Physical interaction between neurofibromin and serotonin 5-HT$_6$ receptor promotes receptor constitutive activity

Wissem Deraredj Nadim[a], Séverine Chaumont-Dubel[b], Fahima Madouri[a], Laetitia Cobret[a], Marie-Ludivine De Tauzia[a], Pawel Zajdel[c], Hélène Bénédetti[a], Philippe Marin[b], and Séverine Morisset-Lopez[a,1]

[a]Centre de Biophysique Moléculaire, CNRS, Unité Propre de Recherche 4301, Université d'Orléans, 45071 Orleans, France; [b]Institut de Génomique Fonctionnelle, CNRS UMR 5203, INSERM U1191, Université de Montpellier, F-34094 Montpellier Cedex 5, France; and [c]Department of Medicinal Chemistry, Jagiellonian University Medical College, 30-688 Kraków, Poland

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Active G protein-coupled receptor (GPCR) conformations not only are promoted by agonists but also occur in their absence, leading to constitutive activity. Association of GPCRs with intracellular protein partners might be one of the mechanisms underlying GPCR constitutive activity. Here, we show that serotonin 5 hydroxytryptamine 6 (5-HT$_6$) receptor constitutively activates the Gs/adenylyl cyclase pathway in various cell types, including neurons. Constitutive activity is strongly reduced by silencing expression of the Ras-GTPase activating protein (Ras-GAP) neurofibromin, a 5-HT$_6$ receptor partner. Neurofibromin is a multidomain protein encoded by the *NF1* gene, the mutation of which causes Neurofibromatosis type 1 (NF1), a genetic disorder characterized by multiple benign and malignant nervous system tumors and cognitive deficits. Disrupting association of 5-HT$_6$ receptor with neurofibromin Pleckstrin Homology (PH) domain also inhibits receptor constitutive activity, and PH domain expression rescues 5-HT$_6$ receptor-operated cAMP signaling in neurofibromin-deficient cells. Furthermore, PH domains carrying mutations identified in NF1 patients that prevent interaction with the 5-HT$_6$ receptor fail to rescue receptor constitutive activity in neurofibromin-depleted cells. Further supporting a role of neurofibromin in agonist-independent Gs signaling elicited by native receptors, the phosphorylation of cAMP-responsive element-binding protein (CREB) is strongly decreased in prefrontal cortex of *Nf1*$^{+/-}$ mice compared with WT mice. Moreover, systemic administration of a 5-HT$_6$ receptor inverse agonist reduces CREB phosphorylation in prefrontal cortex of WT mice but not *Nf1*$^{+/-}$ mice. Collectively, these findings suggest that disrupting 5-HT$_6$ receptor–neurofibromin interaction prevents agonist-independent 5-HT$_6$ receptor-operated cAMP signaling in prefrontal cortex, an effect that might underlie neuronal abnormalities in NF1 patients.

5-HT$_6$ receptor | constitutive activity | G protein-coupled receptor | neurofibromin | neurofibromatosis type 1

Among 14 serotonin [5 hydroxytryptamine (5-HT)] receptor subtypes, the 5-HT$_6$ receptor has emerged as a promising target for the treatment of cognitive impairment associated with several neuropsychiatric disorders, including Alzheimer's disease and schizophrenia: 5-HT$_6$ receptor antagonists consistently enhance mnemonic performance in a broad range of procedures in rodents, and there is preliminary evidence for procognitive properties of 5-HT$_6$ receptor antagonists and/or inverse agonists in humans (1–3).

The 5-HT$_6$ receptor is a Gs-coupled receptor that activates cAMP formation on agonist stimulation in several recombinant systems (4–6) as well as in native systems, such as primary neurons (7) and pig caudate membranes (8). In addition to its coupling to G proteins, the 5-HT$_6$ receptor interacts with the Src family tyrosine kinase Fyn (9), the Jun activation domain-binding protein 1 (10), and the microtubule-associated protein Map1b (11). The 5-HT$_6$ receptor also recruits the mammalian Target of Rapamycin (mTOR) Complex 1, and receptor-operated activation of mTOR signaling in prefrontal cortex (PFC) mediates its deleterious influence on cognition (12). Furthermore, 5-HT$_6$ receptors associate with and activate Cyclin-dependent kinase 5 (Cdk5) in an agonist-independent manner through mechanisms involving receptor phosphorylation by associated Cdk5 to promote migration of neurons and neurite growth (13, 14).

Constitutive activity of 5-HT$_6$ receptor was also established at Gs signaling in recombinant cells overexpressing WT or mutant receptors (5, 6), but the underlying mechanism remains to be established. In light of recent evidence indicating that G protein-coupled receptor (GPCR) constitutive activity can be modulated by G protein-coupled receptor-interacting proteins (GIPs) (15), we focused on neurofibromin, another 5-HT$_6$ receptor partner known to be involved in adenylyl cyclase activation by various GPCRs (12, 16). Neurofibromin is an Ras GTPase-activating protein (Ras-GAP) encoded by the tumor suppressor gene *NF1*. Mutations of the *NF1* gene cause Neurofibromatosis type 1 (NF1), one of the most common autosomal dominant diseases characterized by skin pigmentation (cafe au lait spots and freckling), multiple benign and malignant nervous system tumors, and learning and attention deficits (17). Learning deficits are observed in *Nf1* heterozygous mice (*Nf1*$^{+/-}$) (17, 18) and *Nf1* null *Drosophila melanogaster* (19). Notably, learning impairments in *Nf1* null flies are rescued by expression of a constitutively active form of PKA, suggesting that they are caused by decreased activation of adenylyl cyclase (19). Whether 5-HT$_6$ receptors contribute to neurofibromin-dependent cAMP production remains to be explored. Likewise, the role of neurofibromin association with 5-HT$_6$ receptor in receptor constitutive activity remains to be established.

