Eur J Neurosci. Author manuscript; available in PMC 2015 August 18.

Published in final edited form as:
Eur J Neurosci. 2011 January ; 33(2): 214–223. doi:10.1111/j.1460-9568.2010.07498.x.

Freud-2/CC2D1B mediates dual repression of the serotonin-1A receptor gene

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Abstract

The serotonin-1A (5-HT1A) receptor functions as a pre-synaptic autoreceptor in serotonin neurons that regulates their activity, and is also widely expressed on non-serotonergic neurons as a post-synaptic heteroreceptor to mediate serotonin action. The 5-HT1A receptor gene is strongly repressed by a dual repressor element (DRE), which is recognized by two proteins: Freud-1/CC2D1A and another unknown protein. Here we identify mouse Freud-2/CC2D1B as the second repressor of the 5-HT1A–DRE. Freud-2 shares 50% amino acid identity with Freud-1, and contains conserved structural domains. Mouse Freud-2 bound specifically to the rat 5-HT1A–DRE adjacent to, and partially overlapping, the Freud-1 binding site. By supershift assay using nuclear extracts from L6 myoblasts, Freud-2–DRE complexes were distinguished from Freud-1–DRE complexes. Freud-2 mRNA and protein were detected throughout mouse brain and peripheral tissues. Freud-2 repressed 5-HT1A promoter–reporter constructs in a DRE-dependent manner in non-neuronal (L6) or 5-HT1A-expressing neuronal (NG108-15, RN46A) cell models. In NG108-15 cells, knockdown of Freud-2 using a specific short-interfering RNA reduced endogenous Freud-2 protein levels and decreased Freud-2 bound to the 5-HT1A–DRE as detected by chromatin immunoprecipitation assay, but increased 5-HT1A promoter activity and 5-HT1A protein levels. Taken together, these data show that Freud-2 is the second component that, with Freud-1, mediates dual repression of the 5-HT1A receptor gene at the DRE.

Keywords

epigenetic; major depressive disorder; mental retardation; mouse; raphe; transcription factor

Introduction

The serotonin system originates from neurons of the midbrain raphe nuclei that project widely throughout the brain (Törk, 1990). The serotonin system regulates the development of anxiety, aggression and stress-reactivity phenotypes (Gordon & Hen, 2004; Lesch, 2005), and is implicated in feeding behavior, sleep disorders and emotional function (Jacobs & Azmitia, 1992). The serotonin-1A (5-HT1A) receptor is expressed pre-synaptically on serotonergic raphe neurons as a somatodendritic autoreceptor (Sotelo *et al.*, 1990; Riad *et al.*, 2000), which regulates the activity of the entire serotonin system (Pineyro & Blier,

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1999). The 5-HT1A receptor is also expressed as a heteroreceptor on non-serotonergic neurons in the hippocampus, septum, hypothalamus and cortex to mediate serotonin action (Albert *et al.*, 1990; Pompeiano *et al.*, 1992). Mice lacking 5-HT1A receptors display increased anxiety and fear behaviors, altered sleep patterns and reduced behavioral and neurogenic responses to antidepressants (Boutrel *et al.*, 2002; Gross *et al.*, 2002; Santarelli *et al.*, 2003; Toth, 2003; Tsetsenis *et al.*, 2007). The anxiety phenotype can be rescued by early post-natal expression of 5-HT1A receptors in the forebrain (Gross *et al.*, 2002), suggesting that 5-HT1A receptors mediate early developmental events that set the anxiety phenotype in the adult (Faber & Haring, 1999; Gross & Hen, 2004; Scott & Deneris, 2005; Whitaker-Azmitia, 2005; Alexandre *et al.*, 2006).

In order to elucidate the transcriptional mechanisms that regulate 5-HT1A receptor expression, we have characterized its promoter and identified specific DNA elements that regulate this gene (Albert & Lemonde, 2004). The rat and human 5-HT1A receptor genes are negatively regulated by a strong repressor element, the 31-bp dual repressor element (DRE), located between −1590 and −1519 bp upstream of the rat 5-HT1A translation start site (Ou *et al.*, 2000). Using the DRE as the target sequence in yeast one-hybrid cloning, we identified Freud-1/CC2D1A (Ou *et al.*, 2003; Rogaeva *et al.*, 2007a), which binds to the 5′ 14-bp segment [5′-repressor element (FRE)] within the 5-HT1A DRE and represses the 5-HT1A promoter in raphe RN46A cells, a model of 5-HT1A autoreceptor regulation. However, in non-neuronal L6 myoblasts and other neuronal cell lines, a second protein binds to the 3′ 12-bp [3′-repressor element (TRE)] portion of the DRE to mediate dual repression (Ou *et al.*, 2000). Mutations in the 14-bp segment (FRE) eliminated the binding of Freud-1 and derepressed 5-HT1A receptor expression in RN46A cells. However, in L6 myoblast cells, mutation of both FRE and TRE was required to eliminate DRE-dependent repression of 5-HT1A promoter activity. Thus, although Freud-1 regulates the basal expression of 5-HT1A receptors in raphe RN46A cells, a second unknown repressor mediates dual repression of the 5-HT1A receptor gene in several other cell types.

In the present study we identify the Freud-1-related transcription factor Freud-2/CC2D1B as the second component that binds to the 5-HT1A–DRE. These results, together with our previous results showing the role of human Freud-2 in the regulation of the human 5-HT1A promoter (Hadjighassem *et al.*, 2009), implicate Freud-2 as a key repressor of 5-HT1A receptor expression in brain.

Materials and methods

Polymerase chain reaction and plasmids

A 2.5-kb fragment of mouse Freud-2 cDNA was amplified from an NIH-3T3 cDNA library (Clontech) using specific primers: forward: 5′-CCGCTCGAGCGGCAGGCCCCAGGCTCCAGGACC-3′; reverse: 5′-CCGGAATTCCGGATGCCAGGGCCAAGACCTCG-3′. The polymerase chain reaction products were gel purified and subcloned in pGEMT-Easy vector (Promega, Madison, WI, USA). Freud-2 expression plasmids were created by subcloning the coding sequence of mouse Freud-2 from pGEMT-Easy vector to the EcoRI/XhoI site in either pcDNA3

(Invitrogen, Burlington, ON, Canada) or pGEX-4T-1 (Amersham Bioscience, Baie d'Urfe, QC, Canada). All constructs were verified by DNA sequence analysis.

Expression of Freud-2 protein

In vitro transcription and translation of recombinant glutathione-S-transferase (GST)–Freud-2 or GST were performed using the EcoProT7 system (Novagen, Madison, WI, USA). Briefly, the desired amount of Freud-2 expression vector and vector alone as a control were combined with the EcoPro extract, methionine and water based on the manufacturer's protocol and incubated for 60 min at 37 °C. The expression of Freud-2 was tested by western blot assay using specific peptide antibody against the Freud-2 protein.

