5-HT₆ receptor recruitment of mTOR as a mechanism for perturbed cognition in schizophrenia

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Cognitive deficits in schizophrenia severely compromise quality of life and are poorly controlled by current antipsychotics. While 5-HT₆ receptor blockade holds special promise, molecular substrates underlying their control of cognition remain unclear. Using a proteomic strategy, we show that 5-HT₆ receptors physically interact with several proteins of the mammalian target of rapamycin (mTOR) pathway, including mTOR. Further, 5-HT₆ receptor activation increased mTOR signalling in rodent prefrontal cortex (PFC). Linking this signalling event to cognitive impairment, the mTOR inhibitor rapamycin prevented deficits in social cognition and novel object discrimination induced by 5-HT₆ agonists. In two developmental models of schizophrenia, specifically neonatal phencyclidine treatment and post-weaning isolation rearing, the activity of mTOR was enhanced in the PFC, and rapamycin, like 5-HT₆ antagonists, reversed these cognitive deficits. These observations suggest that recruitment of mTOR by prefrontal 5-HT₆ receptors contributes to the perturbed cognition in schizophrenia, offering new vistas for its therapeutic control.

INTRODUCTION

There is increasing recognition that cognitive deficits in schizophrenia severely compromise the real world function of patients. Despite their efficacy against positive symptoms, currently available antipsychotics are poorly effective against cognitive dysfunction. Accordingly, intensive efforts are being made to improve our understanding of pathological mechanisms underlying cognitive impairment in schizophrenia and to identify novel strategies for its alleviation (Gray & Roth, 2007; Insel, 2010; Millan et al, 2012).

Amongst mechanisms currently under study, 5-HT₆ receptors are of special interest, in as much as their blockade consistently enhances mnemonic performance in a broad range of procedures in rodents (Hirst et al, 2006; Loiseau et al, 2008; Marcos et al, 2008; Rogers & Hagan, 2001; Woolley et al, 2001), while 5-HT₆ receptor antagonists also promote the corticolimbic release of acetylcholine, glutamate and monoamines to favour cognitive processes (Codony et al, 2011). Further, there is preliminary evidence for pro-cognitive properties of 5-HT₆ antagonists in humans (Codony et al, 2011; Johnson et al, 2008; Mitchell & Neumaier, 2005), while 5-HT₆ receptor antagonists also promote the corticolimbic release of acetylcholine, glutamate and monoamines to favour cognitive processes (Codony et al, 2011). Nonetheless, the nature of signalling mechanisms mediating the influence of 5-HT₆ receptors on cognition remains unclear, complicating analysis of their significance to the induction and control of cognitive deficits in schizophrenia and other disorders. 5-HT₆ receptors...
stimulate Gs and adenylyl cyclase (Millan et al., 2008). They also activate extracellular-regulated kinase (ERK)1,2 via the Src-family tyrosine kinase Fyn (Yun et al., 2007). However, these pathways exert a positive influence on cognition (Millan et al., 2012). Hence, it is unlikely that their inactivation would transduce the pro-cognitive effects of 5-HT6 receptor antagonists, raising the possibility that alternative coupling mechanisms are involved.

In light of recent evidence that G protein-coupled receptors (GPCRs) can interact with extrinsic protein networks, including proteins dedicated to signal transduction (Bockaert et al., 2004), we used a proteomic strategy to identify proteins potentially associated with 5-HT6 receptors. Based on identification of several proteins of the mammalian target of rapamycin (mTOR) pathway (including mTOR itself, Raptor, Neurofibromin 1 and Vps34, a class III phosphatidyl inositol 3-kinase), we then undertook an extensive suite of in vivo studies to determine whether 5-HT6 receptor engagement of mTOR contributes to their deleterious influence upon cognition, specifically in developmental models of schizophrenia.