Here, we show that constitutive activity of 5-HT$_6$ receptor at Gs signaling is critically dependent on a physical interaction between the receptor C-terminal domain (CTD) and the

Significance

This study provides evidence of a physical interaction between neurofibromin, an Ras-GTPase activating protein, and a G protein-coupled receptor (GPCR), the serotonin 5 hydroxytryptamine 6 (5-HT$_6$) receptor. It also shows that constitutive activity of a GPCR depends on its association with GPCR-interacting proteins. Finally, it suggests that the 5-HT$_6$ receptor may be considered as a potentially relevant therapeutic target to correct some neurofibromatosis type 1-related cognitive deficits, a genetic disorder caused by mutations in the gene encoding neurofibromin.

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[1]To whom correspondence should be addressed. Email: severine.morisset-lopez@cnrs-orleans.fr.

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neurofibromin Pleckstrin Homology (PH) domain. Moreover, mutations located in the PH domain identified in NF1 patients, which disrupt the association of 5-HT$_6$ receptor with neurofibromin, strongly inhibit agonist-independent receptor-operated Gs signaling that is also impaired in a mouse model of NF1. This study identifies the 5-HT$_6$ receptor–neurofibromin interaction as a molecular substrate that might contribute to neuronal abnormalities and cognitive impairment observed in NF1 patients.

Results

5-HT$_6$ Receptor Recruits Neurofibromin via Its PH Domain and CTD. Our previous studies of the 5-HT$_6$ receptor interactome identified neurofibromin as a candidate receptor partner (12). Immunoprecipitation followed by Western blot analysis confirmed the interaction of endogenously expressed neurofibromin with human (HA)-tagged 5-HT$_6$ receptor expressed in neuroblastoma–glioma NG108-15 cells (Fig. 1*A*). Native 5-HT$_6$ receptor also coimmunoprecipitated with neurofibromin in protein extracts from mice striatum, the brain region expressing the highest receptor density (Fig. 1*A*). Because no 5-HT$_6$ receptor antibody that efficiently immunoprecipitates the mouse receptor was available, we immunoprecipitated 5-HT$_6$ receptors from knock-in mice expressing a GFP-tagged version of the receptor (5-HT$_6$-GFP) using a GFP nanobody and found that neurofibromin coimmunoprecipitates with 5-HT$_6$-GFP receptor (Fig. S1*A*). Furthermore, 5-HT$_6$-GFP receptors and neurofibromin are colocalized at the surface of cell bodies of neurons from various brain regions, including the striatum and the CA3 area of the hippocampus (Fig. S1*B*). Collectively, these findings indicate that native 5-HT$_6$ receptors form complexes with neurofibromin in vivo.

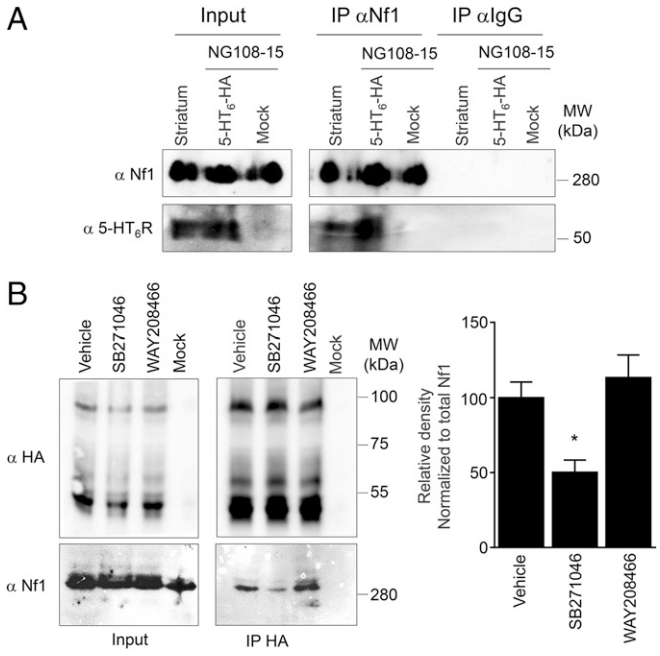

Fig. 1. Association of 5-HT$_6$ receptor with neurofibromin PH domain is dependent on receptor conformational state. (*A*) Coimmunoprecipitation of neurofibromin with native 5-HT$_6$ receptor from mouse striatum and human HA-tagged 5-HT$_6$ receptor expressed in NG108-15. (*B*) Coimmunoprecipitation of neurofibromin with HA-5-HT$_6$ receptor expressed in NG108-15 cells treated with vehicle, WAY208466, or SB271046 for 30 min. The histogram represents the results of densitometric analyses of three independent experiments. Data are means ± SEM. Inputs represent 5% of the total protein amount used for immunoprecipitations (IPs). Representative blots of three independent experiments are illustrated. MW, molecular mass. *$P < 0.05$ vs. vehicle.

