The inducible serotonergic 1C11\(^{\text{5-HT}}\) cell line expresses a defined set of serotonergic receptors of the 5-HT\(_{2B}\), 5-HT\(_{1B/D}\), and 5-HT\(_{2A}\) subtypes, which sustain a regulation of serotonergic associated functions through G-protein-dependent signaling. 1C11\(^{\text{5-HT}}\) cells have been instrumented to assign a signaling function to the cellular prion protein PrPC. Here, we establish that antibody-mediated ligation of PrPC concomitant to agonist stimulation of 5-HT receptors modulates the couplings of all three serotonergic receptors present on 1C11\(^{\text{5-HT}}\) cells. Specific impacts of PrP antibodies were monitored depending on the receptor and pathway considered. PrPC ligation selectively cancels the 5-HT\(_{2A}\)-PLC response, decreases the 5-HT\(_{1B/D}\) negative coupling to adenylate cyclase, and potentiates the 5-HT\(_{2B}\)-PLA\(_2\) coupling. As a result, PrP\(^{\text{I}}\) ligation disturbs the functional interactions occurring between the signaling pathways of the three receptor subtypes. In 1C11\(^{\text{5-HT}}\) cells, antagonizing cross-talks arising from 5-HT\(_{2B}\) and 5-HT\(_{2A}\) receptors control the 5-HT\(_{1B/D}\) function. PrPC\(^{\text{I}}\) ligation reinforces the negative regulation exerted by 5-HT\(_{2B}\) on 5-HT\(_{1B/D}\) receptors. On the other hand, it abrogates the blocking action of 5-HT\(_{2A}\) on the regulatory loop linking 5-HT\(_{2B}\) and 5-HT\(_{1B/D}\) receptors. We propose that the ligation of PrPC affects the potency or dynamics of G-protein activation by agonist-bound serotonergic receptors. Finally, the PrPC\(^{\text{I}}\)-dependent modulation of 5-HT receptor couplings is restricted to 1C11\(^{\text{5-HT}}\) cells expressing a complete serotonergic phenotype. It critically involves a PrPC\(^{\text{I}}\)-caveolin platform implemented on the neurites of 1C11\(^{\text{5-HT}}\) cells during differentiation. Our findings define PrPC\(^{\text{I}}\) as a modulator of 5-HT receptor coupling to G-proteins and thereby as a protagonist contributing to the homeostasis of serotonergic neurons. They provide a foundation for uncovering the impact of prion infection on serotonergic functions.

Some aspects of serotonin (5-hydroxytryptamine (5-HT\(^{1}\)) homeostasis may relate to the plasticity of serotonergic neurons,

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\(^{2}\)The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

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\(^{4}\)The abbreviations used are: 5-HT, 5-hydroxytryptamine; DOI, (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-amino propane; GPCR, G-protein coupled receptor; PLC, phospholipase C; IP\(_3\), inositol 1,4,5-trisphosphate; NO, nitric oxide; NOS, NO synthase; PLA\(_2\), phospholipase A\(_2\); AA, arachidonic acid; PrP\(^{\text{G}}\), cellular prion protein; SERT, 5-HT transporter; 5-CT, 5-carboxamidotryptamine; d4, day 4; FScA, forskolin-stimulated cAMP accumulation.

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and stimulates phospholipase C (PLCβ), thereby initiating a rapid release of inositol 1,4,5-trisphosphate (IP₃) and a rise in intracellular calcium level (6). The 5-HT₂A receptor is also coupled to the ras/mitogen-activated protein kinase cascade (7) and cross-talks with tyrosine kinase pathways to promote cell proliferation (8). In addition, the 5-HT₁B receptor recruits both cellular and inductive nitric-oxide (NO) synthase (NOS) transduction pathways through a type I PDZ domain (9). Finally, a coupling of the 5-HT₂B receptor to the phospholipase A2 (PLA2)/arachidonic acid (AA) pathway was evidenced in 1C115-HT₁D cells and shown to sustain a negative regulation of the 5-HT₁B/D receptor function at day 2 of 1C11 serotonergic differentiation (5). The inhibitory effect exerted by 5-HT₂B receptors on the 5-HT₁B/D coupling to adenylate cyclase however is lifted in 1C115-HT₁D day 4 cells by the concomitant activation of the 5-HT₂A receptor. 5-HT₂A receptors are also coupled with the PLA2 cascade. The signaling pathway through which 5-HT₂A receptors counterbalance the antagonizing action of 5-HT₂B receptors on the 5-HT₁B/D receptor function, necessarily distinct from PLA2, is still unclear.