RESULTS

5-HT6 receptors physically interact with the mTOR complex 1
Due to the low density of 5-HT6 receptors in mammalian brain and the lack of an antibody permitting immunoprecipitation yields compatible with mass spectrometry analysis, we purified receptor-interacting proteins by co-immunoprecipitation with a hemagglutinin (HA)-tagged 5-HT6 receptor expressed in human embryonic kidney (HEK)-293 cells. Functionality of HA-5-HT6 receptors was assessed by the ability of 5-HT and two synthetic 5-HT6 agonists, WAY181187 and WAY208466 (Schechter et al., 2008), to increase cAMP production (Supporting Information Fig S1). Analysis of affinity-purified proteins by SDS–PAGE revealed the presence of proteins that co-immunoprecipitated with the receptor and that were not detected in control immunoprecipitations performed in the presence of HA peptide (Fig 1A). Correspondingly, systematic analysis by high-resolution nanoflow liquid tandem mass spectrometry of gel lanes identified 28 proteins, which specifically co-immunoprecipitated with the 5-HT6 receptor (Fig 1B and Supporting Information Tables S1 and S2). These proteins were considered as potential partners of the receptor, though one cannot rule out the possibility that some of them do not interact with the 5-HT6 receptor but that their presence reflects some affinity for the anti-HA antibody. Compared with what would be expected by chance, the 5-HT6 receptor ‘interactome’ showed a remarkable enrichment in proteins implicated in intracellular signalling pathways, brain development, learning and synaptic plasticity (Fig 1C). These include several proteins of the mTOR pathway such as mTOR itself and Raptor, which together with GβL, constitute the rapamycin-sensitive mTOR complex 1 (mTORC1; Laplante & Sabatini, 2012; Swiech et al., 2008; Wang & Proud, 2011; Zhou & Huang, 2010). mTOR also forms the mTOR complex 2 (mTORC2), which includes specific members (Rictor, mSin1 and Protor1/2) in addition to mTOR and GβL but is insensitive to acute rapamycin treatment (Laplante & Sabatini, 2012; Swiech et al., 2008; Wang & Proud, 2011; Zhou & Huang, 2010). None of the proteins specific to mTORC2 were detected in the 5-HT6 receptor interactome, suggesting a specific recruitment of mTORC1 by this receptor. The 5-HT6 receptor also recruited Tti1 and Tel2, two proteins critical for assembly and activity of mTORC1 and 2 (Kaizuka et al., 2010). In addition, two proteins of the pathways leading to mTOR activation were identified: the Ras GTPase activating protein (GAP) Neurofibromin 1 and the class III phosphatidyl inositol 3-kinase Vps34 (Swiech et al., 2008; Zhou & Huang, 2010; Fig 1B). Immunoprecipitation followed by Western blot analysis confirmed the constitutive interaction of mTOR, Raptor and Neurofibromin 1 with the 5-HT6 receptor in HEK-293 cells and indicated that their recruitment was not further increased upon receptor activation by 5-HT (Fig 1D). Importantly, mTOR specifically co-immunoprecipitated with native 5-HT6 receptor expressed in mice brain (Fig 1E), indicating that they form a complex in vivo. Further supporting a specific association of mTORC1 with the 5-HT6 receptor, neither mTOR nor Raptor co-immunoprecipitated with the 5-HT7 receptor (another Gs-coupled 5-HT receptor) expressed in HEK-293 cells (Fig 1D). Likewise, mTOR did not co-immunoprecipitate with a truncated 5-HT6 receptor deleted of the 49 carboxy-terminal residues (5-HT6Δ49Ct, Supporting Information Fig S2), indicating a role of the 5-HT6 receptor C-terminus in its physical association with mTORC1. Nonetheless, other parts of the receptor sequence were necessary to mTORC1 recruitment as mTOR did not bind to the receptor C-terminus in GST pull-downs using the entire receptor C-terminal sequence as bait (Supporting Information Fig S2C).

5-HT6 receptors activate the mTOR pathway in HEK-293 cells via a mechanism requiring both the canonical phosphatidylinositol 3-kinase/Akt/Rheb pathway and 5-HT6 receptor/mTOR physical interaction
Exposure of 5-HT6 receptor-expressing HEK-293 cells to 5-HT (1 μM) induced a transient (<5 min) increase of mTOR phosphorylation at Ser2481, which reflects mTOR activation status, and of its substrate p70 ribosomal S6 kinase (p70S6K) at Thr389 (Fig 2A). 5-HT induced a more sustained (∼30 min) increase in the phosphorylation of two downstream substrates of mTOR, 4EBP1 (at Ser65) and S6 (at Ser240/244). The 5-HT-induced phosphorylation of mTOR and its substrates was prevented by SB258585 (10 μM), a specific 5-HT6 receptor antagonist, and was reproduced by WAY181187 and WAY208466 (1 μM each, Fig 2B). 5-HT6 receptor-elicited mTOR signalling was comparable to that induced by insulin-like growth factor 1 or epidermal growth factor (Supporting Information Fig S3) and was strongly decreased in cells expressing 5-HT6Δ49Ct receptor, which did not co-immunoprecipitate with mTOR but fully activated cAMP production compared with cells expressing the wild-type receptor (Supporting Information Fig S2).

In line with activation of mTORC1 by 5-HT6 receptors, phosphorylation of p70S6K (Thr389), 4EBP1 (Ser65) and S6 (Ser240/244) by WAY181187 were prevented by rapamycin, a
specific mTORC1 inhibitor, whereas, as expected, phosphorylation of mTOR (Ser2448) was unaffected (Fig 2B). Moreover, and consistent with the 5-HT-elicited transient activation of Akt (assessed by phosphorylation at Ser473, Fig 2A) that paralleled mTOR phosphorylation, mTOR activation was dependent on the canonical class I phosphatidylinositol 3-kinase (PI3K)/Akt signalling: phosphorylation of both mTOR (Ser2448) and S6 (Ser240/244) was strongly reduced in cells pretreated with the PI3K inhibitors wortmannin (100 nM) or LY294002 (20 μM, Fig 3A). Activated Akt can phosphorylate tuberin (TSC2) (Dan et al, 2002; Inoki et al, 2002; Manning et al, 2002), which together with hamartin (TSC1) constitutes the tuberous complex (TSC1/2). TSC1/2 is a GAP for Rheb (Ras homolog enriched in brain), a major upstream activator of mTORC1 (Garami et al, 2003; Inoki et al, 2003; Tee et al, 2003). Phosphorylation of TSC2 by Akt inhibits GAP activity of the complex, resulting in increased levels of Rheb-GTP that in turn stimulates mTOR (Garami et al, 2003; Inoki et al, 2003; Tee et al, 2003). Rheb was not identified by mass spectrometry in the 5-HT₆ receptor complex purified from HEK-293 cells (Fig 1B and Supporting Information Table S1), likely due to its low expression in these cells. Nonetheless, GST pull-down followed by Western blotting showed recruitment of Rheb from mice brain by the 5-HT₆ receptor C-terminus (Supporting Information Fig S2C). Moreover, Rheb contributed to 5-HT₆ receptor-dependent mTOR signalling in HEK-293 cells: both mTOR (S2448) and S6 (S240/244) phosphorylations elicited by WAY181187 were prevented by expression of a Rheb-dominant-negative mutant (I39K, Fig 3B). In contrast, over-expression of wild-type Rheb itself increased phosphorylation of these residues, which was not further enhanced by WAY181187 exposure (Fig 3B).