Coimmunoprecipitation studies and bioluminescence resonance energy transfer (BRET) experiments showed that both PH domain and CTD of neurofibromin interact with the 5-HT$_6$ receptor (*SI Materials and Methods*, *SI Results*, and Figs. S2 and S3*A*). Further supporting a specific interaction between neurofibromin and 5-HT$_6$ receptor, no interaction was detected between PH domain and 5-HT$_4$ or 5-HT$_7$ receptor, two other Gs-coupled 5-HT receptors (*SI Materials and Methods*, *SI Results*, and Fig. S3*B*).

Association of YFP-tagged 5-HT$_6$ receptor (5-HT$_6$-YFP) with PH-Rluc and CTD-Rluc, already detected in absence of receptor ligand, was not further enhanced by exposure of cells to WAY208466, a specific 5-HT$_6$ receptor agonist (Fig. S2*D*). Treatment of cells with SB271046, a 5-HT$_6$ receptor antagonist/inverse agonist, induced a strong decrease in BRET signal between 5-HT$_6$-YFP and PH-Rluc, whereas it did not affect association of 5-HT$_6$-YFP with CTD-Rluc (Fig. S2*D*). Likewise, coimmunoprecipitation of neurofibromin with 5-HT$_6$ receptor was not significantly enhanced by treating cells with WAY208466, whereas SB271046 strongly reduced the coimmunoprecipitation of neurofibromin with 5-HT$_6$ receptor (Fig. 1*B*), suggesting a predominant contribution of PH domain vs. CTD in the association of both proteins. We, thus, focused on this interaction in additional experiments.

Neurofibromin Interacts with the CTD of 5-HT$_6$ Receptor. Previous studies have identified the CTD of GPCRs as a key domain involved in the recruitment of GIPs (20). Using several truncation mutants (deleted of C-terminal residues) of the receptor (Fig. S4*A*), we showed an essential role of 22 N-terminal residues of the receptor CTD in 5-HT$_6$ receptor–neurofibromin interaction (Fig. 2 *A* and *B*, *SI Materials and Methods*, *SI Results*, and Fig. S4*B*). We then generated a cell-permeable interfering peptide composed of the transduction domain of the HIV TAT protein fused to these residues (TAT Ct22 peptide) to block 5-HT$_6$ receptor–neurofibromin interaction. Treatment of cells with the TAT Ct22 peptide but not with a scrambled peptide abolished the specific BRET saturation signal observed in cells coexpressing 5-HT$_6$-YFP and PH-RLuc (Fig. 2*C*). Collectively, these results show the importance of the 22 N-terminal residues of 5-HT$_6$ receptor CTD in mediating the interaction with the PH domain of neurofibromin.

Neurofibromin Depletion Inhibits 5-HT$_6$ Receptor Constitutive Activity at Gs Signaling. Stable silencing of neurofibromin expression using an shRNA strongly inhibited constitutive activation of cAMP production in HeLa cells induced by expression of the human 5-HT$_6$ receptor (Fig. 3*A* and Fig. S5*A*). The amplitude of this inhibition represented about 80% of the reduction of constitutive activity induced by treating cells expressing control shRNA with a maximally effective concentration of SB271046 (Fig. 3*A* and Fig. S5*A*). This effect did not result from a decrease in 5-HT$_6$ receptor expression in neurofibromin-depleted cells as shown by Western blot analysis (Fig. 3*A*, *Right*) and ELISA assessing 5-HT$_6$ receptor plasma membrane expression (o.d. arbitrary unit = 1.60 ± 0.05 and 1.35 ± 0.20, $P > 0.05$ in HeLa cells expressing control shRNA and neurofibromin shRNA, respectively). In contrast, depleting cells of neurofibromin did not significantly affect cAMP production induced by WAY208466 (Fig. 3*A*). Neurofibromin depletion in cells expressing 5-HT$_4$ receptor, another Gs-coupled 5-HT receptor that displays strong constitutive activity (21) but does not interact with neurofibromin PH domain, did not alter basal cAMP level or the amplitude of the inhibitory effect of Ro1162617, a 5-HT$_4$ receptor inverse agonist (Fig. S5*B*). This finding suggests that neurofibromin specifically modulates 5-HT$_6$ receptor constitutive activity.

Silencing neurofibromin expression in both a neuronal cell line (NG108-15) and primary striatal neurons resulted in decreased basal cAMP levels, whereas it did not affect agonist-induced cAMP accumulation (Fig. 3 *B*, *Left* and *C*, *Left*). Notably, in mouse striatal neurons depleted of neurofibromin, basal cAMP level was similar to that measured in control siRNA-transfected neurons treated with