Nuclear extracts and electrophoretic mobility shift assay

Nuclear proteins were extracted from L6 cells as previously described (Lemonde *et al.*, 2004). For electrophoretic mobility shift assay, sense and antisense oligonucleotides of the 5′ or 3′ rat 5-HT1A–DRE with CC/GG 3′-overhang were hybridized and labeled with [α-^{32}P]-dCTP using Klenow fragment DNA polymerase (Ou *et al.*, 2000). Labeled probe was incubated with L6 protein (60 μg/reaction or *in vitro* transcribed mouse Freud-2 or vector as control), with or without competitor DNA in a 25-μL reaction containing gel shift DNA binding buffer (20 mM HEPES, 0.2 mM EDTA, 0.2 mM EGTA, 100 mM KCl, 5% glycerol and 2 mM dithiothreitol, pH 7.9) and 2 μg poly(deoxy(I-C)) at 22 °C. Unlabeled double-stranded 31-bp DRE as well as 12-bp and 14-bp segments of the 31-bp DRE (Ou *et al.*, 2000) were used as competitors. For supershift assay, polyclonal rabbit anti-Freud-2 antibody (C-F2) was generated against a peptide from a common immunogenic region between human and mouse Freud-2 near the C-terminus (DGRKPTGGKLF) (Fig. 1) with an N-terminal cysteine residue added for conjugation with maleimide-activated keyhole limpet hemocyanin. In addition, for western blot of cell extracts, a polyclonal rabbit antibody was generated against recombinant bacterially-purified human GST–Freud-2 (Cedarlane). The C-F2 and full-length Freud-2 antibodies were purified using the Montage antibody purification PROSEP-G spin column (Millipore). Specificity of the C-F2 antibody was determined by enzyme-linked immunosorbent assay using bacterially- expressed human and mouse GST–Freud-2, and by immunoprecipitation of L6 cell extracts compared with pre-immune serum. The full-length Freud-2 antibody was screened for specificity by western blot using bacterially-expressed GST–Freud-2; no cross-reactivity with GST–Freud-1 was detected. Previously we showed that this antibody to full-length Freud-2, but not anti-GST or pre-immune serum, specifically recognizes Freud-2 in electrophoretic mobility shift assay supershift, western blot, immunohistochemistry and immunofluorescence (Hadjighassem *et al.*, 2009). Purified C-F2 antibody (3 μL) was used in a 25-μL reaction and incubated for 20 min at 37 °C. [^{32}P]-labeled 31-DRE probe (60 000–100 000 dpm/sample) was added and incubated for more 20 min at room temperature. The DNA/protein complexes were separated on a 5% polyacrylamide gel at 4 °C, gel dried and exposed to film overnight at 80 °C with an intensifying screen.

Northern blot and western blot analyses

Mouse Multiple Tissues Northern blot (MTN#7762-1; Clontech Laboratories, Mountain View, CA, USA) was used. It was probed with an 800-bp mouse Freud-2 cDNA fragment

using a Strip-EZ DNA kit (Ambion, Austin, TX, USA). Northern blot assay was performed as described previously (Mao *et al.*, 2004). For western blot analysis, tissues were dissected from male C57BL6 mice and homogenized in homogenization buffer [10 mM Tris, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM protease inhibitor (Roche)] on ice. Homogenized tissues were filtered and centrifuged at 4 °C for 5 min at 200 ***g*** followed by the addition of 2% sodium dodecyl sulfate and 1% Nonidet P-40. Samples were sonicated on ice (three times, power setting 3, 10 s, 10 s off) and centrifuged at 10 000 ***g*** for 10 min at 4 °C. The supernatant was then transferred to new tubes and centrifuged at 10 000 ***g*** for 15 min at 4 °C. Supernatants were then transferred to new tubes and kept at −80 °C. Extracts (60 μg) were loaded on 8% sodium dodecyl sulfate gel, electrophoresed and blotted onto nitrocellulose membrane. The membrane was incubated in 5% western blocking reagent (Roche) in 1 × Tris-buffered saline at 4 °C overnight, and then incubated with PROSEP-G-purified anti-full-length Freud-2 antibody (1 : 2000) overnight. The membrane was then incubated with horseradish peroxidase-linked anti-rabbit (1 : 2000; New England Biolabs, Pickering, ON, Canada) as secondary antibody for 30 min at 22 °C, washed and the reactive bands were visualized using the enhanced chemiluminescence protocol (Amersham) and exposure to film. 5-HT1A receptors were detected using 1 : 500 rabbit polyclonal anti-5-HT1A antibody raised against the second intracellular domain of the rat 5-HT1A receptor (Cedarlane) (Jacobsen *et al.*, 2008). The 5-HT1A antibody specifically recognized the 5-HT1A receptor in western blot of 5-HT1A-negative human embryonic kidney 293 cells transfected with rat 5-HT1A receptor cDNA vs. vector-transfected cells. Blots were reprobed with 1 : 2000 anti-beta actin antibody (Sigma) as loading control.

Cell culture and transient transfection

The L6 myoblast and NG108-15 cells were cultured and transfected as previously described (Ou *et al.*, 2000). Briefly, cells were grown in Dulbecco's modified Eagle's medium (Life Technologies, Gaithersburg, MD, USA) containing 10% fetal calf serum at 37 °C in 5% CO_2. The medium was replaced 12 h before transfection and cells (except NG108-15) were transiently transfected by calcium phosphate coprecipitation (Charest *et al.*, 1993) using 20 μg/plate of luciferase constructs and 10 μg/plate of beta-galactosidase expression plasmid pCMVβgal. NG108-15 cells were transfected by Lipofectamine2000 reagent (Invitrogen) at 50–60% confluency in Primaria six-well plates (Falcon, Franklin Lakes, NJ, USA) with 1.5 μg plasmid/well. RN46A cells were cultured as previously described (Ou *et al.*, 2000). RN46A cells were transfected with a 1 : 1.5 ratio of plasmid : Lipofectamine2000 reagent (Invitrogen) using 7.5–10 μg/plate of luciferase plasmid and an equal amount of protein expression construct or empty vector, with 2 μg/plate pCMVβgal.

Luciferase and β-galactosidase assay

The 5-HT1A promoter luciferase reporter plasmid constructs have been described previously (Ou *et al.*, 2000; Lemonde *et al.*, 2004). For reporter assays, triplicate samples after 48 h of transfection were washed three times with cold phosphate-buffered saline and extracted with 150 μL of reporter lysis buffer (Promega). Supernatants were collected, assayed for luciferase activity using Spectramax M2 (Molecular Devices, Sunnyvale, CA, USA) and measured by Softmax Pro 4.8. Activities were obtained from at least three independent experiments in which transfections were performed in triplicate and corrected for

transfection efficiency by calculating the ratio of luciferase : β-galactosidase activity and normalized to vector-transfected extracts. Data are presented as mean ± SEM. Statistical significance was evaluated using a two-tailed unpaired *t*-test or one-way ANOVA, with Newman–Keuls post-test.

Short-interfering RNA transfection

Stealth short-interfering RNA (siRNA) targeting human Freud-2 (CC2D1BHSS153336) (5′-CCCUGCAGCAGAGGCUGAACAAGUA-3′) and stealth short-interfering RNA negative control duplexes were used (Invitrogen). NG108-15 cells were transfected using Lipofectamine2000 (Invitrogen) with a final siRNA concentration of 100 nM. For luciferase assay, 5 μL of specific Freud-2 siRNA or siRNA negative control (CG scrambled) was cotransfected with 1.5 μg of rat 5-HT1A luciferase construct (5-HT1A) in NG108-15 cells and incubated for 72 h. Luciferase activity was normalized to that of β-galactosidase and normalized to control. All data are presented as the mean ± SEM of at least three independent experiments. For western blot, cells were processed as described above.

Chromatin immunoprecipitation

Chromatin immunoprecipitation assays were performed as described previously (Rogaeva & Albert, 2007). Briefly, cells were washed 3 × in phosphate-buffered saline and cross-linked for 15 min at 25 °C in phosphate-buffered saline, 1 mM $MgCl_2$ and 1% formaldehyde. Cell lysates were sheared with 212–300-μm glass beads added using a 60 Sonic Dismembrator (Fisher, Ottawa, ON, Canada) (15 × 20 s each), immunoprecipitated overnight at 4 °C with anti-full-length Freud-2 (1 : 2000) and decross-linked (16 h, 65 °C), followed by 1-h proteinase K treatment (Sigma) and DNA purification. The polymerase chain reaction primers designed to amplify a 265-bp region containing 5-HT1A 5′-DRE and 3′-DRE were: forward: 5′-ATTTTATAGAGTCTTTGGGTTTTC; reverse: 5′-AGGGCTTCTTTTTCTATGGAC. The amplification cycles were 94 °C for 3 min, 40 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 20 s, and termination was at 72 °C for 10 min using a Rotor-Gene RG-3000 cycler (Corbett Life Science, Sydney, Australia).