5-HT₆ receptors activate mTOR signalling in the prefrontal cortex and the striatum

Corroborating observations in HEK-293 cells, systemic administration of WAY181187 (10 mg/kg i.p.) to mice increased levels of phosphorylated mTOR (Ser2448) and S6 (Ser240/244), as assessed by Western blotting analysis, in the prefrontal cortex.
(PFC), a cerebral region involved in the modulation of social cognition by 5-HT₆ receptor ligands (Loiseau et al., 2008; Fig 4A). This result was confirmed by immunofluorescence staining, which revealed a strong increase in the number of phospho-Ser²₄₄₈-mTOR and phospho-Ser²₄₀/₂₄₄-S6-positive cells in the PFC of animals treated with WAY181187 (WAY1) or WAY208466 (WAY2) versus vehicle-treated animals. Phosphorylation of S6 by WAY181187 and WAY208466 was abolished by SB258585 (10 mg/kg, i.p., delivered 15 min before the agonists), which itself did not significantly affect S6 phosphorylation (Supporting Information Fig S4). Further supporting engagement of the mTOR pathway by 5-HT₆ receptor activation, phosphorylation of S6 by WAY181187 was likewise prevented by acute rapamycin administration (10 mg/kg, i.p., 15 min before agonist injections, Fig 4A and B). Notably, this systemic administration of rapamycin was followed by its long-lasting (>2 h after injection) accumulation within the brain (Supporting

Figure 2. Transient stimulation of mTOR signalling upon 5-HT₆ receptor activation in HEK-293 cells. Representative immunoblots of three independent experiments are shown. Data, expressed as ratios of phosphorylated to total proteins, represent the means ± SEM of values obtained in three independent experiments.

A. HEK-293 cells expressing HA-5-HT₆ receptor were challenged with 5-HT (1 μM) for the indicated times. mTOR signalling was assessed by sequential immunoblotting with antibodies against phosphorylated mTOR (Ser²₄₄₈), p70S6K (Thr³₈⁹), S6 (Ser²₄₀/₂₄₄), 4EBP1 (Ser⁶⁵) and Akt (Ser⁴⁷³) and antibodies recognizing the corresponding proteins independently of their phosphorylation state. Immunoreactive signals were quantified by densitometry.

B. Cells were exposed for 2 min to 1 μM of either 5-HT, WAY181187 (WAY1) or WAY208466 (WAY2). They were pretreated with SB258585 (10 μM) or rapamycin (1 μM) for 10 min before agonist exposure (p < 0.05, *p < 0.01 vs. basal, ‡p < 0.05 vs. the corresponding condition in absence of SB258585 or rapamycin, ANOVA followed by Newman–Keuls test).
Information Fig S5). Consistent with data on receptor localization in PFC (Codony et al., 2011; Woolley et al., 2004), phospho-S6 immunoreactivity was detected in both GABAergic neurons [mostly dopamine and cAMP-regulated phosphoprotein (DARPP32)-negative] and non-GABAergic neurons (Supporting Information Figs S6 and S7). Robust 5-HT6 receptor-mediated activation of mTOR signalling was also detected in striatum (Supporting Information Fig S4), the brain structure expressing the highest density of 5-HT6 receptors. Therein, more than 90% of phospho-Ser240/244-S6-stained cells were positive for DARPP32 and correspond to GABAergic medium-sized spiny neurons (Supporting Information Fig S6).

Rapamycin administration prevents deficits of social cognition and novel object discrimination induced by the 5-HT6 receptor agonist WAY181187

As previously shown (Loiseau et al., 2008) and consistent with a role of 5-HT6 receptors in social cognition (i.e. the complex set of processes used to acquire, interpret and store information about a subject’s social environment, including the identity, intentions and behaviour of others), systemic administration of WAY181187 (10 mg/kg, i.p.) to adult rats significantly impaired social recognition in a procedure where a juvenile rat was presented to an adult for two consecutive 5 min sessions (Fig 4C). This action of WAY181187, which was abrogated by SB258585 (Loiseau et al., 2008), was prevented by rapamycin (10 mg/kg, i.p., administered 15 min before the agonist), which alone did not affect social recognition (Fig 4C). Rapamycin, like SB258585 (Supporting Information Fig S8A), also abrogated the deficit induced by WAY181187 in the social novelty discrimination procedure (Fig 4D). Consistent with a specific role of mTOR in the cognitive impairment induced by 5-HT6 receptor activation, rapamycin did not block the deficit in social recognition induced by the muscarinic receptor antagonist scopolamine (1.25 mg/kg s.c., Supporting Information Fig S9). In agreement with the documented improvement of recognition...
Figure 4. Enhanced mTOR signalling in PFC mediates the cognitive impairments induced by administration of a 5-HT₆ receptor agonist.

A. Western blot analysis of phosphorylation of mTOR (S²⁴⁴⁸) and S6 (S²⁴⁰/²⁴⁴) in PFC of mice treated with vehicle or WAY181187 (10 mg/kg, i.p., 30 min). Rapamycin (10 mg/kg, i.p.) was administered 15 min before WAY181187. Data, expressed as ratios of phosphorylated mTOR or S6 to total mTOR or S6, are means ± SEM (n = 4).