Understanding how serotonergic neurons manage 5-HT-related inputs with respect to the diversity of other signals constitutes another important challenge. Recently, the 1C11 cell line allowed us to identify transduction pathways coupled with the cellular prion protein (PrPc). This protein is the normal counterpart of a pathogenic protein termed scrapie prion protein, which is involved in a group of fatal neurodegenerative disorders known as transmissible spongiform encephalopathies. PrPc is expressed in all cell types and is particularly disorders known as transmissible spongiform encephalopathies. PrPc is expressed in all cell types and is particularly associated with the surface of neurons to which it is anchored by a glycosylphosphatidylinositol moiety. Whatever the state of differentiation of 1C11 cells, PrPc is endogenous expressed at roughly similar levels (10). Moreover, antibody-mediated ligation of PrPc, which is assumed to mimic the interaction with a yet to be identified ligand, induces NADPH oxidase-dependent reactive oxygen species production and activation of the extracellular regulated kinases 1/2 (ERK1/2), two members of the mitogen-activated protein kinase family (11). In cells harboring a fully differentiated phenotype exclusively, PrPc associates with the membrane protein caveolin and the tyrosine kinase Fyn within a signaling platform that governs the downstream effectors, i.e. reactive oxygen species and ERK1/2 (11, 12). The implementation of the PrPc-caveolin-Fyn complex depends upon the expression of neuronal and neurotransmitter-associated functions. It may relate to the proper structural organization of the partners within subcellular microdomains. Noticeably, the PrPc-Fyn coupling occurs on the neurites of 1C11-derived neuronal progenies where bioaminergic receptors are most likely localized (13).

In an attempt to shed some light on the mechanisms sustaining the homeostasis of serotonergic neurons, we took interest in relations between 5-HT-mediated pathways and PrPc. Our initial goal was to monitor in 1C115-HT₁D serotonergic cells the functionality of 5-HT receptors under PrPc ligation. We show here that PrPc modulates the couplings and cross-talks of the 5-HT₁B/D, 5-HT₂A and 5-HT₂B receptors in 1C115-HT₁D serotonergic cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dibutyryl cyclic AMP and cyclohexane carboxylic acid were from Sigma-Aldrich Chimie (St-Quentin Fallavier, France). Neurochemicals were from RBI (Natick, MA). All other chemicals, of the purest grade available, were from classical commercial sources. [³⁵S]-labeled DOI (8.4 TBq/mmol) and [¹⁴C]A8 (57 mCi/mmol) were obtained from PerkinElmer Life Sciences. Forskolin was purchased from Calbiochem.

Agnostics and antagonists of 5-HT₂ receptor subtypes were selected according to Porter et al., Cussac et al., and Jerman et al. (14–16). All tissue culture reagents and Hank's balanced salt solution were from Invitrogen. PrP monoclonal antibodies (SAF61, SAF32, and BAR221, all IgG) with distinct binding epitopes (11) were obtained from the Service de Pharmacologie et d’Immunologie, Commissariat à l’Énergie Atomique (Saclay, France). Polyclonal goat IgG antibodies against the 5-HT transporter (SERT) were from Santa Cruz Biotechnology.

**RESULTS**

**PrPc Ligation Modifies PLA2 and PLC (but Not NOS) Activities Coupled to 5-HT Receptors**—To assess potential cross-talks between PrPc and the signaling pathways coupled with the 1C115-HT₁D serotonergic receptors, we monitored the levels of various second messengers production in response to 5-HT receptor activation combined to antibody-mediated ligation of PrPc. A first set of experiments was centered on the two 5-HT₂ subtypes, i.e. 5-HT₂A and 5-HT₂B receptors. Treatment of 1C115-HT₁D day 4 cells with 100 nM DOI, a selective 5-HT₂ receptor agonist, stimulates PLC (18), PLA2 (5), and NOS (9) activities. When 1C115-HT₁D day 4 cells were incubated with anti-PrP antibodies (SAF61, SAF32, or BAR221) only, no PLC, PLA2, or NOS activation was obtained (Fig. 1, A–C). Now, upon simultaneous exposure to DOI and PrPc antibodies, the level of NOS activity did not differ from that obtained in response to DOI alone (Fig. 1A). By contrast, as shown in Fig. 1B, PrPc ligation combined with the addition of DOI caused a significant 1.7-fold increase in PLA2 stimulation as compared with DOI treatment alone. PrPc ligation also had an impact on the DOI-induced PLC activation. Concomitant exposure of 1C115-HT₁D d4 cells to DOI and PrPc antibodies reduced by 3-fold the level of DOI-induced PLC activation.