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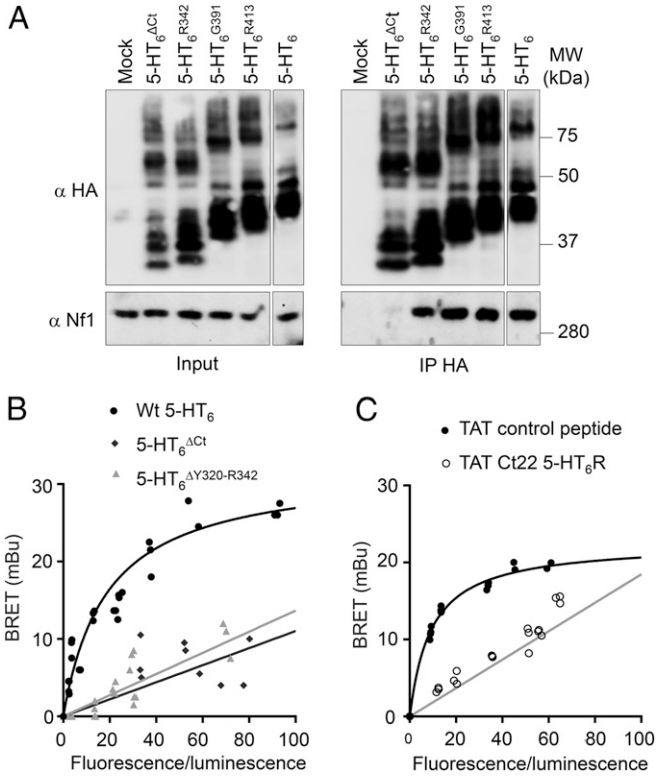

Fig. 2. The juxtamembrane segment of 5-HT_6 receptor CTD interacts with neurofibromin PH domain. (*A*) The corresponding constructs encoding HA-tagged 5-HT_6 receptor were transfected in HEK-293 cells as indicated, and receptors were immunoprecipitated using anti-HA antibody-conjugated beads. Representative immunoblots of three independent experiments are illustrated. The different gel lanes for each panel originate from a unique original Western blot. Inputs represent 5% of the total protein amount used for immunoprecipitation (IP). (*B*) BRET titration curves were generated in HEK-293 cells expressing a constant amount of PH-RLuc (donor) and increasing amounts of 5-HT_6-YFP (acceptor) as indicated. Plotted results are from three independent experiments. (*C*) Cells transfected with 5-HT_6-Luc construct and increasing concentrations of PH-YFP construct were treated for 2 h with TAT control or TAT Ct22 peptide (10 μM) before BRET measurement. Plotted results are from three independent experiments. MW, molecular mass.

SB271046. Moreover, the inverse agonist did not further decrease cAMP level, indicating a complete loss of 5-HT_6 receptor constitutive activity in neurofibromin-depleted neurons (Fig. 3*C*).

Disruption of the 5-HT_6 Receptor–Neurofibromin Interaction Inhibits Receptor Constitutive Activity. Pretreatment of HEK-293 cells expressing 5-HT_6 receptor with the TAT Ct22 peptide, which disrupts receptor–neurofibromin interaction (Fig. 2*C*), strongly reduced the basal cAMP level (otherwise inhibited by incremental concentrations of SB271046) compared with that measured in cells treated with the TAT control peptide (Fig. 3*D*). Furthermore, expression of the PH domain but not the region homologous to the yeast Sec14p protein (Sec) domain in neurofibromin-depleted HeLa cells fully restored the level of 5-HT_6 receptor constitutive activity measured in presence of neurofibromin (Fig. 3*E*), indicating that the receptor's constitutive activity at Gs signaling is critically dependent on the physical interaction between 5-HT_6 receptor CTD (residues 320–342) and neurofibromin PH domain. Neither basal nor agonist-stimulated cAMP production were altered in cells treated with FTI277, a specific Ras inhibitor, ruling out a role of Ras GTPase activity of neurofibromin in its control of 5-HT_6 receptor constitutive activity (Fig. S6).

Impact of *Nf1* Mutations on 5-HT_6 Receptor Constitutive Activity. Mutations located in neurofibromin PH domain identified in NF1 patients (Fig. S7*A*) differentially affect its interaction with 5-HT_6 receptor. The A1764S and T1787M PH domain mutants produced comparable BRET signals to those obtained with WT PH domain (Fig. 4*A*). In contrast, no specific BRET signal was detected in cells expressing R1748A, K1750A, Δ1719–1736, Δ1746–1750, or ΔK1750 mutant (Fig. 4*A* and Fig. S7*B*; Fig. S7*C* also shows expression of WT and mutant PH domains in HEK-293 cells). Corroborating their differential capabilities to interact with 5-HT_6 receptor, expression of A1764S and T1787M mutants rescued the deficit in receptor constitutive activity induced by neurofibromin depletion and thus, reproduced the effect of reexpression of WT PH domain, whereas the Δ1746–1750 and ΔK1750 mutants did not restore cAMP levels resulting from agonist-independent receptor activation (Fig. 4*B*).

5-HT_6 Receptor Constitutive Activity Is Impaired in a Mouse Model of Neurofibromatosis. Whereas 5-HT_6 receptor expression was not modified in *Nf1*$^{+/-}$ mice (Fig. 4*C*), we found a lower cAMP level in PFC compared with in WT mice (0.587 ± 0.026 vs. 0.441 ± 0.041 pmol/mg protein; $n = 3$, respectively). Treatment of PFC microdisks from WT mice with SB271046 (1 μM) decreased basal cAMP level but did not further reduce cAMP level in *Nf1*$^{+/-}$ mice (Fig. 4*D*). Further supporting a role of 5-HT_6 receptor constitutive activity in brain cAMP production, (*S*)-1-[(3-chlorophenyl)sulfonyl]-4-(pyrrolidine-3-yl-amino)-1H-pyrrolo[3,2-c]quinolone (CPPQ), a recently described 5-HT_6 receptor neutral antagonist (22), did not decrease cAMP production in either NG108-15 cells (Fig. S8*A*) or microdisks from WT and *Nf1*$^{+/-}$ mice (Fig. 4*D*). Exposure to WAY208466 slightly but not significantly enhanced cAMP production in microdisks from WT mice (Fig. 4*D*). A significant agonist-induced stimulation of cAMP production was only measured in PFC from *Nf1*$^{+/-}$ mice that exhibit a reduced level of receptor constitutive activity.

