indicate a direct effect.

Increased presynaptic 5-HT1A autoreceptor expression in Deaf-1 knock-out mice would tend to reduce raphe firing rate and decrease serotonin release. Consistent with this, we observed reduced 5-HT levels in dorsal raphe neurons from Deaf-1−/− mice, suggesting an inhibition of serotonin synthesis despite the lack of change in TPH2 mRNA. Oppositely, in 5-HT1A−/− mice, the lack of 5-HT1A autoreceptors results in increased raphe basal firing and fluoxetine-induced 5-HT release (39–41). Similarly, specific suppression of raphe 5-HT1A autoreceptor expression also increases raphe firing activity and 5-HT release (42–44). Interestingly, the number of serotonin-positive cells was not altered in adult Deaf-1 knock-out mice, suggesting that Deaf-1 deficiency does not significantly alter survival or differentiation of serotonin neurons. However, dysregulation of 5-HT1A autoreceptor levels and serotonin neurotransmission induced by the lack of Deaf-1 during development could impact the behavioral phenotype of adult knock-out mice, as previously observed for 5-HT1A-null mice (13).

By contrast to the up-regulation of 5-HT1A autoreceptors, 5-HT1A mRNA and 5-HT1A-positive cells were reduced in the frontal cortex of Deaf-1 knock-out mice, suggesting that Deaf-1 acts as an enhancer in the cortex, consistent with a dual regu-
5-HT1A Receptor Dysregulation in Deaf-1−/− Mice

A

B

C

D

E

FIGURE 7. Reduced serotonin in 5-HT1A-positive cells of Deaf-1 knock-out mice. A, representative images of immunofluorescent labeling of 5-HT-positive cells in DRN of wild-type (+/+ ) at magnifications of ×10 and ×20 compared with Deaf-1 knock-out (−/− ) DRN at magnification of ×20. Scale bars, 50 μm. B, quantification of 5-HT-positive cells in wild-type and Deaf-1 knock-out DRN. Cell counts are presented as mean percentage of total cells ± S.E. (error bars) from combined rostral and caudal DRN. C, reduced 5-HT intensity/cell in Deaf-1−/− DRN. 5-HT intensity is presented as percent brightness above background. n ≥ 3 mice/group, * p < 0.05 by two-tailed unpaired t test. D, TPH2 mRNA expression in raphe region. Multiplex real-time PCR shows TPH2 RNA levels in the dorsal raphe region dissected from wild-type (+/+ ) and Deaf-1 knock-out (−/− ) mice. Data are normalized to GAPDH using the 10^CtG–CtT method and are presented as mean ± S.E., n = 3 mice/group. E, 5-HT and 5-HIAA content in raphe region. Sections of dorsal raphe tissue from three to five Deaf-1−/− or Deaf-1+/+ mice were pooled and the total content of 5-HT and metabolite 5-HIAA quantified by HPLC analysis. **, p < 0.01 by two-tailed unpaired t test.

lation of pre- and postsynaptic 5-HT1A receptors by Deaf-1 in vivo. Although Deaf-1 enhanced 5-HT1A receptor transcription in nonserotonergic neurons, Hes1, which represses at the 26-bp 5-HT1A palindrome sequence, retained repressor activity in these cells (19, 45). In contrast to Deaf-1-deficient mice, Hes1−/− mice display widespread up-regulation of 5-HT1A receptor RNA and protein in embryonic midbrain and hindbrain regions (45). Thus, the loss of Deaf-1 regulation results in a dual action to decrease serotonergic neurotransmission: induction of 5-HT1A autoreceptors to decrease 5-HT neuronal activity and decreased cortical 5-HT1A receptors to reduce serotonin responsiveness.

Deaf-1 and Human 5-HT1A C(−1019)G Polymorphism— Reduced activity of the serotonin system is implicated in major depressive disorder (3), and antidepressant therapies act directly or indirectly to enhance serotonergic neurotransmission (4). Upon chronic treatment with SSRIs, 5-HT1A autoreceptors become desensitized whereas postsynaptic 5-HT1A receptors remain responsive to enhanced serotonergic neurotransmission, correlating with clinical improvement (4, 46–49). Genetic suppression of 5-HT1A autoreceptor expression by only 30% in mice reduces depression and stress reactivity phenotypes and permits an accelerated response to chronic SSRI treatment, suggesting that relatively small changes in autoreceptor expression can have a major impact on behavioral phenotype (42). The 5-HT1A C(−1019)G polymorphism is associated with depression, with the G allele preventing Deaf-1 regulation of 5-HT1A transcription, which could reduce serotonergic neurotransmission (16). Based on our results, the human 5-HT1A G/G(−1019) genotype would be predicted to result in opposite changes in 5-HT1A receptor RNA and protein levels in pre- and postsynaptic brain regions. Recent studies support an up-regulation of 5-HT1A autoreceptors in antidepressant-free depressed patients with the G/G genotype (21, 22, 50), but not in healthy subjects (51). This suggests that compensatory mechanisms may be recruited in healthy subjects to normalize 5-HT1A autoreceptor expression whereas they are incompletely effective in depressed subjects. However, the effect of the G/G genotype on postsynaptic 5-HT1A receptors is less clear. The G/G genotype in depressed subjects was associated with no change or increased 5-HT1A receptors in some regions such as the hippocampus (21, 22, 50), which could reflect a compensatory up-regulation due to lower serotonergic neurotransmission in these subjects (52) or a decrease in forebrain Deaf-1 levels, leading to reduced transcription of the 5-HT1A heteroreceptors (53). Several studies report reduced 5-HT1A RNA or binding in the prefrontal cortex of subjects with major depression (24, 53–56), consistent with reduced transcription of the 5-HT1A receptor. Similarly, in female cynomolgus monkeys, depressed status was associated with reduced 5-HT1A receptor levels in several forebrain regions (especially cingulate cortex, with lesser change in hippocampus and amygdala) with no change in the raphe region (57). In addition to the presence of the G(−1019) allele, reduction in Deaf-1...
expression has also been associated with reduced 5-HT1A receptor expression in the prefrontal cortex of female depressed subjects (53). Thus, both the C(−1019)G polymorphism and Deaf-1 levels may differentially influence 5-HT1A receptor levels in the brains of healthy versus depressed subjects, conferring distinct phenotypes and treatment responses. However, association studies are limited by frequency of the risk allele in the population size, heterogeneity of the genetic background, as well as environmental influences, and are not always replicated.

Hence, better animal models are needed to address the etiology and treatment of depression. Deaf-1-deficient mice provide an animal model of perturbed 5-HT1A receptor regulation that corresponds to the expected effects of G/G(−1019) genotype in humans. In addition to its association with depression, anxiety, and suicide, the C(−1019)G polymorphism has been associated with reduced response to antidepressant treatment (14, 58–64). In mice and rats, 5-HT1A receptors are implicated in the activity of chronic SSRI treatment to induce hippocampal neurogenesis and to reduce anxiety and depression-like behaviors (11, 65). It is possible that the combination of reduced cortical 5-HT1A receptors and increased 5-HT1A autoreceptors in the Deaf-1-null mice may attenuate SSRI action. However, unlike the Deaf-1-null mice, the human G allele would affect not only Deaf-1 repression but also that of Hes1 and Hes5 (45). Nevertheless, Deaf-1-deficient mice provide a useful model for investigating the role of Deaf-1 binding at the C(−1019)G polymorphism and its impact on 5-HT1A receptor expression, behavior, and antidepressant response (16).

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