B. Immunofluorescent detection of cells positive for phospho-Ser²⁴⁴⁸-mTOR and phospho-Ser²⁴⁰/²⁴⁴-S6 in the PFC. Scale bar: 40 μm.

C,D. Inhibition by rapamycin of alteration of social cognition in rats treated with WAY181187. Data are means ± SEMs and represent the difference in duration of social interaction between the two sessions (T2-T1, n = 6 rats per group, C) and the ratios of time spent investigating the novel juvenile rat to time spent investigating the familiar one during the choice trial (n = 7–8, D).

E. Reversal by rapamycin (10 mg/kg s.c.) of the deficit of NOD (assessed by the d² score) induced by systemic (2.5 mg/kg i.p., left panel) and local (0.63 μg per side in PFC, right panel) administration of WAY181187. Data are means ± SEMs (n = 6–12). *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle/vehicle, §§§ p < 0.001 versus vehicle/WAY181187.
memory by 5-HT₆ receptor antagonism (Woolley et al, 2004), systemic administration of WAY181187 to rats also impaired visual episodic-like memory in a non-spatial novel object discrimination (NOD) procedure, which was reversed by both rapamycin (Fig 4E) and SB258585 (Supporting Information Fig S8B). Neither WAY181187, SB258585 nor rapamycin, alone or in combination, significantly altered total object exploration in this task (Supporting Information Fig S8B and S10), confirming their specific action on cognition. As previously shown for social cognition (Loiseau et al, 2008), local delivery of WAY181187 (bilaterally, 0.63 µg/side) into the PFC also impaired NOD, an effect prevented by peripheral rapamycin administration (Fig 4E). Collectively, these results indicate that mTOR signalling mediates the cognitive impairments induced by activation of 5-HT₆ receptors in PFC in two cognitive paradigms with clear relevance to the impairment of social cognition, attention and episodic memory observed in schizophrenia.

**Rapamycin administration prevents cognitive deficits in two developmental models of schizophrenia, neonatal phencyclidine treatment and post-weaning rearing in isolation**

We next examined the role of 5-HT₆ receptor-mediated mTOR signalling in rats that had received repeated injections of the psychotomimetic phencyclidine (PCP) during the neonatal period (Fig 5A), a treatment that provokes cognitive and other behavioural changes characteristic of schizophrenia in the mature animal (Jones et al, 2011). A marked increase in phospho-S6-positive neurons was detected in PFC of adult rats treated as neonates with PCP compared with vehicle-treated rats (Fig 5B). Immunofluorescent detection and quantification of cells positive for phospho-Ser240/244-S6 in the PFC and the striatum of vehicle- or PCP- (10 mg/kg, s.c.) treated rats that received either acute (30 min before brain perfusion) injection of SB258585 (2.5 mg/kg i.p.), rapamycin (10 mg/kg i.p.) or vehicle. Error bars represent SEM (n = 4). Quantification was performed on 224 µm x 168 µm images. *p < 0.001 versus vehicle-treated rats. Scale bar: 40 µm.

**Figure 5. 5-HT₆ receptor-elicited mTOR signalling in PFC underlies impairment of social discrimination in rats treated with phencyclidine during the neonatal period.**

A. Schema of the experimental paradigm used.

B. Immunofluorescent detection and quantification of cells positive for phospho-Ser240/244-S6 in the PFC and the striatum of vehicle- or PCP- (10 mg/kg, s.c.) treated rats that received either acute (30 min before brain perfusion) injection of SB258585 (2.5 mg/kg i.p.), rapamycin (10 mg/kg i.p.) or vehicle. Error bars represent SEM (n = 4). Quantification was performed on 224 µm x 168 µm images. *p < 0.001 versus vehicle-treated rats. Scale bar: 40 µm.

C. Vehicle- or PCP-treated rats were treated with SB258585 (2.5 mg/kg i.p.), rapamycin (10 mg/kg i.p.) or vehicle 30 min before the first session of the trial. Data, expressed as ratios of time spent investigating the novel juvenile rat to time spent investigating the familiar one during the 5-min choice trial, are means ± SEM obtained in n = 7–8 rats per group. *p < 0.05 versus vehicle/vehicle, §p < 0.05 versus PCP/vehicle.
animals (Fig 5B). Conversely, underpinning the specificity of this change, neonatal PCP treatment did not increase S6-phosphorylation in striatum (Fig 5B). S6 phosphorylation in PFC of PCP-treated rats was abolished by acute (30 min, i.p.) treatment with rapamycin (10 mg/kg) or SB258585 (2.5 mg/kg). These findings suggest that neonatal treatment with PCP leads to delayed increase in 5-HT₆ receptor-mediated mTOR signalling specifically in the PFC, a structure critically implicated in the cognitive deficits relevant to those seen in schizophrenia. Correspondingly, the deficit in social discrimination observed in PCP-treated rats was abolished by either SB258585 or rapamycin (Fig 5C).

Systemic administration of rapamycin (10 mg/kg) or SB258585 (10 mg/kg) also reversed the NOD deficit produced by housing rats in social isolation from the day of weaning (Fig 6A–D), an alternative neurodevelopmental model of schizophrenia (Fone & Porkess, 2008; Marsden et al, 2011). As observed in PCP-treated rats, we found an increase in mTOR signalling (assessed by S6 phosphorylation) in the PFC of isolated rats compared with group-housed animals, a difference abolished by administration of rapamycin or SB258585 (Fig 6E).