**Measurement of NOS Activity**—NOS activity was evaluated by direct electrophotometrical measurement of NO as described by Kaaij et al. (19). Cells were washed twice and incubated at 37 °C in perfusate as described (19). NO production was directly measured in a porphyrinic microsensor positioned on the surface of 1C115-HT₁D cells 10 min after 5-HT₇ agonist and/or anti-PrP antibody addition by a nanoinjector (20).

**Measurement of Phospholipase A2 Activity**—PLA2 activity was assessed through measurement of [¹⁴C]arachidonic acid release 10 min after receptor and/or PrP ligation as in Ref. 5.

**Measurement of 5-HT₁B/D Receptor Response**—The functionality of the 5-HT₁B/D receptor was assessed as in Ref. 5. The 5-HT₁B/D receptor-mediated response was followed by measuring (15 min, 37 °C) the 5-carboxamidotryptamine (5-CT)-induced inhibition of cAMP accumulation in the presence of 1 μM forskolin and 0.1 mM rolipram, a phosphodiesterase inhibitor. For each experiment, data were expressed according to the response obtained with 1 μM forskolin (100%).

**Antibody-mediated PrPc Ligation**—Ligation of PrPc at the surface of 1C115-HT₁D cells was carried out using SAF61, SAF32, or BAR221 antibodies at 10 μg/ml as in Ref. 11.

**Cell Permeabilization**—1C115-HT₁D cells were permeabilized prior to antibody-mediated neutralization of G proteins according to Launay et al. (7).

**Caveolin Immunosequestration—**1C115-HT₁D cells were bombarded with tungsten microprojectiles coated with antibodies to caveolin-1 as in Ref. 12. Uncoated microprojectiles were used as a control.

**Data Analysis and Statistics**—Statistical analyses on small groups were performed using non-parametric tests. The significance criterion of p < 0.001 was adopted. All values are given as arithmetic means ± standard errors of the means (S.E.).
IP$_3$ production triggered by the sole addition of 100 nM DOI, thereby remaining at the basal level (Fig. 1C). In all experiments, identical results could be obtained using three distinct antibodies against PrP (SAF61, SAF32, or BAR221). Of note, no effect could be recorded on the DOI-induced activation of NOS, PLA2, or PLC under simultaneous incubation of 1C115-HT day 4 cells with irrelevant antibodies to the membraneous serotonin transporter (SERT) used as a negative control (Fig. 1, A–C). These overall data argue against the occurrence of functional interactions between PrP$^C$ and 5-HT$_2$ receptor-NO pathway. In turn, they emphasize PrP$^C$-dependent modulations of the PLC and PLA2 couplings associated to 5-HT$_{2A}$ and/or 5-HT$_{2B}$ receptors.

PrP$^C$ Ligation Preferentially Enhances the Efficacy of PLA2 Coupling to the 5-HT$_{2B}$ Receptor—The effect of PrP ligation on 5-HT$_2$ receptors-induced PLA2 activity was further investigated using SAF61 antibodies. In 1C115-HT day 4 cells, both 5-HT$_{2A}$ and 5-HT$_{2B}$ receptors promote PLA2 activation and subsequent AA release (5). Because DOI activates both 5-HT$_2$ receptor subtypes, we selected a set of 5-HT$_2$ agonists with distinct specificities toward the 5-HT$_{2A}$ and 5-HT$_{2B}$ subtypes. The level of AA production monitored in 1C115-HT day 4 cells submitted to PrP$^C$ ligation was compared with the maximal response (Emax) elicited by the agonist alone. As shown in Fig. 1D, PrP$^C$ ligation had no significant effect on the PLA2 activity induced by treatment with 100 nM MK212, preferentially targeting 5-HT$_2A$ receptors with various specificities toward the 5-HT$_2$ or 5-HT$_2B$ subtypes were measured with or without PrP$^C$ concomitant ligation using SAF61 antibodies. Values are given as a percentage of the PLA2 maximal response obtained with 5-HT. Data shown are the means ± S.E. of five independent experiments. A6, antibody.