Results

Freud-2: molecular cloning and domains

To identify the second protein that binds the 5-HT1A DRE, we repeated the yeast one-hybrid screening of a murine brain cDNA library, but did not identify any new positive clones. Screening of the Genbank database for Freud-1 homologs using the Blast2 sequence search engine identified Freud-2/CC2D1B cDNA, which encodes an 812-amino acid protein with 50% amino acid identity to mouse Freud-1/CC2D1A (long form) and 80% amino acid identity to human 216 M. R. Hadjighassem *et al.* Freud-2 (Fig. 1). The highest similarity was within the conserved domains including the four DM-14 domains, a helix-loop-helix (HELIX-LOOP-HELIX) domain, and a protein kinase C conserved domain (C2) (Fig. 1). The DM-14 domain is characteristic of the Freud gene family, but its function remains unknown (Rogaeva *et al.*, 2007a). The C2 domain (protein kinase C conserved region 2) mediates calcium-dependent lipid binding of protein kinase C, phospholipases, and protein–protein interactions (Nalefski & Falke, 1996; Sondermann & Kuriyan, 2005), and is required

for DNA binding and repression by Freud-1 (Ou *et al.*, 2003). Thus, Freud-2 is the second member of the Freud gene family.

Freud-2 binding to the rat serotonin-1A 31-bp dual repressor element

Like Freud-1, we hypothesized that Freud-2 binds to the 5-HT1A–DRE. To address this possibility, *in vitro* transcribed and translated GST–Freud-2 cDNA or GST vector control was incubated with labeled 5-HT1A–DRE for electrophoretic mobility shift assay (Fig. 2A). A single specific protein–DNA complex was detected with GST–Freud-2, but not GST, and this complex was competed by unlabeled 5-HT1A–DRE oligonucleotides (lane 3 vs. 2), indicating a specific interaction. The Freud-2–DRE complex was also competed by either a 50- or 100-fold molar excess of unlabeled 12-bp (TRE) oligonucleotides (lanes 4 and 5), but not with excess 14-bp FRE primers (lanes 6 and 7), suggesting that Freud-2 binds preferentially to the 12-bp (TRE) portion of 5-HT1A–DRE. Anti-GST antibody did not shift the complex in this assay. We further examined whether Freud-2 is the second protein in L6 cells that binds to 5-HT1A DRE in non-neuronal cells. Nuclear extract from L6 myoblasts was incubated with labeled 5-HT1A–DRE in the presence or absence of C-F2 anti-Freud- 2 antibody in electrophoretic mobility shift assay (Fig. 2B). Two protein–DNA complexes were detected. As reported previously, Freud-1 forms the lower protein–DNA complex and is displaced using anti-Freud-1 antibody (Ou *et al.*, 2000, 2003). In the presence of anti-Freud-2 antibody only the upper complex was super-shifted, indicating that Freud-2 is the second protein that binds to 5-HT1A 31-bp DRE, recognizing the 12-bp TRE segment.

Freud-2 mRNA and protein distribution

To address the distribution of Freud-2 in tissues, we examined the RNA and protein expression profile of Freud-2 using northern and western blot analyses, respectively (Fig. 3). Freud-2 mRNA was ubiquitously expressed in mouse brain and peripheral tissues, with the highest levels in testes (Fig. 3A). Freud-2 protein was detected with anti-C-F2 antibody, which is selective for Freud-2 and did not recognize Freud-1 (data not shown). Freud-2 protein was detected as a 120-kDa species in all brain and tissue regions examined, including the cortex and cerebellum, as well as in the midbrain (Fig. 3B), consistent with the apparent molecular weight of 120–130 kDa observed for the predominant long form of Freud-1 (Rogaeva & Albert, 2007). Although both long and short Freud-2 cDNA species have been reported in GenBank, we did not detect the short isoform of Freud-2 in these tissues. Taken together, the tissue distribution of Freud-2 RNA and protein in mouse is widespread, consistent with a general role for Freud-2 in repressing 5-HT1A receptor expression.

Freud-2 repression of serotonin-1A heteroreceptor expression

The transcriptional regulatory activity of Freud-2 was compared in non-neuronal 5-HT1A-negative L6 myoblasts, 5-HT1A-expressing NG108-15 (heteroreceptor model) and RN46A neuronal cells (autoreceptor model) (Storring *et al.*, 1999; Ou *et al.*, 2000; Czesak *et al.*, 2006). L6 cells were cotransfected with Freud-2 or vector and 2300-bp rat 5-HT1A promoter–luciferase reporter constructs containing the DRE (Fig. 4). These reporter constructs included the wild-type sequence (5-HT1A) or the corresponding 5-HT1A–DRE mutants m1, m2 and m3, which lack the FRE site, TRE site or both FRE and TRE sites (Ou

et al., 2000). Freud-2 significantly repressed the activity of the wild-type 5-HT1A–luciferase construct in L6 cells compared with control. Mutation in either the 14-bp FRE that recognizes Freud-1 or the 12-bp TRE segment of 5-HT1A–DRE did not block Freud-2-induced repression (Fig. 5). However, mutation of both FRE and TRE (m3 construct) resulted in strong derepression of the basal activity of the 5-HT1A promoter, similar to the derepression observed previously upon deletion of the entire DRE (Ou *et al.*, 2000). These results are consistent with our previous data (Ou *et al.*, 2000), suggesting that in cells that lack 5-HT1A receptors, either Freud-2 or Freud-1 could suppress the transcription of the 5-HT1A receptor gene. The lack of effect of the m2 mutant on Freud-2 repression suggests that additional DNA sequences participate in Freud-2 binding and repression.

We further investigated Freud-2 activity in 5-HT1A receptor-expressing cells. In NG108-15 cells, Freud-2 significantly repressed the activity of the 5-HT1A promoter construct. Similarly, in 5-HT1A-positive H19-7 hippocampal cells (Eves *et al.*, 1992), both Freud-1 and Freud-2 strongly repressed the 5-HT1A promoter (data not shown). As observed in L6 cells, Freud-2-induced repression was not blocked in the m2 mutant of the TRE site. Similarly, the m3 mutant derepressed basal 5-HT1A promoter activity and Freud-2 repression was blocked; surprisingly, Freud-2 enhanced activity of this construct in these cells, suggesting that Freud-2 may have indirect actions at other sites of the 5-HT1A promoter. These data suggest that Freud-2 may bind to the TRE and partial sequence in the FRE that overlaps with the Freud-1 site. In RN46A cells, Freud-2 lacked repressor activity at 5-HT1A reporter constructs, in both the wild-type and m3 mutant (Fig. 6A and B). However, no repression by Freud-1 or the combination of Freud-1 and Freud-2 was observed using this construct (Fig. 6C), perhaps due to the strong basal repression in these cells. The basal repression of this construct was attenuated by mutation of the FRE (not shown) or both FRE and TRE (m3, Fig. 6B), but not TRE alone (not shown), indicating mediation by Freud-1, which recognizes the FRE (Ou *et al.*, 2003). This is consistent with our previous finding in RN46A nuclear extracts of only the Freud-1–DRE complex and no Freud-2–DRE complex (Ou *et al.*, 2000, 2003). The activity of Freud- 1 and Freud-2 was addressed in RN46A cells using the −1790Δ repressor element-1 construct, a 5-HT1A promoter construct lacking the repressor element-1 site (Lemonde *et al.*, 2004), which had greater basal activity than the complete 5-HT1A promoter construct (Fig. 6D). Both Freud-1 and Freud-2 repressed this construct, but the combination showed a level of repression consistent with Freud-1 being the dominant repressor in RN46A cells. Taken together, these data suggest that both Freud-1 and Freud-2 repress 5-HT1A transcriptional activity, but that Freud-1 appears to be the dominant repressor in RN46A cells.