To further confirm the role of 5-HT_6 receptor constitutive activity in activation of the cAMP pathway in vivo, we next measured the phosphorylation level of cAMP-responsive element-binding protein (CREB) in PFC. Reminiscent of the impact of neurofibromin on cAMP level, CREB phosphorylation (at Ser^{133}) was strongly reduced in PFC of *Nf1*$^{+/-}$ mice (−84 ± 13%) compared with WT mice (Fig. 4*E*). Systemic administration of SB271046 (10 mg/kg i.p.) also reduced the level of phosphorylated CREB in PFC of WT mice but to a lesser extent (−31 ± 6%; $n = 3$), whereas it had no effect on CREB residual phosphorylation level measured in PFC of *Nf1*$^{+/-}$ mice (Fig. 4*E*). This finding suggests that neurofibromin-dependent 5-HT_6 receptor constitutive activity at Gs signaling only partially contributes to basal CREB phosphorylation. As expected, treatment of WT mice with the neutral antagonist CPPQ (10 mg/kg i.p.) did not alter CREB phosphorylation (Fig. S8*B*), whereas administration of WAY208466 (10 mg/kg i.p.) induced a slight but not significant increase in CREB phosphorylation in both WT and *Nf1*$^{+/-}$ mice (Fig. 4*E*). Collectively, these results indicate that cAMP signaling is strongly affected in *Nf1*$^{+/-}$ mouse brain and that inhibition of 5-HT_6 receptor constitutive activity contributes to this reduced cAMP production and the associated CREB phosphorylation.

Discussion

Using an unbiased proteomic strategy, we previously identified neurofibromin as a candidate partner of recombinant 5-HT_6 receptor expressed in HEK-293 cells (12). In line with the common influence of 5-HT_6 receptor and neurofibromin in brain development, learning and memory, and brain cAMP signaling (23, 24), we further characterized in this study 5-HT_6 receptor–neurofibromin interaction and showed that they form a complex in mouse brain. Moreover, we showed that 5-HT_6 receptor–neurofibromin interaction is a dynamic process that depends on receptor conformational state and can be