To explore the functional relation between PrP and the 5-HT$_{2B}$ receptor, we investigated whether PrP ligation affects the binding properties of the 5-HT$_{2B}$ Receptor. We selected a panel of agonists and antagonists that differentially bind to 5-HT$_{2A}$ or 5-HT$_{2B}$ receptors (14–16). This set of drugs allows discriminating between both 5-HT$_2$ receptor subtypes and more precisely specifies the 5-HT$_{2B}$ receptor. We determined the pharmacological profile of the 5-HT$_{2B}$ receptor in 1C115-HT day 4 cells by assessing the binding affinities to each drug, used as a competitor of DOI binding. The binding of all drugs to 1C115-HT day 4 cells was then followed under simultaneous incubation with antibodies to PrP. As shown in Fig. 2A, the binding constants of the drugs to 1C115-HT cells exposed to PrP antibodies (SAF61) did not significantly differ from those monitored without PrP ligation. In view of the exclusive specificity of this set of drugs toward the 5-HT$_{2B}$ receptor, we conclude that PrP antibodies have no impact on the binding properties of the 5-HT$_{2B}$ receptor.

In another set of experiments, we evaluated the effect of the same panel of drugs on PLA2 activity in 1C115-HT day 4 cells. In the case of agonists, the pEC50 values were deduced from the dose-response curves. As for antagonists, their capacities to inhibit the DOI-dependent stimulation of PLA2 activity were monitored to calculate pK$_{a}$ values. Comparison between the binding constants of the drugs (pK$_{a}$) and the apparent equilibrium constants deduced from their effects on PLA2 activity (pEC50 or pK$_{a}$) yield a correlation ratio of 0.787 (n = 19, p < 0.0001) (Fig. 2B). The dose effect of the drugs on PLA2 activity was then monitored in 1C115-HT day 4 cells submitted to concomitant PrP$^C$ ligation using SAF61 antibodies. The pEC50 and pK$_{a}$ values thus obtained were plotted against the pK$_{a}$ values of the drugs determined in 1C115-HT cells exposed to PrP antibodies (Fig. 2C). A highly significant ratio (0.957, n = 19, p < 0.0001) was found between the effects of the agonists and

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antagonists on PLA2 activity and their binding. This correlation was over one order of magnitude higher than that obtained without PrP antibodies. Because the set of selected drugs specifies the 5-HT$_{2B}$ receptor (14–16), this comparative analysis directly demonstrates that PrP$^C$ ligation enhances the coupling potency (EC$_{50}$) of 5-HT$_{2B}$ receptors to the PLA2 pathway.

PrP$^C$ Ligation Abrogates the 5-HT$_{2A}$ Receptor-dependent PLC Coupling in 1C11$^{5-HT}$ D4 Cells—To further characterize the negative impact of PrP$^C$ ligation on the DOI-induced PLC activation, we first sought to evaluate the relative contribution of 5-HT$_{2A}$ and 5-HT$_{2B}$ receptors to the DOI-dependent IP$_3$ production in 1C11$^{5-HT}$ day 4 cells. In contrast to the 5-HT$_{2A}$ receptor, a functional coupling of the 5-HT$_{2B}$ receptor to PLC$\beta$ has never been evidenced in vivo (21, 22). However, we described previously a 5-HT$_{2B}$ receptor-dependent IP$_3$ accumulation both in LMTK cells stably transfected with a cDNA encoding this receptor or in 1C11$^{5-HT}$ cells at day 2 of serotonergic differentiation when 5-HT$_{2A}$ receptors are not yet present (9, 18). As already mentioned in Fig. 1C, the addition of 100 nM DOI on 1C11$^{5-HT}$ d4 cells promoted a rapid IP$_3$ accumulation reaching a plateau at 15 min with a level of maximal response (Emax) corresponding to a 4-fold increase as compared with the basal level of IP$_3$ production. When the cells were simultaneously exposed to 100 nM DOI and to 100 nM MDL100.907, a selective antagonist of 5-HT$_{2B}$ receptors, IP$_3$ accumulation remained at the basal level (Fig. 3A). Furthermore, as shown in Fig. 3A, treatment of 1C11$^{5-HT}$ d4 cells by 100 nM BW23C86, which specifically activates 5-HT$_{2B}$ receptors, did not induce any PLC$\beta$ activation either (32 ± 2.5% of the DOI reference level, not significantly different from 26 ± 3.7% corresponding to the basal level). These data indicate that the PLC$\beta$ stimulation monitored in 1C11$^{5-HT}$ d4 cells in response to 100 nM DOI (Fig. 3A) depends on 5-HT$_{2A}$ receptors only. They also emphasize that between day 2 and day 4 of the serotonergic differentiation program of 1C11$^{5-HT}$ cells 5-HT$_{2B}$ receptors lose their ability to recruit the PLC$\beta$/IP$_3$ pathway.