Endogenous Freud-2 represses serotonin-1A receptor expression

To further address the role of endogenous Freud-2 in the regulation of 5-HT1A gene transcription and expression, we knocked down Freud-2 expression using specific siRNA against human Freud-2 (Freud-2 siRNA) in NG108-15 cells, to monitor the effects on both 5-HT1A promoter activity and endogenous 5-HT1A receptor levels. In these cells, maximal depletion was obtained using 5 μL of Freud-2 siRNA, resulting in a significant 60 ± 5% reduction in Freud-2 protein ($P < 0.01$ by one-way ANOVA, Newman–Keuls post-test) levels compared with siRNA control (Fig. 7A, inset). To assess the effects on 5-HT1A

promoter activity, NG108-15 cells were cotransfected with the 5-HT1A promoter construct and Freud-2 siRNA, scrambled siRNA–control as a negative control, or without siRNA (Fig. 7A). In the presence of Freud-2 siRNA, 5-HT1A promoter activity was significantly enhanced compared with control, indicating that reduction of Freud-2 protein levels derepresses the 5-HT1A promoter. The modest derepression observed may be due to repression by other repressors [e.g. Freud-1, repressor element-1 silencing transcription factor/neural restrictive silencing factor (REST/NRSF) (Lemonde *et al.*, 2004)] or to incomplete knockdown of Freud-2. The effect of Freud-2 knockdown on endogenous 5-HT1A receptor expression was examined by western blot (Fig. 7B). In the presence of Freud-2 siRNA, Freud-2 protein levels were decreased and 5-HT1A receptor protein was significantly increased compared with siRNA control or untreated NG108-15 cells. These data indicate that endogenous Freud- 2 represses the 5-HT1A promoter and negatively regulates the 5-HT1A receptor level in NG108-15 cells.

Finally, to address whether endogenous Freud-2 binds to the 5-HT1A DRE repressor region, chromatin immunoprecipitation assays were performed using NG108-15 cells in the presence or absence of siRNA to deplete Freud-2 (Fig. 8). In untreated cells, a strong association of Freud-2 with the 5-HT1A DRE region was observed upon immunoprecipitation with anti-Freud-2 antibody C-F2, but not in its absence. Upon treatment with Freud-2 siRNA, Freud-2 binding to the DRE region was reduced, but was unchanged in the presence of control siRNA. Taken together, these experiments indicate that endogenous Freud-2 negatively regulates 5-HT1A heteroreceptor expression in non-serotonergic cells via binding to the DRE repressor region of the 5-HT1A receptor gene.

Discussion

The 5-HT1A autoreceptors in the raphe nuclei play key roles in regulating serotonergic activity (Sotelo *et al.*, 1990; Riad *et al.*, 2000; Richardson-Jones *et al.*, 2010), whereas 5-HT1A heteroreceptors are widely expressed in the brain (Albert *et al.*, 1990; Pompeiano *et al.*, 1992) and forebrain 5-HT1A receptors are implicated in anxiety and depression (Gross *et al.*, 2002). Knockout of the 5-HT1A receptor gene results in an anxiety phenotype in several mouse strain backgrounds (Toth, 2003), whereas transgenic mice overexpressing the receptor display reduced anxiety behavior (Kusserow *et al.*, 2004). The anxiety phenotype of 5-HT1A-null mice can be rescued by early post-natal induction of forebrain 5-HT1A receptor expression, whereas rescue of receptor expression after post-natal day 21 failed to rescue the anxiety phenotype (Gross *et al.*, 2002). By contrast, knockdown of the pre-synaptic 5-HT1A receptor results in a depression-like phenotype and impaired response to antidepressant, with no anxiety phenotype (Richardson-Jones *et al.*, 2010). These studies indicate that the level of expression of 5-HT1A receptors, in the adult and during development, is a key determinant of the anxiety–depression phenotype in mice. Hence we have addressed the specific DNA elements and transcription factors that regulate 5-HT1A receptor expression.

Freud-2/CC2D1B: a repressor of serotonin-1A receptor expression

We have used 5′-deletion mapping of the 5-HT1A promoter to identify the DRE as a strong, conserved repressor element that, when deleted, leads to a 10-fold induction of 5-HT1A promoter activity in neuronal and non-neuronal cells (Storring *et al.*, 1999; Ou *et al.*, 2000). Freud- 1 binds at the 5′ portion of the DRE (FRE) to repress 5-HT1A receptor expression in a variety of cell types, including in 5-HT1A-expressing raphe RN46A cells. In this study, we identify Freud-2/CC2D1B as a repressor of 5-HT1A receptor transcription via binding to the 3′ portion of the 5-HT1A–DRE (TRE). Although Freud-2 specifically bound to the TRE (Fig. 2), mutational inactivation of the TRE (Ou *et al.*, 2000) did not block Freud-2 repression, but the combined mutation of both FRE and TRE did block Freud-2 repression (Figs 4–6). Thus, Freud-2 appears to recognize the TRE and a part of the FRE to repress the activity of the 5-HT1A receptor promoter. Freud-1 and Freud-2 binding to the DRE appears to be mutually exclusive, as in L6 cell extracts we observed distinct Freud-1–DRE (Ou *et al.*, 2003) and Freud-2–DRE (Fig. 2B) complexes, with no evidence for a third complex containing both, consistent with competition between Freud-1 and Freud-2 for binding.

Freud-2 repressed the 5-HT1A receptor promoter in 5-HT1A receptor-negative L6 cells, non-serotonergic 5-HT1A-expressing NG108-15 cells and serotonergic raphe RN46A cells (Figs 4–6). In non-serotonergic NG108-15 cells, Freud-2 regulated basal 5-HT1A receptor expression, as depletion of Freud-2 using small interfering RNA (siRNA) modestly derepressed the 5-HT1A promoter and enhanced the level of 5-HT1A protein. Freud-2 siRNA also reduced the amount of Freud-2 bound to the genomic 5-HT1A DRE region, correlating with increased 5-HT1A receptor expression and indicating that Freud-2 binding to the DRE is required to repress 5-HT1A receptors in NG108-15 cells. The limited derepression observed in Freud-2 siRNA-treated cells may reflect the partial reduction (60%) of Freud-2 protein, or repression maintained by Freud-1.

We previously found that Freud-1 represses 5-HT1A autoreceptor expression in RN46A cells, and that specific depletion of Freud-1 using transfection of antisense Freud-1 cDNA derepressed the 5-HT1A promoter to increase 5-HT1A receptor expression in RN46A cells, but not L6 cells (Ou *et al.*, 2003). This suggests that Freud-1 is a primary determinant of basal 5-HT1A receptor expression in RN46A cells. Consistent with this, Freud-1 is strongly expressed in raphe neurons and colocalized with serotonin and 5-HT1A receptor immunostaining (Ou *et al.*, 2003). By contrast, Freud-2 RNA and protein are weakly expressed in human dorsal raphe nucleus as visualized by reverse transcription–polymerase chain reaction, immunohistochemistry and western blot of post-mortem brain tissue (Hadjighassem *et al.*, 2009). Thus, based on its localization and competition by Freud-1, Freud-2 is expected preferentially to regulate 5-HT1A heteroreceptors, rather than 5-HT1A autoreceptors *in vivo*.

Both Freud-1 and Freud-2 RNA and protein are detected in a variety of brain and peripheral tissues (Ou *et al.*, 2003; Basel-Vanagaite *et al.*, 2006; Rogaeva & Albert, 2007), indicating a ubiquitous role for these factors in repression of 5-HT1A receptors. 5-HT1A receptor RNA and protein are widely expressed in brain, including the pyramidal and interneurons of the cortex (Aznar *et al.*, 2003; Palchaudhuri & Flugge, 2005) as well as in the septum, hippocampus and hypothalamus (Albert *et al.*, 1990; Pompeiano *et al.*, 1992). The

combination of Freud-1 and Freud-2 provides a dual mechanism for regulating the expression level of 5-HT1A receptors in 5-HT1A-positive neurons, where Freud-2 is expressed in non-serotonergic neuronal cells, whereas Freud-1 is expressed in both serotonergic and non-serotonergic cells. By contrast, in most peripheral tissues, 5-HT1A RNA is undetectable (Albert *et al.*, 1990). In 5-HT1A receptor-negative tissues, Freud-1 and Freud-2 may participate in silencing 5-HT1A receptor expression in concert with repressor element-1 silencing transcription factor neural restrictive silencing factor (REST/NRSF), which recognizes a consensus repressor element-1 site located immediately 3′ of the DRE to repress the 5-HT1A promoter (Lemonde *et al.*, 2004).