**DISCUSSION**

In the present study, we performed an unbiased proteomic analysis of the 5-HT₆ receptor complex in an effort to identify novel signalling mechanisms underlying its control of cognition. Six of the 28 proteins found to reproducibly interact with 5-HT₆ receptors expressed in HEK-293 cells are proteins of the mTOR pathway. These include two core components of mTORC1, mTOR itself and Raptor, which constitutively binds to mTOR and is involved in recruiting substrates for phosphorylation by the kinase domain of mTOR (Wang & Proud, 2011). Physical association of mTOR with native 5-HT₆ receptors expressed in mice brain was further validated by co-immunoprecipitation,
providing strong evidence that 5-HT₆ receptor and mTORC1 form a complex in vivo. To our knowledge, this is the first demonstration of a physical interaction of mTORC1 with a GPCR.

Although several proteins of the mTOR pathway [e.g. GβL, the third core component of mTORC1 (Wang & Proud, 2011)] were not identified in our proteomic screen, the possibility that additional partners involved in mTOR signalling interact with 5-HT₆ receptors should not be discounted. Their absence in the identified interactome might reflect a transient interaction with the receptor complex, loss of interaction during receptor solubilization and/or insufficient expression in the heterologous system used. For instance, pull-downs performed from brain tissue, which express high levels of Rheb (presumably higher than in HEK-293 cells), clearly identified Rheb as a 5-HT₆ receptor interacting protein, whereas Rheb was not identified by mass spectrometry in the initial proteomic screen.

The precise architecture of the complex formed by 5-HT₆ receptors and mTORC1 also remains to be established. Mass spectrometry analyses identified many more peptides in the mTOR sequence (42 unique peptides corresponding to 20.2% sequence coverage) than in the Raptor sequence (five unique peptides corresponding to 4.7% sequence coverage). This suggests that 5-HT₆ receptors recruit larger amounts of mTOR than Raptor, since it is unlikely that these values only reflect a difference in the number of suitably ionizable tryptic peptides in both proteins. Accordingly, mTOR might be a direct partner of the 5-HT₆ receptor, whereas Raptor might be recruited indirectly via mTOR. In addition, we showed that the 49 C-terminal amino acids of the 5-HT₆ receptor were necessary for interacting with mTOR but that the receptor C-terminal domain alone was not capable of recruiting mTOR. This suggests that several domains of the receptor, including both its extreme C-terminus and residues located upstream to its C-terminal domain, contribute to receptor-mTOR interaction.

The remarkable enrichment in proteins of the mTOR pathway in the 5-HT₆ receptor interactome was complemented by observations of its engagement by the receptor both in transfected HEK-293 cells and in vivo, providing the first evidence of a functional link between serotonergic transmission and mTOR signalling. The PI3K/Akt/Rheb pathway classically involved in mTOR activation by insulin and other growth factors (Swiech et al, 2008; Wang & Proud, 2011; Zhou & Huang, 2010) was likewise implicated in the 5-HT₆ receptor-elicited mTOR signalling in HEK-293 cells. Moreover, the deletion of the 49 C-terminal residues of receptor, which prevented its interaction with mTOR, strongly reduced its ability to activate mTOR, without affecting its coupling to Gαₛ. This suggested that 5-HT₆ receptor-elicited mTOR signalling was critically dependent of their physical association with mTOR. The lack of a unique, well-localized mTOR-binding motif in the 5-HT₆ receptor sequence precluded the use of an ‘interfering peptide’ in order to disrupt its interaction with mTOR and to further explore the functional impact of this coupling. In any event, it is possible that constitutive physical interaction of 5-HT₆ receptors with mTOR facilitates its engagement, via PI3K and Akt, upon receptor stimulation. It may also help in confining 5-HT₆ receptor-elicited mTOR signalling to specific cellular domains, thereby permitting local regulation of mTOR down-stream targets by 5-HT₆ receptors.

Peripheral administration of a 5-HT₆ receptor agonist markedly activated mTOR in striatal medium spiny neurons, which express the highest density of 5-HT₆ receptors in the brain (Codony et al, 2011; Woolley et al, 2004). Activation of mTOR in striatum via Rhes, a striatal-specific small G protein, is known to mediate l-DOPA-induced dyskinesia (Santini et al, 2009; Subramaniam et al, 2011). It has also been proposed that blockade of striatal mTOR signalling caused by the sequestration of Rhes by mutant Huntingtin might underlie the pronounced atrophy of the striatum in Huntington disease (Subramaniam & Snyder, 2011). Whether these processes are regulated by 5-HT₆ receptors remains to be established.

Administration of a 5-HT₆ receptor agonist also stimulated mTOR signalling in neurons of PFC, the cerebral structure involved in the control of social cognition (Loiseau et al, 2008). Neurons exhibiting enhanced mTOR signalling upon 5-HT₆ receptor activation included GABAergic neurons consistent with data on receptor localization, which indicated that almost 20% of 5-HT₆-immunoreactive neurons in cerebral cortex were GABAergic (Codony et al, 2011).

The mTOR pathway plays a crucial role in neurodevelopmental processes, including cell proliferation, synaptogenesis and growth of dendrites and axons and its perturbation has been implicated in the cognitive deficits of two rare genetic forms of autism spectrum disorders, tuberous sclerosis and fragile X syndrome (Auerbach et al, 2011; Ehninger et al, 2008; Ehninger & Silva, 2011; Swiech et al, 2008). These findings, together with our observations that 5-HT₆ receptors engage mTOR in neurons of the PFC, a structure critical for numerous cognitive functions, encouraged us to explore the role of prefrontal mTOR in cognitive impairments induced by 5-HT₆ agonists. We found that systemic administration of the mTOR inhibitor rapamycin prevented cognitive deficits induced by a 5-HT₆ agonist (also administered at the periphery) in models of social cognition (primarily involving olfactory cues) and NOD (visual cues). Further supporting a role of prefrontocortical mTOR activation, rapamycin administration also abolished the deficit in NOD induced by a local injection of a 5-HT₆ agonist in the PFC.