As shown in Fig. 3A, IP$_3$ accumulation in 1C11$^{5-HT}$ d4 cells remained at a basal level under simultaneous exposure to PrP antibodies and to 100 nM DOI. Again, similar impacts on the DOI-induced IP$_3$ release were recorded using three distinct anti-PrP antibodies (SAF61, SAF32, and BAR221), whereas irrelevant antibodies against the SERT protein had no effect (Fig. 3A). This observation demonstrates that PrP$^C$ ligation specifically abrogates the 5-HT$_{2A}$ receptor-dependent IP$_3$ response to DOI. Such a behavior led us to examine whether the constitutive IP$_3$ production measured could be attributed either to the intrinsic activity of the 5-HT$_{2A}$ receptor, to that of the 5-HT$_{2B}$ receptor, or to the added constitutive activities of the two receptors. Incubation of 1C11$^{5-HT}$ d4 cells with inverse agonists (ritanserin (5 nM), which broadly acts on all 5-HT$_2$ subtypes, or LY266070 (1 nM), which selectively targets 5-HT$_{2B}$ receptors) fully cancelled the basal IP$_3$ accumulation (Fig. 3A). This result indicates that 5-HT$_{2B}$ receptors retain their agonist-independent intrinsic activity for PLC coupling in 1C11$^{5-HT}$ d4 cells.

Noteworthy, whereas PrP$^C$ ligation per se had no impact on the basal level of phosphoinositide hydrolysis (Fig. 3A), a slight but significant increase in IP$_3$ production was observed under concomitant exposure of 1C11$^{5-HT}$ d4 cells to BW23C86 and PrP antibodies (45% of the DOI reference level using SAF61) as compared with the value obtained with
Prion Protein Modulates 5-HT Receptor Coupling to G-proteins

**Fig. 3.** Modulation of 5-HT1B/5-HT1D- and 5-HT2A-related signals by PrP<sup>C</sup> ligation and PrP<sup>C</sup>-induced imbalance of 5-HT<sub>2B</sub> and 5-HT<sub>1B/D</sub> receptor cross-talks in 1C11<sup>5-HT</sup> day 4 cells. A: the maximal PLC responses to a panel of 5-HT<sub>3</sub> agonists (DOI, BW723C86), antagonist (MDL100.907), and/or reverse agonists (ritanserin, LY266070) were measured with or without concomitant PrP<sup>C</sup> ligation (SAF61, SAF32, BAR221). Control experiments were carried out in parallel using irrelevant antibodies against the SERT protein. PrP antibodies drastically reduced the DOI-induced IP<sub>3</sub> release to basal level (*), and significantly elevated the level of IP<sub>3</sub> response to BW723C86 (**). Values are given as percentage of the maximal PLC response obtained with DOI alone. B, the effect of PrP<sup>C</sup> ligation on the 5-HT<sub>1B/D</sub> receptor function was assessed by monitoring the inhibition of forskolin-induced accumulation of cAMP in response to the agonist 5-CT with or without concomitant exposure to anti-PrP antibodies. The impact of 5-HT<sub>1D</sub> activation alone (BW723C86, DOI+ MDL100.907) or in combination with 5-HT<sub>2A</sub> receptor activation (DOI) on the 5-HT<sub>1B/D</sub> receptor negative coupling to adenylyl cyclase was evaluated with or without concomitant PrP<sup>C</sup> ligation. Anti-SERT antibodies were used as negative control. Anti-PrP antibodies (SAF61, SAF32, BAR221) significantly reduced the extent of FScA inhibitions obtained under 5-HT<sub>1B/D</sub> activation alone with 5-CT (*), combined activation of all three (5-HT<sub>1B/D</sub>, 5-HT<sub>2A</sub>, and 5-HT<sub>2B</sub>) receptors with 5-CT plus DOI (**), or simultaneous activation of 5-HT<sub>1B/D</sub> and 5-HT<sub>2A</sub> receptors (5-CT and BW723C86, ***). Values are given as percentage of the maximal cAMP accumulation obtained with forskolin alone. Data shown in each panel are the means ± S.E. of five independent experiments.