Potential roles of Freud-2 in vivo

As a homolog of Freud-1, Freud-2 complements Freud-1 action to repress the 5-HT1A promoter. Freud-1 also recognizes a highly conserved DRE in the second intron of the DRD2 gene to repress basal expression of the dopamine D2 receptor (Rogaeva *et al.*, 2007b). However, unlike Freud-1, Freud-2 does not bind the D2–DRE (data not shown), consistent with the poor conservation of the TRE region compared with the identical FRE region, which is nearly identical to the 5-HT1A FRE sequence. Hence Freud-2 may have additional gene targets and functions that differ from those of Freud-1.

Recent linkage analysis of non-syndromal mental retardation cohorts has revealed a deletion mutation of the Freud-1/CC2D1A gene locus in this disorder, suggesting a role for Freud-1 in cognitive development (Basel-Vanagaite *et al.*, 2006) that may be shared by Freud-2. The non-syndromal mental retardation Freud-1 mutant lacks the C-terminal portion of the protein including the protein kinase C conserved region 2, and is thus predicted to lack repressor activity, resulting in derepression of 5-HT1A and dopamine D2 receptor genes. Similarly, a mutation in Freud-2 might also result in derepression of the 5-HT1A receptor gene particularly in cortical or hippocampal neurons, which could contribute to mental retardation. Increased 5-HT1A receptor expression results in reduced anxiety, but also impaired cognitive ability (Bert *et al.*, 2005), which could contribute to mental retardation. Inactivation of the Freud-2 gene could induce post-synaptic 5-HT1A heteroreceptor expression, and may contribute to cognitive impairment and antianxiety behavior; however, to date, linkage of Freud-2 to mental retardation has not been reported.

Taken together, in this study we demonstrate that Freud-2/CC2D1B binds to the 5-HT1A–DRE at a site adjacent to and partly overlapping the Freud-1 site, and represses 5-HT1A receptor expression in non-neuronal and neuronal 5-HT1A-expressing cells. Freud-2 is expressed in many serotonin brain target regions as well as in peripheral tissues, but is weakly expressed in raphe nuclei (Hadjighassem *et al.*, 2009). These findings suggest that Freud-2 preferentially regulates 5-HT1A receptor expression in these regions compared with Freud-1, which regulates 5-HT1A receptor autoreceptor expression. Thus, these transcription factors provide complementary regulation of the 5-HT1A receptor gene.

Acknowledgments

We thank Drs Xiaoming Ou (University of Mississippi), Mohammad Farajollahi and Chris Bown for helpful discussions and Dr Farajollahi for the enzymelinked immunosorbent assay. The Canadian Institutes of Health Research grant FRN 36437 to P.R.A. supported this research.

Abbreviations

5-HT1A	serotonin-1A
C-F2	antibody to C-terminal human Freud-2 peptide
DRE	dual repressor element
FRE	5′-repressor element
GST	glutathione-Stransferase
siRNA	short-interfering RNATRE, 3′-repressor element

References

Albert PR, Lemonde S. 5-HT1A receptors, gene repression, and depression: guilt by association. Neuroscientist. 2004; 10:575–593. [PubMed: 15534042]

Albert PR, Zhou QY, Van Tol HH, Bunzow JR, Civelli O. Cloning, functional expression, and mRNA tissue distribution of the rat 5-hydroxytryptamine1A receptor gene. J Biol Chem. 1990; 265:5825–5832. [PubMed: 2156831]

Alexandre C, Popa D, Fabre V, Bouali S, Venault P, Lesch KP, Hamon M, Adrien J. Early life blockade of 5-hydroxytryptamine 1A receptors normalizes sleep and depression-like behavior in adult knock-out mice lacking the serotonin transporter. J Neurosci. 2006; 26:5554–5564. [PubMed: 16707806]

Aznar S, Qian Z, Shah R, Rahbek B, Knudsen GM. The 5-HT1A serotonin receptor is located on calbindin- and parvalbumin-containing neurons in the rat brain. Brain Res. 2003; 959:58–67. [PubMed: 12480158]

Bains W, Ponte P, Blau H, Kedes L. Cardiac actin is the major actin gene product in skeletal muscle cell differentiation in vitro. Mol Cell Biol. 1984; 4:1449–1453. [PubMed: 6493226]

Basel-Vanagaite L, Attia R, Yahav M, Ferland RJ, Anteki L, Walsh CA, Olender T, Straussberg R, Magal N, Taub E, Drasinover V, Alkelai A, Bercovich D, Rechavi G, Simon AJ, Shohat M. The CC2D1A, a member of a new gene family with C2 domains, is involved in autosomal recessive non-syndromic mental retardation. J Med Genet. 2006; 43:203–210. [PubMed: 16033914]

Bert B, Dere E, Wilhelmi N, Kusserow H, Theuring F, Huston JP, Fink H. Transient overexpression of the 5-HT1A receptor impairs water-maze but not hole-board performance. Neurobiol Learn Mem. 2005; 84:57–68. [PubMed: 15936683]

Boutrel B, Monaca C, Hen R, Hamon M, Adrien J. Involvement of 5-HT1A receptors in homeostatic and stress-induced adaptive regulations of paradoxical sleep: studies in 5-HT1A knock-out mice. J Neurosci. 2002; 22:4686–4692. [PubMed: 12040075]

Charest A, Wainer BH, Albert PR. Cloning and differentiation-induced expression of a murine serotonin1A receptor in a septal cell line. J Neurosci. 1993; 13:5164–5171. [PubMed: 8254366]

Czesak M, Lemonde S, Peterson EA, Rogaeva A, Albert PR. Cell-specific repressor or enhancer activities of Deaf-1 at a serotonin 1A receptor gene polymorphism. J Neurosci. 2006; 26:1864–1871. [PubMed: 16467535]

Eves EM, Tucker MS, Roback JD, Downen M, Rosner MR, Wainer BH. Immortal rat hippocampal cell lines exhibit neuronal and glial lineages and neurotrophin gene expression. Proc Natl Acad Sci USA. 1992; 89:4373–4377. [PubMed: 1316607]

Faber KM, Haring JH. Synaptogenesis in the postnatal rat fascia dentata is influenced by 5-HT1a receptor activation. Brain Res Dev Brain Res. 1999; 114:245–252. [PubMed: 10320763]

Gordon JA, Hen R. The serotonergic system and anxiety. Neuromolecular Med. 2004; 5:27–40. [PubMed: 15001810]

Gross C, Hen R. The developmental origins of anxiety. Nat Rev Neurosci. 2004; 5:545–552. [PubMed: 15208696]

Gross C, Zhuang X, Stark K, Ramboz S, Oosting R, Kirby L, Santarelli L, Beck S, Hen R. Serotonin1A receptor acts during development to establish normal anxiety-like behaviour in the adult. Nature. 2002; 416:396–400. [PubMed: 11919622]

Hadjighassem MR, Austin MC, Szewczyk B, Daigle M, Stockmeier CA, Albert PR. Human Freud-2/CC2D1B: a novel repressor of postsynaptic serotonin-1A receptor expression. Biol Psychiatry. 2009; 66:214–222. [PubMed: 19423080]

Jacobs BL, Azmitia EC. Structure and function of the brain serotonin system. Physiol Rev. 1992; 72:165–229. [PubMed: 1731370]

Jacobsen KX, Vanderluit J, Slack RS, Albert PR. HES1 regulates 5-HT1A receptor gene transcription at a functional polymorphism: essential role in developmental expression. Mol Cell Neurosci. 2008; 38:349–358. [PubMed: 18499474]