We then reasoned that enhanced mTOR signalling in the PFC, under the control of 5-HT₆ receptors, might underlie cognitive deficits of schizophrenia. Consistent with this hypothesis, we observed an enhanced mTOR signalling (blocked by a 5-HT₆ antagonist) in PFC of adult rats treated with PCP at a neonatal stage or housed in isolation after weaning from the dam. These two well-characterized and complementary developmental models of schizophrenia reproduce in adulthood several features of schizophrenia such as deficits in social cognition (neonatal PCP administration) and episodic memory (isolation rearing; Jones et al, 2011; Marsden et al, 2011). These cognitive deficits were abolished by an acute administration of rapamycin, mirroring the effects of 5-HT₆ antagonists. In both models, the increased mTOR signalling was specifically detected in PFC, and not in striatum, contrasting with the effects induced by agonist treatment. These findings are consistent with...
the dysfunction of prefrontal GABAergic transmission in schizophrenia, which is associated with gene expression abnormalities in a subclass (paravalbumin-positive) of PFC GABAergic interneurons (Hashimoto et al, 2003). They support the notion that certain cognitive defects in schizophrenia reflect a disruption of PFC function and connectivity, itself related to anomalous activity of GABAergic interneurons and a loss of their synchronized modulation of pyramidal neurons (Lewis & Gonzalez-Burgos, 2008). Alterations in network connectivity might ultimately be related to a deregulation of mTOR signalling in the PFC, occurring at a critical period in post-natal brain development and persisting into adulthood.

In conclusion, the present observations demonstrate that 5-HT6 receptors recruit and activate mTOR to compromise cognition both in pharmacological paradigms and in developmental models of schizophrenia (Fig 7). Accordingly, activation of mTOR may fulfil a broader role in the cognitive impairment of CNS disorders than hitherto appreciated. Indeed, our findings encourage an extension of clinical trials of mTOR inhibitors from patients presenting genetic forms of autism-related disorder, like tuberous sclerosis (Auerbach et al, 2011; Ehninger et al, 2008; Ehninger & Silva, 2011), to more numerous populations of schizophrenic patients. They reciprocally suggest that 5-HT6 antagonists might profitably be evaluated in autism. Irrespective of the outcome, the present work may help to resolve and to inter-link two persistent conundrums: namely, the molecular substrates mediating the detrimental influence of 5-HT6 receptors on cognition and the cellular events accounting for cognitive deficits in schizophrenia.

MATERIALS AND METHODS

Plasmids, chemicals and antibodies
The human 5-HT6 receptor cDNA, purchased from IMAGE (30915608), was subcloned in pPRKs vector into the BsrGI and HindIII sites and fused amino-terminally to a HA tag. The 5-HT6 receptor construct was kindly obtained by Dr E. Ponimaskin (Göttingen, Germany), Flag-Rheb1 (WT and I39K mutant) cDNAs by Dr J. Avruch (Boston, MA). The cDNA encoding 5-HT6 receptor C-terminus (last 120 residues) was amplified by PCR and subcloned in the pGEX-3X vector (GE Healthcare) using the BamHI and EcoRI restriction sites.

SB258585 (4-iido-N-[4-methoxy-3-[4-methyl-piperazin-1-yl]-phenyl]benzenesulphonamide) was obtained from Tocris. WAY181187 (2-(1-[(6-chloroimidazo[2,1-b]thiazol-5-sulfonyl)-1H-indol-3-yl]ethanamine)) and WAY208466 (N-[2-[3-(3-fluorophenylsulfonyl)-1H-pyridolo[2,3-b]pyridin-1-yl]ethyl]-N,N-dimethylamine) were synthesized by Gilbert Lavielle (IDRS, France). 5-HT (creatinine sulfate), scopalamine and HA peptide were purchased from Sigma–Aldrich, rapamycin from LC Laboratories, wortmannin and LY294002 from Calbiochem. The agarose-conjugated anti-HA antibody and the mouse anti-flag antibody were obtained from Sigma–Aldrich, the rabbit anti-HA antibody from LC Laboratories, wortmannin and LY294002 from Calbiochem. The agarose-conjugated anti-HA antibody and the mouse anti-flag antibody were obtained from Sigma–Aldrich, the rabbit anti-HA antibody from Invitrogen, the mouse anti-NeuN antibody from Millipore, the mouse anti GABA antibody from Chemicon, the rabbit anti-Neurofibrin 1 antibody from Santa-Cruz Biotechnology and the rabbit anti 5-HT6 receptor antibody from GeneTex, Inc. The mouse monoclonal anti-DARPP32 antibody was described elsewhere (Snyder et al, 1992). Rabbit polyclonal antibodies against phospho-Ser189-mTOR, phospho-Ser240/244-S6, phospho-Thr189-P70S6K, phospho- Ser65-4EBP1, phospho-Ser473-Akt, total mTOR, S6, P70S6K, 4EBP1, Rheb, Raptor and Akt were from Cell Signaling Technology.

Cell culture and transfection
HEK-293 cells, grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% dialysed, heat-inactivated foetal calf serum and antibiotics, were transfected using polyethyleneimine (PEI, Sigma–Aldrich) and used 24 h after transfection.