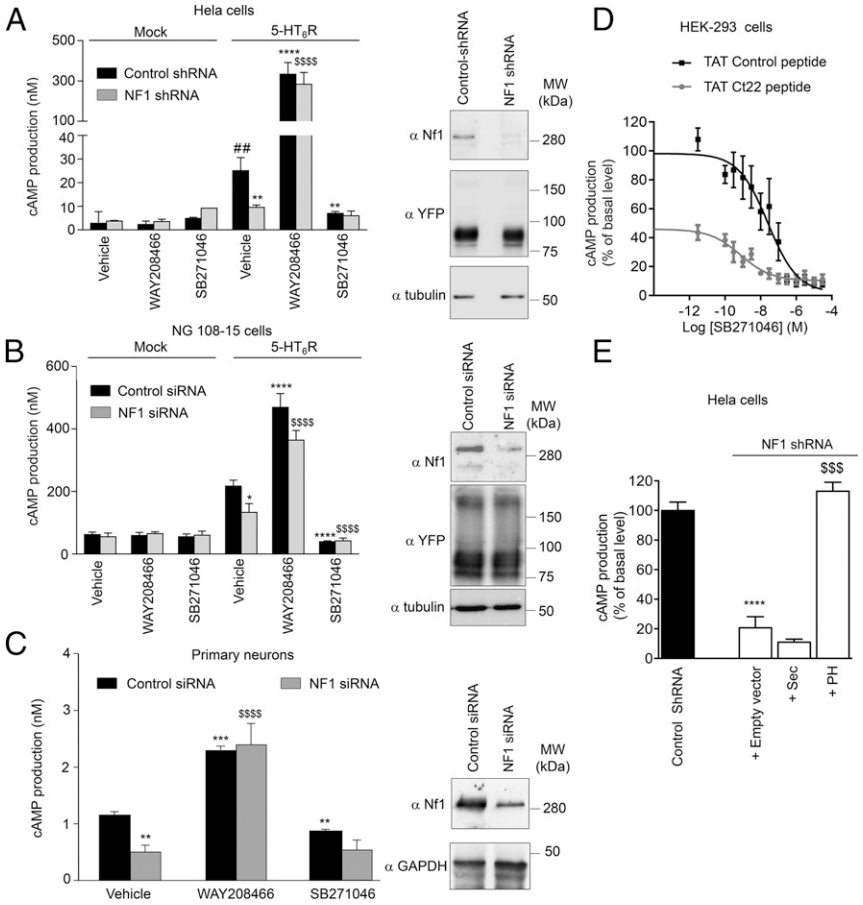

Fig. 3. Neurofibromin promotes 5-HT$_6$ receptor constitutive activity at Gs/cAMP signaling. (*A*) HeLa cells stably expressing neurofibromin shRNA or control shRNA were transfected with the plasmid encoding human HA-5-HT$_6$ receptor and stimulated with vehicle, WAY208466, or SB271046 (1 μM). cAMP production was quantified by time-resolved fluorescence resonance energy transfer (TR-FRET). (*B*) NG108-15 cells, transiently transfected with control or neurofibromin siRNA and either empty vector (Mock) or plasmid encoding 5-HT$_6$-YFP, were treated as in *A*. (*C*) Primary striatal neurons were transfected with control or neurofibromin siRNA and treated as in *A*. In *A*–*C*, data illustrated in histograms are means ± SEM of values obtained in four independent experiments, and *A*, *Right*, *B*, *Right*, and *C*, *Right* show efficient silencing of neurofibromin expression by shRNA/siRNA. MW, molecular mass. $^{*}P < 0.05$ vs. vehicle in cells expressing control siRNA and 5-HT$_6$ receptor or vehicle in neurons expressing control siRNA; $^{**}P < 0.01$ vs. vehicle in cells expressing control siRNA and 5-HT$_6$ receptor or vehicle in neurons expressing control siRNA; $^{***}P < 0.001$ vs. vehicle in cells expressing control siRNA and 5-HT$_6$ receptor or vehicle in neurons expressing control siRNA; $^{****}P < 0.0001$ vs. vehicle in cells expressing control siRNA and 5-HT$_6$ receptor or vehicle in neurons expressing control siRNA; $^{\#\#}P < 0.01$ vs. vehicle in mock cells; $^{\$\$\$\$}P < 0.0001$ vs. vehicle in cells transfected with neurofibromin siRNA and expressing 5-HT$_6$ receptor. (*D*) HEK-293 cells expressing WT receptors were treated with TAT control or Ct22 peptide (10 μM) for 2 h before SB271046 application. (*E*) HeLa cells stably expressing neurofibromin shRNA were cotransfected with 5-HT$_6$ receptor construct and plasmids encoding Flag-tagged Sec or PH domains of neurofibromin. Data in *D* and *E* are means ± SEM of values obtained in three independent experiments. $^{****}P < 0.0001$ vs. control shRNA; $^{\$\$\$}P < 0.001$ vs. empty vector in cells expressing neurofibromin shRNA.

prevented by a specific antagonist (SB271046), which thus behaves as an inverse agonist to disrupt this spontaneous (constitutive) association. Using BRET, we provided evidence that the PH domain and the CTD of neurofibromin independently interact with the receptor, suggesting that they contribute to the association of the receptor with full-length neurofibromin. Nonetheless, the higher affinity of PH domain for the receptor compared with CTD and the ability of the 5-HT$_6$ inverse agonist to prevent association of 5-HT$_6$ receptor with PH domain but not with CTD suggest a prominent role of PH domain in the interaction between both protein partners. PH-like domains have long been associated with proteins involved in signal transduction (25). Neurofibromin PH domain is adjacent to the central GTPase-activating protein-related domain and the Sec14-homologous Sec domain that interacts with glycerophospholipids (26). A hairpin-like protrusion of PH domain interacts with a helical lid segment of Sec domain and has been proposed to promote its binding to the lipid cage. However, neurofibromin PH domain alone cannot bind to lipids (26), whereas it is sufficient to associate with the 5-HT$_6$ receptor, thus providing a demonstration of an Sec-independent function of this domain. Finally, we show that 22 N-terminal residues of the receptor CTD are essential for the association of neurofibromin with 5-HT$_6$ receptor, consistent with numerous findings, which identified GPCR CTD as a key domain contributing to their association with GIPs (20). This 22-residue sequence comprises a stretch of four prolines that might be important for binding to neurofibromin PH domain (26).

In an effort to characterize the functional impact of the 5-HT$_6$ receptor–neurofibromin interaction, we showed that it promotes receptor constitutive activity at Gs signaling without affecting agonist-elicited cAMP production. Notably, neurofibromin similarly affects constitutive activity of human and mouse receptors, despite their divergent pharmacology and signal transduction properties. Indeed, down-regulating neurofibromin expression as well as any strategy leading to the disruption of the interaction between 5-HT$_6$ receptor CTD and neurofibromin PH domain strongly reduced agonist-independent receptor-operated cAMP signaling in both heterologous cells expressing recombinant human 5-HT$_6$ receptor and mice neurons expressing native receptors. Furthermore, reexpressing WT PH domain, but not mutant PH domains unable to interact with 5-HT$_6$ receptor, rescued a normal level of receptor constitutive activity in neurofibromin-depleted cells. Collectively, these results suggest that 5-HT$_6$ receptor association with neurofibromin maintains the receptor in a specific conformation, allowing the constitutive activation of Gαs protein and thereby, cAMP production through activation of adenylyl cyclase. Several lines of evidence support a role of PH-like domains present in other proteins in GPCR-operated signal transduction. For instance, the C-terminal one-half of GPCR Kinase 2 PH domain serves as a platform for G protein βγ-subunit binding (27). Whether the neurofibromin PH domain associated with 5-HT$_6$ receptor favors recruitment of Gs protein remains to be explored. A recent study has shown that the regulation by neurofibromin of cAMP signaling requires Ras activation and operates through the activation of atypical PKC zeta (28), whereas other studies performed in *Drosophila* suggest that neurofibromin controls cAMP production in an Ras-independent manner (19, 29). Our results showing that expression of PH domain alone is sufficient to rescue normal 5-HT$_6$ receptor constitutive activity in neurofibromin-deficient cells are consistent with an Ras-independent mechanism. They also rule out a role for the CTD, which was previously shown to regulate adenylyl cyclase activity in *Drosophila* (16), in the constitutive activation of adenylyl cyclase by 5-HT$_6$ receptor.