Kusserow H, Davies B, Hortnagl H, Voigt I, Stroh T, Bert B, Deng DR, Fink H, Veh RW, Theuring F. Reduced anxiety-related behaviour in transgenic mice overexpressing serotonin 1A receptors. Brain Res Mol Brain Res. 2004; 129:104–116. [PubMed: 15469887]

Lemonde S, Rogaeva A, Albert PR. Cell type-dependent recruitment of trichostatin A-sensitive repression of the human 5-HT1A receptor gene. J Neurochem. 2004; 88:857–868. [PubMed: 14756806]

Lesch KP. Serotonergic gene inactivation in mice: models for anxiety and aggression? Novartis Found Symp. 2005; 268:111–140. discussion 140–146, 167–170. [PubMed: 16206878]

Mao H, Zhao Q, Daigle M, Ghahremani MH, Chidiac P, Albert PR. RGS17/RGSZ2, a novel regulator of Gi/o, Gz, and Gq signaling. J Biol Chem. 2004; 279:26314–26322. [PubMed: 15096504]

Nalefski EA, Falke JJ. The C2 domain calcium-binding motif: structural and functional diversity. Protein Sci. 1996; 5:2375–2390. [PubMed: 8976547]

Ou XM, Jafar-Nejad H, Storring JM, Meng JH, Lemonde S, Albert PR. Novel dual repressor elements for neuronal cell-specific transcription of the rat 5-HT1A receptor gene. J Biol Chem. 2000; 275:8161– 8168. [PubMed: 10713139]

Ou XM, Lemonde S, Jafar-Nejad H, Bown CD, Goto A, Rogaeva A, Albert PR. Freud-1: a novel calcium-regulated repressor of the 5-HT1A receptor gene. J Neurosci. 2003; 23:7415–7425. [PubMed: 12917378]

Palchaudhuri M, Flugge G. 5-HT1A receptor expression in pyramidal neurons of cortical and limbic brain regions. Cell Tissue Res. 2005; 321:159–172. [PubMed: 15947971]

Pineyro G, Blier P. Autoregulation of serotonin neurons: role in antidepressant drug action. Pharmacol Rev. 1999; 51:533–591. [PubMed: 10471417]

Pompeiano M, Palacios JM, Mengod G. Distribution and cellular localization of mRNA coding for 5-HT1A receptor in the rat brain: correlation with receptor binding. J Neurosci. 1992; 12:440–453. [PubMed: 1531498]

Riad M, Garcia S, Watkins KC, Jodoin N, Doucet E, Langlois X, el Mestikawy S, Hamon M, Descarries L. Somatodendritic localization of 5-HT1A and preterminal axonal localization of 5-HT1B serotonin receptors in adult rat brain. J Comp Neurol. 2000; 417:181–194. [PubMed: 10660896]

Richardson-Jones JW, Craige CP, Guiard BP, Stephen A, Metzger KL, Kung HF, Gardier AM, Dranovsky A, David DJ, Beck SG, Hen R, Leonardo ED. 5-HT(1A) autoreceptor levels determine vulnerability to stress and response to antidepressants. Neuron. 2010; 65:40–52. [PubMed: 20152112]

Rogaeva A, Albert PR. The mental retardation gene CC2D1A/Freud-1 encodes a long isoform that binds conserved DNA elements to repress gene transcription. Eur J Neurosci. 2007; 26:965–974. [PubMed: 17714190]

Rogaeva A, Galaraga K, Albert PR. The Freud-1/CC2D1A family: transcriptional regulators implicated in mental retardation. J Neurosci Res. 2007a; 85:2833–2838. [PubMed: 17394259]

Rogaeva A, Ou XM, Jafar-Nejad H, Lemonde S, Albert PR. Differential repression by Freud-1/CC2D1A at a polymorphic site in the dopamine-D2 receptor gene. J Biol Chem. 2007b; 282:20897–20905. [PubMed: 17535813]

Santarelli L, Saxe M, Gross C, Surget A, Battaglia F, Dulawa S, Weisstaub N, Lee J, Duman R, Arancio O, Belzung C, Hen R. Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. Science. 2003; 301:805–809. [PubMed: 12907793]

Scott MM, Deneris ES. Making and breaking serotonin neurons and autism. Int J Dev Neurosci. 2005; 23:277–285. [PubMed: 15749252]

Sondermann H, Kuriyan J. C2 can do it, too. Cell. 2005; 121:158–160. [PubMed: 15851022]

Sotelo C, Cholley B, El Mestikawy S, Gozlan H, Hamon M. Direct immunohistochemical evidence of the existence of 5-HT1A autoreceptors on serotoninergic neurons in the midbrain raphe nuclei. Eur J Neurosci. 1990; 2:1144–1154. [PubMed: 12106075]

Storring JM, Charest A, Cheng P, Albert PR. TATA-driven transcriptional initiation and regulation of the rat serotonin 5-HT1A receptor gene. J Neurochem. 1999; 72:2238–2247. [PubMed: 10349831]

Törk I. Anatomy of the serotonergic system. Ann NY Acad Sci. 1990; 600:9–34. discussion 34–35. [PubMed: 2252340]

Toth M. 5-HT1A receptor knockout mouse as a genetic model of anxiety. Eur J Pharmacol. 2003; 463:177–184. [PubMed: 12600709]

Tsetsenis T, Ma XH, Lo Iacono L, Beck SG, Gross C. Suppression of conditioning to ambiguous cues by pharmacogenetic inhibition of the dentate gyrus. Nat Neurosci. 2007; 10:896–902. [PubMed: 17558402]

Whitaker-Azmitia PM. Behavioral and cellular consequences of increasing serotonergic activity during brain development: a role in autism? Int J Dev Neurosci. 2005; 23:75–83. [PubMed: 15730889]

Fig. 1.

Alignment of human and mouse Freud-2. Shown is the amino acid alignment of predicted amino acid sequences for human (upper sequence) and mouse (lower sequence) Freud-2, with mismatched amino acids underlined. Solid lines between arrowheads represent the DM-14 domains and the boxes show the conserved helix-loop-helix and protein kinase C conserved region 2 (C2 domains). Domains were identified by NCBI blast (http://www.ncbi.nlm.nih.gov/BLAST) conserved domain alignment. The helix-loop-helix domain was identified by HELIX-TURN-HELIX.

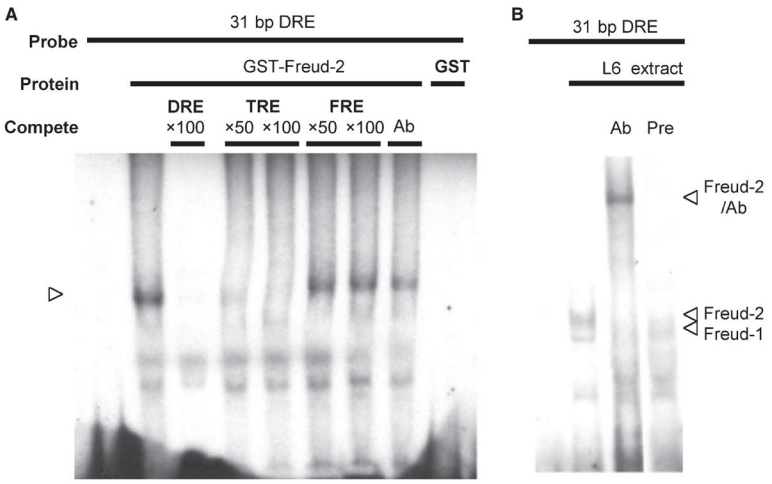

Fig. 2.