BW723C86 alone (32% of the DOI reference level). This enhancing effect of PrP<sup>C</sup> ligation on the BW723C86-related IP<sub>3</sub> response was reproducibly recorded using two other anti-PrP antibodies (SAF32, BAR221). In contrast, the IP<sub>3</sub> accumulation measured in 1C11<sup>5-HT</sup> d4 cells simultaneously exposed to BW723C86 and anti-SERT antibodies (37 ± 6% of the DOI reference level) was not statistically different from that obtained in cells treated with BW723C86 alone. These overall experiments suggest that PrP<sup>C</sup> ligation specifically promotes a partial restoration of the agonist-induced PLC coupling of the 5-HT<sub>2B</sub> receptor at day 4 of differentiation. In any case, PrP<sup>C</sup> ligation was unable to fully rescue the IP<sub>3</sub> coupling that is associated to the 5-HT<sub>2B</sub> receptor at day 2 because the maximal IP<sub>3</sub> response to combined BW723C86 treatment and PrP<sup>C</sup> ligation remained unequivocally below that measured in 1C11<sup>5-HT</sup> d2 cells exposed to DOI (17) or to the more potent agonist BW723C86 (data not shown). As a whole, our findings highlight that (i) PrP<sup>C</sup> ligation concomitant to agonist stimulation switches off the coupling of 5-HT<sub>2A</sub> receptors to the PLC pathway and that (ii) PrP antibodies do not affect the basal level of IP<sub>3</sub> accumulation specifically associated to the 5-HT<sub>2B</sub> receptor but allow a slight restoration of the agonist-dependent 5-HT<sub>1B/D</sub> receptor-PLC coupling.

**PrP<sup>C</sup> Ligation Down-regulates the 5-HT<sub>1B/D</sub> Receptor Negative Coupling to Adenylyl Cyclase**—At last, we examined the effect of PrP antibodies on the 5-HT<sub>1B/D</sub> receptors-mediated signaling. 5-HT<sub>1B/D</sub> receptors are linked to G<sub>i</sub>-mediated inhibition of adenylyl cyclase activity. Their functionality can be deduced from the capacity of agonists to inhibit forskolin-stimulated cAMP accumulation (FScA). A typical response of 1C11<sup>5-HT</sup> d4 cells to 5 nm 5-CT, a 5-HT<sub>1B/D</sub> agonist, is shown on Fig. 3B. A 52% inhibition of FScA is obtained following 5-CT addition. FScA does not vary upon ligation of PrP<sup>C</sup> alone (Fig. 3B). Therefore, PrP<sup>C</sup> by itself is not coupled with G<sub>i</sub>-mediated inhibition of adenylyl cyclase. Interestingly, upon simultaneous incubation of 1C11<sup>5-HT</sup> d4 cells with 5-CT and PrP antibodies, a 2-fold decrease in the extent of FScA inhibition was observed (Fig. 3B). Similar reduced FScA inhibition values were obtained using three distinct anti-PrP antibodies (27, 33, and 21% with SAF61, SAF32, and BAR221, respectively) as compared with the reference 5-CT-induced FScA response (52% inhibition). The above results demonstrate that PrP<sup>C</sup> ligation lowers the efficacy (Emax) of the 5-HT<sub>1B/D</sub> receptor negative coupling to adenylyl cyclase.