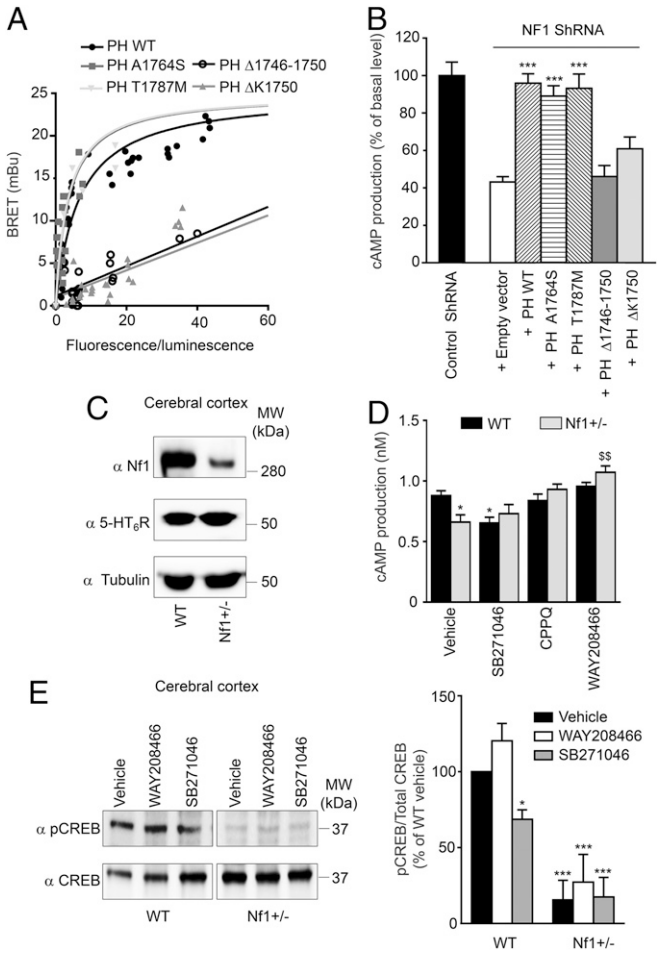

Fig. 4. Effects of PH domain mutations identified in NF1 patients and neurofibromin depletion in mice on 5-HT_6 receptor-operated cAMP signaling. (*A*) HEK-293 cells were transfected with a constant amount of 5-HT_6-RLuc construct and increasing concentrations of YFP-tagged PH domain constructs as indicated. Plotted results are from three independent experiments. (*B*) HeLa cells stably expressing neurofibromin shRNA were transiently transfected with 5-HT_6 receptor construct in the absence or presence of YFP-tagged PH domain constructs as indicated. Data are means ± SEM of values obtained in a representative experiment performed in triplicate. Two other independent experiments performed on different cultures yielded similar results. ***$P < 0.001$ vs. neurofibromin-depleted cells transfected with empty vector. (*C*) Western blot analysis of neurofibromin and 5-HT_6 receptor expression in PFC of WT and *Nf1*$^{+/-}$ mice. (*D*) cAMP production in PFC microdisks from WT and *Nf1*$^{+/-}$ mice treated with vehicle, SB271046, CPPQ, or WAY208466 (1 μM each). Data are means ± SEM of values obtained in three different mice. *$P < 0.05$ vs. microdisks from WT mice treated with vehicle; $^{\$\$}P < 0.01$ vs. microdisks from *Nf1*$^{+/-}$ mice treated with vehicle. (*E*) CREB phosphorylation (at Ser^{133}) in PFC of both mice strains treated for 30 min with vehicle, WAY208466, or SB271046 (10 mg/kg i.p. each) was analyzed by sequential immunoblotting using antiphospho-CREB and total CREB antibodies. Representative Western blots are illustrated in *C* and *E*. The data in *E* are means ± SEM of values measured in four mice per group. MW, molecular mass. *$P < 0.05$ vs. WT mice treated with vehicle; ***$P < 0.001$ vs. WT mice treated with vehicle.

This study provides an example of modulation of GPCR constitutive activity by a GIP. Another example is the modulation of agonist-independent activity of group I metabotropic glutamate receptors (mGluR1a and mGluR5) by the constitutively expressed Homer3 protein (30). In contrast to the 5-HT_6 receptor–neurofibromin interaction, the association of Homer3 with the CTD of mGluR1a or mGluR5 prevents the constitutive activation of these receptors, whereas the activity-dependent short Homer1a isoform behaves as a dominant negative regulator of the interaction between mGluR1a or mGluR5 and Homer3 and thereby, promotes constitutive activity of these receptors. Therefore, this work provides an example of constitutive activation of G protein signaling by a GPCR dependent on its physical association with a GIP.