Purification and identification of 5-HT6 receptor-interacting proteins by mass spectrometry
HEK-293 cells expressing HA-5-HT6 receptor were lysed in a buffer containing HEPES 20 mM, pH 7.4, 150 mM NaCl, 1% NP40, 10% glycerol, 4 mg/ml dodecylmaltoside and a protease inhibitor cocktail (Roche), for 1 h at 4°C. Samples were centrifuged at 15,000 g for 30 min at 4°C. Solubilized proteins (10 mg per condition) were immunoprecipitated with the agarose-conjugated HA antibody (100 µl per condition). Control immunoprecipitations were performed in the presence of HA peptide (100 µM). Immunoprecipitated proteins were eluted in Laemmli sample buffer, separated by SDS–PAGE and
stained with Page Blue Stain (Fermentas). Gel lanes were cut into 20 gel pieces and proteins digested in-gel using trypsin (Gold, Promega). Generated peptides were analysed online by nano-flow HPLC-nanoelectrospray ionization using a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) coupled with an Ultimate 3000 HPLC ( Dionex). Desalting and pre-concentration of samples were performed on-line on a Pepmap\textsuperscript{R} precolumn (0.3 mm × 10 mm, Dionex). A gradient consisting of 0–40% A in 30 min, 80% B in 15 min (A = 0.1% formic acid, 2% acetonitrile in water; B = 0.1% formic acid in acetonitrile) at 300 nL/min was used to elute peptides from the capillary (0.075 mm × 150 mm) reverse-phase column (Pepmap\textsuperscript{R}). LC-MS/MS experiments comprised cycles of five events; an MS\textsuperscript{1} scan with Orbitrap mass analysis at 30,000 resolution followed by CID of the five most abundant precursors. Fragment ions generated by CID were detected in the linear trap. Normalized collision energy of 35 eV and activation time of 30 ms were used for CID. All Spectra were recorded under positive ion mode using the Xcalibur 2.0.7 software (Thermo Fisher Scientific). The mass scanning range (m/z) was 400–2000 and the capillary temperature was 200°C. Source parameters were adjusted as follows: ion spray voltage, 2.40 kV; capillary voltage, 40 V and tube lens, 120 V. Spectra were acquired with the instrument operating in the information-dependent acquisition mode throughout the HPLC gradient.

All MS/MS spectra were searched against the Homo sapiens entries of either SwissProt or TrEMBL databases (http://www.uniprot.org) by using the Mascot v 2.2 algorithm (Matrix Science, http://www.matrixscience.com/) with trypsin enzyme specificity and one trypsin missed cleavage. Methionine oxidation was set as variable modification for searches. A peptide mass tolerance of 5 ppm and a fragment mass tolerance of 0.5 Da were allowed for identification. Management and validation of mass spectrometry data, allowing discrimination of specific HA-tagged 5-HT\textsubscript{6} receptor partners, were performed using the myProMS v2.3. Web server (Poullet et al, 2007). Experiments were repeated four times to assess biological reproducibility. Only proteins identified with two or more peptides (threshold Mascot scores given corresponding to p < 0.01 for two peptides/protein and p < 0.001 for three peptides or more/protein, respectively) in each replicate and not detected in control immunoprecipitations were considered as potential partners of 5-HT\textsubscript{6} receptor.

GO annotations and functional enrichment in the 5-HT\textsubscript{6} receptor complex were analysed using BINGO (version 2.44), a Cytoscape plugin assessing overrepresentation of Gene Ontology categories in biological networks (Maere et al, 2005). The list of proteins interacting specifically with the 5-HT\textsubscript{6} receptor was used as the input set and the whole annotation as the reference set. The p-value was calculated with the hypergeometric test. For multi-testing correction, we applied the Benjamini & Hochberg False Discovery Rate (FDR) correction with a level of significance of 0.05. To minimize the impact of multi-testing issues, we used the GOSlim ontologies.

**GST pull-down**
Ten micromolars of fusion proteins (produced in BL21 cells), immobilized onto glutathione sepharose beads (GE Healthcare), were incubated overnight at 4°C with 5 mg of solubilized proteins from mice brain. After five washes with 0.5 M NaCl, retained proteins were eluted from the beads and analysed by Western blotting.

**Western blotting**
Equal amounts of protein (30 µg) for each sample were resolved onto 10% polyacrylamide gels. Proteins were transferred to Hybond C nitrocellulose membranes (GE Healthcare). Membranes were immuno-blotted with primary antibodies (anti-phospho-Ser\textsuperscript{244} mTOR, anti-phospho-Ser\textsuperscript{244} mTOR, anti-phospho-Ser\textsuperscript{244} mTOR, phospho-Ser\textsuperscript{173} Akt, total S6 and total Akt, Raptor and Neurofibromin 1, 1:1000 dilution; anti-phospho-Ser\textsuperscript{473} Akt, phospho-Thr\textsuperscript{308} S6K, phospho-Thr\textsuperscript{389} P70S6K, phospho-Ser\textsuperscript{451} 4EBP1, total mTOR, P70S6K, 4EBP1, Rheb, 1:500 dilution; anti HA and Flag, 1:400 dilution) and then with either anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1:3000, GE Healthcare). Immunoreactivity was detected with an enhanced chemiluminescence method (ECL\textsuperscript{TM} plus detection reagent, GE Healthcare) and immunoreactive bands were quantified by densitometry using the ImageJ software. In protein phosphorylation analyses, the amount of each phosphoprotein was normalized to the amount of the corresponding total protein detected in the sample. Data were analysed using the GraphPad Prism software (v. 4.0b) and statistical significance determined by one-way ANOVA followed by Newman–Keuls test.