Specific binding of Freud-2 to the 5-HT1A–TRE. (A) Direct binding of recombinant Freud-2 to the 5-HT1A DRE. [^{32}P]-labeled 5-HT1A–DRE (31-bp DRE) as probe was incubated with *in vitro* transcribed and translated GST–Freud-2 (lanes 2–8) or GST vector (lane 9) as control. A single specific band (arrowhead) was detected with GST–Freud-2 (lane 2). Binding of Freud-2 to 31-DRE was abolished in the presence of a 100-fold molar excess of unlabeled DRE (lane 3) or 50- or 100-fold 12-bp TRE (3′ portion of 5-HT1A–DRE; lanes 4 and 5) but not 14-bp FRE (5′-portion of DRE; lanes 6 and 7). (B) Freud-2 is the second DRE-binding protein complex in L6 cells. L6 nuclear extracts were incubated with [^{32}P]-labeled 31-bp 5-HT1A DRE oligonucleotides and two protein–DNA complexes were observed; the lower band represents Freud-1 (as shown previously) and the upper complex contains Freud-2 (arrowhead) as shown by supershift of that complex after incubation using the C-F2 antibody (Materials and methods) against Freud-2 (Freud-2/Ab, arrowhead), but not using pre-immune antibody (Pre).

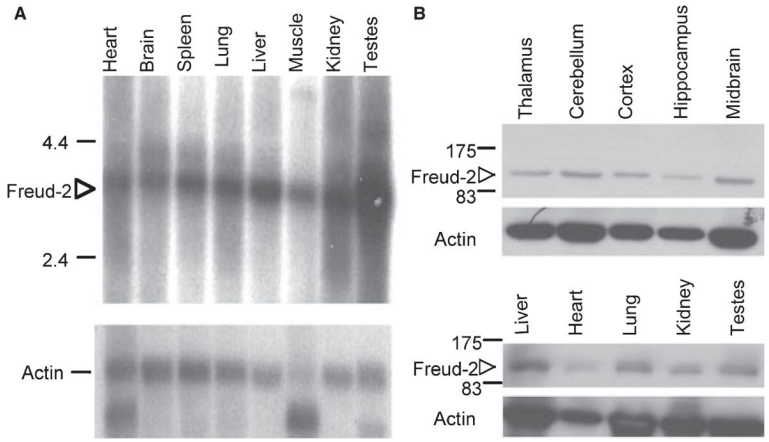

Fig. 3.

Tissue distribution of Freud-2 RNA and protein expression. (A) Freud-2 mRNA expression in mouse tissues. RNA was prepared from the indicated mouse tissues for northern blot analysis using mouse Freud-2 cDNA probe. An arrowhead indicates Freud-2 RNA hybridization, which migrated with an approximate size of 4 kb. The blot was reprobed with beta-actin cDNA as a loading control; the faster migrating species in muscle samples represents alpha-actin cross-hybridization (Bains *et al.*, 1984). (B) Freud-2 protein expression in mouse brain. The indicated regions of mouse brain (above) or peripheral tissue (below) were homogenized and subjected to western blot analysis. Freud-2 protein expression (arrowhead) was detected by antibody against full-length Freud-2. The blots were reprobed with antibody to beta-actin as loading control.

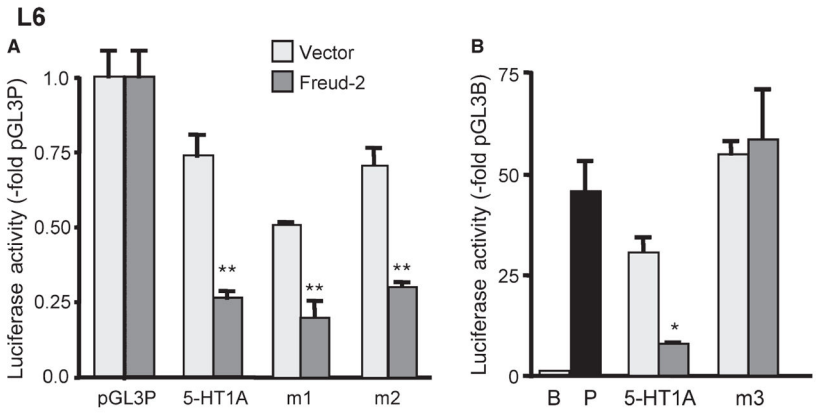

Fig. 4.

Repression of the rat 5-HT1A receptor gene by Freud-2 protein in L6 myoblast cells. (A) Freud-2 repression at the 5-HT1A–DRE. The DRE-containing 2300-bp rat 5-HT1A promoter luciferase reporter pGL3B construct (5-HT1A), or inactivating mutations of the 14-bp FRE (m1), 12-bp TRE (m2) or double mutant of FRE and TRE (m3) were transiently transfected in L6 myoblast cells in the presence of vector (pcDNA3) or Freud-2; relative luciferase activity was measured and normalized to pGL3P SV40 positive control (P) or luciferase vector pGL3B (B). Freud-2 significantly repressed the transcriptional activity of 5-HT1A receptor gene, and this suppression was eliminated in the presence of FRE + TRE mutant (m3). Variation in transfection efficiency was corrected by cotransfection of a β-galactosidase plasmid with each construct. Data represent the mean + SEM of three independent experiments. $^{**}P < 0.01$ compared with vector-transfected and $^{*}P < 0.05$ (m3 vs. 5-HT1A) by one-way ANOVA, Newman–Keuls post-test.

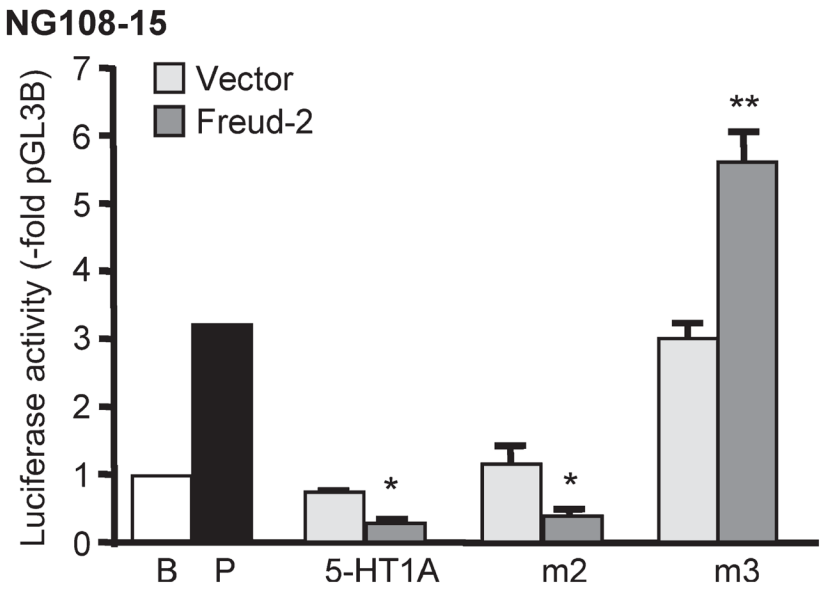

Fig. 5.

Repression of 5-HT1A receptor gene by Freud-2 in NG108-15 neuroglioma cell line. The 2300-bp 5-HT1A promoter luciferase construct (5-HT1A), m2 (TRE mutant) and m3 (double FRE/TRE mutant) were transiently transfected with vector (pcDNA3) or Freud-2 in NG108-15 cells. Luciferase activity was normalized to luciferase vector pGL3B (B); pGL3P was positive control (P). Freud-2 repressed the 5-HT1A and m2 promoter constructs. Note that the 5-HT1A receptor promoter was derepressed in the m3 double mutant and that Freud-2 further enhanced its activity. Data represent the mean + SEM of three independent experiments; $^{**}P < 0.005$, $^{*}P < 0.05$ compared with vector-transfected by unpaired *t*-test.