The inability of neurofibromin mutants (mutated in PH domain) identified in NF1 patients to interact with 5-HT_6 receptor and consequently, promote receptor constitutive activity suggests that agonist-independent 5-HT_6 receptor-operated signaling might be altered in some NF1 patients. Corroborating this hypothesis, we found that CREB phosphorylation and to a lesser extent, cAMP level are diminished in *Nf1*$^{+/-}$ mice compared with WT mice and that the prototypic 5-HT_6 receptor inverse agonist SB271046 does not further decrease this reduced cAMP level and CREB phosphorylation, whereas it affects basal cAMP level and CREB phosphorylation in WT mice. Further supporting a role of 5-HT_6 receptor constitutive activity vs. receptor activation secondary to the release of its endogenous agonist (5-HT), cAMP production and CREB phosphorylation in mouse brain were not altered by the 5-HT_6 receptor neutral antagonist CPPQ. Nonetheless, it is likely that other mechanisms in addition to reduction of 5-HT_6 receptor constitutive activity at Gs signaling contribute to the strong decrease in CREB phosphorylation level in *Nf1*$^{+/-}$ mice. The alteration of cAMP signaling in *Nf1*$^{+/-}$ mice is also consistent with previous observations in *Nf1* null *Drosophila*, which suggest that the associated learning deficits result from an inhibition of cAMP-mediated PKA activity (31, 32).

CREB has been identified as a key regulator of cell survival, proliferation, and differentiation in the developing brain, and it has been involved in learning, memory, and neuronal plasticity in adult brain (33). Inhibition of cAMP signaling and the resulting CREB phosphorylation caused in part by the loss of 5-HT_6 receptor constitutive activity might thus contribute to neuronal abnormalities and cognitive impairment observed in NF1 patients. However, 5-HT_6 receptor blockade by 5-HT_6 receptor antagonists, improves cognition in a wide range of paradigms in rodents rather than it produces cognitive impairment. Another pathway engaged by 5-HT_6 receptor that might contribute to cognitive impairment in NF1 patients is the mTOR pathway. Loss of neurofibromin Ras-GAP activity in NF1 leads to aberrant mTOR activation, which is essential for NF1-associated malignancies (34). Moreover, nonphysiological mTOR activation has been involved in cognitive deficits observed in several neurodevelopmental disorders (35). mTOR activation, under the control of 5-HT_6 receptor, likewise underlies deficits in social cognition and episodic memory in rat developmental models of schizophrenia (12). mTOR activation by 5-HT_6 receptors is critically dependent of its physical association with 5-HT_6 receptor CTD. Neurofibromin might compete with mTOR to associate with 5-HT_6 receptor because of the proximity of their target motif in the receptor sequence and thus, prevent engagement of mTOR signaling by the receptor. Consistent with this hypothesis, neurofibromin mutants unable to interact with 5-HT_6 receptor identified in some NF1 patients would favor the recruitment of mTOR and receptor-operated mTOR signaling. Therefore, the contribution of 5-HT_6 receptor in the enhanced mTOR signaling in NF1 certainly warrants additional exploration. Likewise, it would be of considerable interest to explore in future studies the impact of treatment with 5-HT_6 receptor antagonists and mTOR inhibitors on the associated cognitive deficits.

In conclusion, this study provides evidence for a physical interaction between neurofibromin and a GPCR, the 5-HT_6 receptor, and an example of modulation of constitutive activity of a GPCR through its interaction with a GIP. It also reveals a function of neurofibromin independent of its well-characterized Ras-GAP activity. Although the precise pathophysiological significance of 5-HT_6 receptor–neurofibromin interaction and the resulting 5-HT_6 receptor constitutive activity remain to be established, these findings suggest that it might be considered as a potentially relevant therapeutic target to correct some NF1-related deficits.

Materials and Methods

Reagents, plasmid constructs (Table S1), antibodies, and animals are detailed in *SI Materials and Methods*. Methods used for cell cultures, transfections, coimmunoprecipitation, and Western blot are described in *SI Materials and Methods*. The procedures involving mice were approved by the local ethics committee on animal experimentations of the CNRS in Orleans, France (agreement CECO3 n°1041).

BRET Measurements. Forty-eight hours after transfection, HEK-293T cells resuspended in HBSS saline buffer (Invitrogen) were incubated for 15 min at 25 °C in the absence or presence of the indicated ligands. Coelenterazine H substrate (Molecular Probes) was added at a final concentration of 5 μM. Luminescence and fluorescence were detected using a Mithras LB 940 Multireader (Berthold). BRET measurement and calculation are described in *SI Materials and Methods*.

cAMP Determination. cAMP accumulation in cell cultures was measured using the LANCE cAMP Detection Kit (Perkin-Elmer Life Sciences) as described in *SI Materials and Methods*.

For cAMP measurement in brain tissue, the LANCE Ultra cAMP Kit (Perkin-Elmer Life Sciences) was used. Brains were rapidly extracted after animals were killed. Tissue samples were punched out from PFC slices (300-μm thick, obtained with a microtome) to obtain circular microdisks of 1.5-mm diameter. The microdisks were treated for 10 min with drugs diluted in the stimulation buffer (HBSS containing 5 mM Hepes, 1 mM 3-isobutyl-1-methylxanthine, 1% BSA). After removal of buffer containing drugs, the microdisks were lysed and sonicated in cAMP detection buffer (provided in the kit). After centrifugation, samples were dispensed in white 384-well microtiter plates and incubated with solution containing ULight-anti–cAMP and Eu-cAMP for 1 h. Plates were read on a Victor Microplate Reader.

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