**Drug administration**
Experiments were carried out on male Swiss mice (30–35 g) or Wistar rats (220–240 g, Janvier) under standard laboratory conditions and conformed to European ethics standards (86/609-EEC) and to decrees of the French National Ethics Committee (No 87/848) for the care and use of laboratory animals. Studies using Lister Hooded rats (Charles River, UK) were performed in compliance with the UK Home Office Animals (Scientific Procedures) 1986 ACT and local University of Nottingham ethical committee approval. For systemic administration of drugs, animals were injected i.p. or s.c. with drugs as indicated in the figure legends. WAY181187, WAY208466, SB258585 and rapamycin were dissolved in 5% DMSO/5% Tween 80. For combined treatments, rapamycin or SB258585 were administered 15–30 min before the 5-HT\textsubscript{6} agonist. Animals that did not receive drugs were injected with an equivalent amount of vehicle. WAY181187 was also delivered locally [0.63 µg per side in 1 µL vehicle (10% hydroxypropyl-β-cyclodextrine in a CSF)] by means of cannula implanted bilaterally in the PFC (AP: +3.0, L: ±0.7, DV: –2.3), 1 week before the experiments. For administration of phencyclidine at the neonatal stage, rats were received at 3 days of age, grouped in 10 male pups per adult mother. Phencyclidine or vehicle (saline) was administered on post-natal days (PND) 7, 9 and 11. All pups of the same litter received the same treatment. The rats were weaned from their mother at PND 21, after which they were separated at random and housed in mixed-litter groups of four. Measurement of mTOR activation and social novelty discrimination experiments were carried out at 8–10 weeks of age.

**Analysis of in vivo mTOR activation**
Activation of mTOR was assessed by Western blotting and immunohistochemistry using anti-phosphoSer\textsuperscript{244} mTOR and anti-phospho-Ser\textsuperscript{244} mTOR antibodies. For Western blotting, mice were decapitated and their heads were immediately frozen in liquid nitrogen (for 12 s). The frozen heads were cut into 210-µm thick slices with a cryostat. Microdiscs (1.4 mm diameter) were punched out bilaterally from the median PFC and homogenized by the addition of a boiling solution of 1% SDS w/v and 1 mM Na\textsuperscript+ orthovanadate in water, immediate...
mTOR inhibitor rapamycin prevented deficits of social cognition and NOD induced by a 5-HT₆ agonist in the rat. Moreover, rapamycin, like 5-HT₆ antagonists, rescued cognitive deficits in two complementary development rodent models of schizophrenia that reproduce in adulthood many features of the disease, neonatal treatment with phencyclidine and post-weaning housing in isolation.

**IMPACT:**
These studies identify a signalling complex physically associated with the 5-HT₆ receptor that underlies its deleterious influence upon cognition. They suggest a critical role of mTOR activation not only in rare autism-related genetic disorders, such as Fragile X mental retardation syndrome and tuberous sclerosis, as previously suggested, but also in schizophrenia, a more frequent, multi-factorial and debilitating disorder. These findings encourage an extension of clinical trials of mTOR inhibitors from patients presenting genetic forms of autism to populations of schizophrenic patients and reciprocally suggest that 5-HT₆ antagonists might be evaluated in autism.

**Behavioural studies**
The social recognition test (ability of an adult rat to recognize a younger conspecific during the second of two 5-min sessions) was performed using a procedure without an intersession delay, as previously described (Loiseau et al, 2008). For the social novelty discrimination test (ability of an adult rat to differentiate novel and familiar juveniles), one juvenile rat was introduced in the adult cage in a first 30-min session. The same juvenile rat was then presented together with a second novel juvenile to the adult in a second 5-min session. Time of investigation of each juvenile during the second session was recorded and the ratio of time spent in active social investigation of the novel to the time spent in investigating the familiar during the second session was calculated. We used a procedure without an inter-session interval to maximize discrimination performance (ratio ≥ 3 in vehicle-treated group). The specificity of drug effects on social recognition or social discrimination was analysed by two-way ANOVA, followed by either Newman–Keuls test (social recognition) or Dunnett’s test (social discrimination). The NOD test was performed as previously described (Watson et al, 2011). The inter-trial interval between familiarization and choice trials was 5 min, such that vehicle-treated animals were able to discriminate the novel and familiar objects. In experiments evaluating the impact of social isolation, each litter of Lister hooded rats was weaned on PND 24 and approximately half of each litter housed in either groups (3–4 per cage) or social isolation for 6 weeks. Isolated and group-housed rats were then subjected to the NOD test on PND 66. Injections were carried out 30 min prior to the familiarization trial. The interval between familiarization and choice trials was 2 h. Raw data...
were analysed by ANOVA with the exploration of novel and familiar object as the repeated within-subject factor and the treatment as the between-subject factor. D2 scores (exploration of novel object – exploration of familiar object/total object exploration) were analysed by two-way ANOVA followed by Newman–Keuls test.

Author contributions
JM, SCD, PD and FG performed proteomic analysis, biochemistry and cell biology experiments; MS performed protein identification and validation by mass spectrometry; DH analysed mTOR activity in brain microdiscs; FL, AD, JMR and DW performed behavioural studies; MJM, CMC, KF, JB and PM designed the experiments and supervised the study; MJM and PM wrote the manuscript.

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Supporting Information is available at EMBO Molecular Medicine online.

The authors declare that they have no conflict of interest.

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