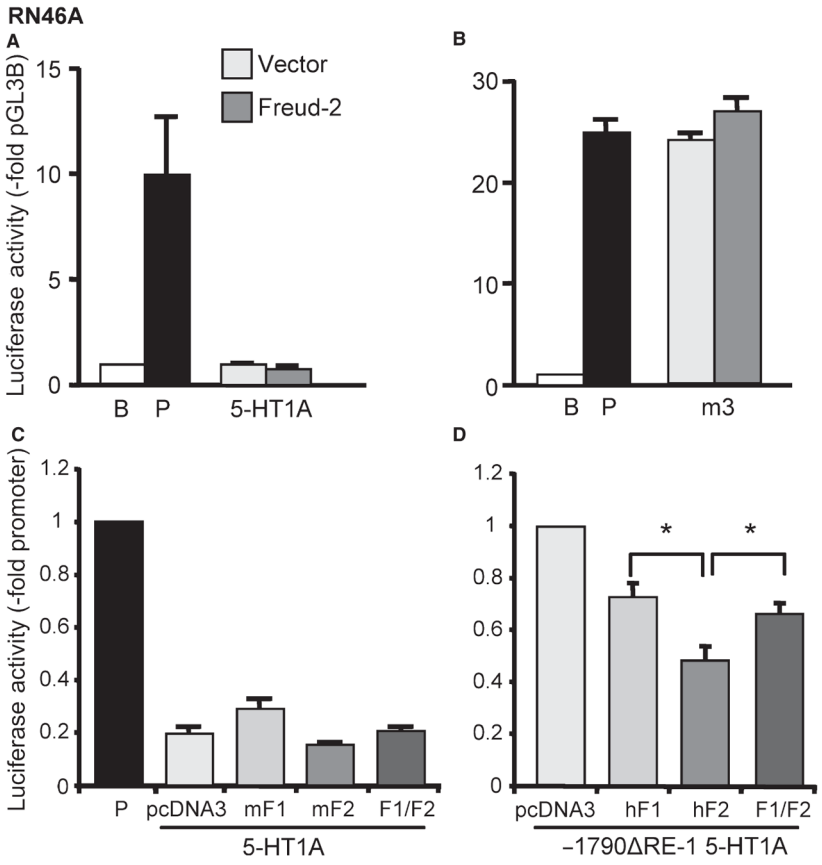

Fig. 6.

Freud-1 and Freud-2 repression in RN46A raphe cells. The 2300-bp rat 5-HT1A (A and C), m3 double FRE/TRE mutant (B) and human -1790ΔRE-1 (lacking repressor element-1 repressor site, D) reporter constructs were transfected in RN46A raphe cells with vector (pcDNA3), mouse or human Freud-1 (mF1, hF1), mouse or human Freud-2 (mF2, hF2) or both (F1/F2) expression plasmids. Luciferase activity was normalized to pGL3B (B); pGL3P was positive control (P). Data represent the mean + SEM of at least three independent experiments for each panel. For D: $P < 0.01$ for hF1, hF2 or F1/F2 compared with pcDNA3; *$P < 0.05$ between hF2 and hF1 or F1/F2 by one-way ANOVA, with Newman–Keuls post-test.

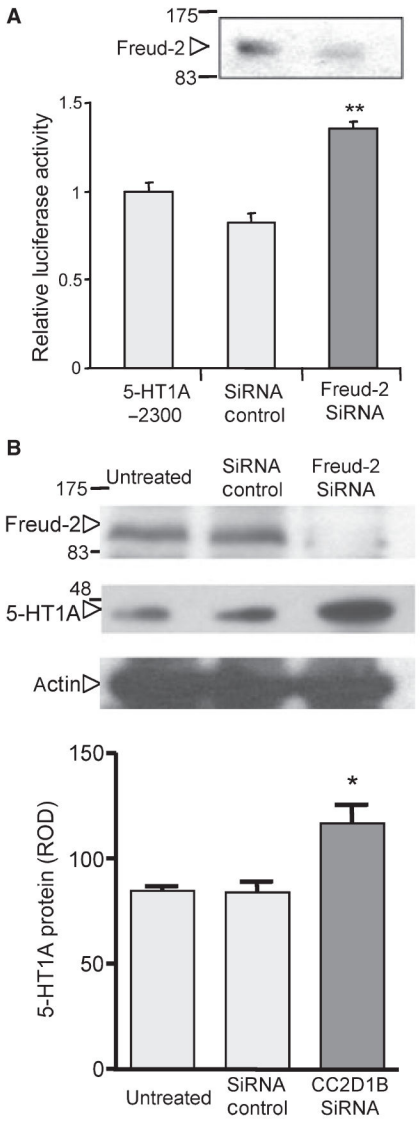

Fig. 7.

Freud-2 depletion by siRNA derepresses the 5-HT1A promoter and increases 5-HT1A receptor expression in NG108-15 neuroglioma cells. (A) Derepression of 5-HT1A promoter activity by Freud-2 siRNA. Rat 2300-bp 5-HT1A luciferase construct (1.5 μg) was transfected alone (5-HT1A) or cotransfected with 5 μL of specific Freud-2 siRNA36 (Freud-2 siRNA) or CGscrambled siRNA (control) in NG108-15 cells and incubated for 72 h. Inset: western blot using antibody to full-length Freud-2 of cell extracts from cells transfected with control vs. Freud-2 siRNA. Luciferase activity is expressed relative to 5-HT1A reporter alone. Data represent the mean + SEM of three independent experiments. $^{**}P < 0.01$ in comparison to control (5-HT1A-2300) or siRNA control by one-way ANOVA, Newman–Keuls post-test. (B) Increased level of 5-HT1A protein upon depletion of Freud-2 by siRNA. NG108-15 cells were transfected with siRNA control, siRNA to Freud-2 or not transfected (untreated), and whole cell extracts were examined by western blot using antibodies to Freud-2 (1 : 2000), 5-HT1A and actin as indicated. Below, the level of 5-HT1A

protein in three independent experiments is plotted as mean + SE. A significant increase in 5-HT1A receptor protein was observed in Freud-2 siRNA vs. siRNA control or untreated (*$P < 0.05$, one-way ANOVA, Newman–Keuls post-test), indicating that depletion of Freud-2 induces an increase in 5-HT1A receptors.

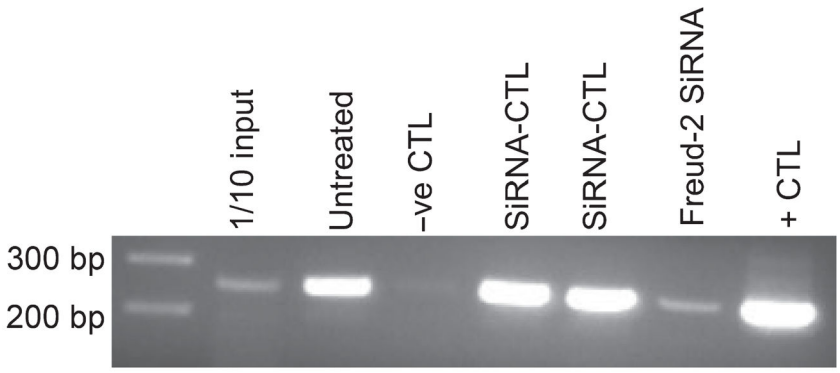

Fig. 8.

Endogenous Freud-2 is bound to the DRE of the rat 5-HT1A receptor gene. NG108-15 cells grown in 10-cm plates were untreated or treated with siRNA control (siRNA-CTL, two plates) or Freud-2 siRNA (Freud-2 siRNA), incubated for 72 h and analyzed by chromatin immunoprecipitation (ChIP) assay using anti-Freud-2 antibody (1 : 2000) and amplifying a region containing the 5′-DRE and 3′-DRE of the rat 5-HT1A receptor gene. As controls, ChIP assay was performed on untreated NG108-15 cells in the absence (−ve CTL) of anti-Freud-2 antibody, with 1/10 dilution as input; rat 2300-bp 5-HT1A promoter luciferase plasmid (15 ng) was amplified as positive control (+CTL) and revealed a specific 265-bp band of amplified DNA containing the 5-HT1A 5′-DRE and 5-HT1A 3′-DRE. Immunoprecipitation using anti-Freud-2 antibody revealed the association of endogenous Freud-2 with the genomic 5-HT1A–DRE, which was reduced in the presence of Freud-2 siRNA.