**PrP<sup>C</sup> Interferes with the Cross-talks between the Three 5-HT Autoreceptors in 1C11<sup>5-HT</sup> Cells**—Because PrP<sup>C</sup> ligation modulates the couplings of each of the 5-HT<sub>2A</sub>, 5-HT<sub>2B</sub>, and 5-HT<sub>1B/D</sub> receptor subtypes in 1C11<sup>5-HT</sup> cells, we anticipated that PrP<sup>C</sup> might affect the functional interactions between these three autoreceptors. Indeed, we previously established that both the 5-HT<sub>2A</sub> and 5-HT<sub>2B</sub> receptors exert an antagonistic role on the 5-HT<sub>1B/D</sub> receptor function (5). In that former study, we took advantage of the sequential onset of the 5-HT<sub>B</sub> and 5-HT<sub>1B/D</sub> receptors at day 2 of the 1C11<sup>5-HT</sup> differentiation program (prior to that of the 5-HT<sub>2A</sub> receptor at day 4) and observed that selective activation of the 5-HT<sub>2B</sub> receptor can-...
cells the coupling of 5-HT \(_{1B/D}\) receptors to adenylate cyclase via the PLA2/AA pathway (5). In 1C11\(^{5-HT}\) day 4 cells, a restoration of the 5-HT \(_{1B/D}\) receptor functionality was obtained upon simultaneous activation of both 5-HT\(_{2}\) receptor subtypes by DOI (Ref. 5 and Fig. 3B). We concluded from these observations that the 5-HT \(_{2B}\) receptor-dependent negative regulation of 5-HT\(_{1B/D}\) function is lifted by the 5-HT\(_{2A}\) receptor.

Here, we wanted to evaluate how PrPC ligation impacts the regulation of the functionality of 5-HT \(_{1B/D}\) receptors by interfering with the coupling of the three serotonergic receptors. As shown in Fig. 3B, stimulation by 100 nm DOI of both 5-HT\(_2\) receptor subtypes in 1C11\(^{5-HT}\) d4 cells, in the absence of PrP antibodies, yield a 5-CT-mediated inhibition of FScA equal to 49%. Upon combined exposure of 1C11\(^{5-HT}\) d4 cells to DOI and to SAF61 anti-PrP antibodies, the 5-HT\(_{1B/D}\) response to its agonist 5-CT was reduced to 18% inhibition of FScA (Fig. 3B).

The other two anti-PrP antibodies used in this study (SAF32 and BAR221) also significantly decreased the FScA inhibition recorded with cells exposed to both DOI and 5-CT, albeit with a less pronounced effect than SAF61 (35 and 25%, respectively). By contrast, anti-SERT antibodies did not induce any change in the extent of FSCA inhibition recorded with combined DOI and 5-CT treatment (Fig. 3B). Thus, as we suspected from our experiments above, PrPC ligation specifically introduces an imbalance between the functional interactions involving the three receptor subtypes. Such an imbalance may integrate three levels of regulation. (i) PrPC ligation directly reduces the efficacy of the 5-HT\(_{1B/D}\) receptor coupling to adenylate cyclase (27% of FScA inhibition with SAF61 versus 52% in the absence of antibodies). (ii) PrP antibodies may abrogate the 5-HT\(_{2A}\) receptor-mediated signal(s) that antagonize the negative regulation by 5-HT\(_{2B}\) receptors of 5-HT\(_{1B/D}\) function. This signal(s), distinct from PLA2 in 1C11\(^{5-HT}\) d4 cells, still has to be identified (5). Noteworthy, the remaining 5-HT\(_{1B/D}\)-associated activity monitored upon activation of both 5-HT\(_2\)-receptor subtypes plus PrPC ligation (18% of FScA inhibition using SAF61) is close to that measured upon selective 5-HT\(_{2B}\) receptor stimulation by BW723C86 without concomitant ligation of PrPC (15% of FScA inhibition, Fig. 3B). Thus, PrPC ligation can interfere with the DOI-induced 5-HT\(_{2A}\) response in 1C11\(^{5-HT}\) d4 cells by disrupting not only the PLC coupling but also the regulatory loop that targets 5-HT\(_{1B/D}\) receptors. The latter loop is distinct from the PLC pathway because 5-HT\(_{2A}\) receptors retain their capacity to relieve the inhibitory effect exerted by the 5-HT\(_{2B}\) receptor on the 5-HT\(_{1B/D}\) function when PLC activity is switched off using the selective inhibitor U73122 (data not shown). (iii) Finally, by enhancing the coupling efficacy of the 5-HT\(_{2B}\) receptor to the PLA2 pathway, PrPC ligation strengthens the inhibitory effect of 5-HT\(_{2B}\) receptors on the 5-HT\(_{1B/D}\) function. In 1C11\(^{5-HT}\) d4 cells, the 5-CT mediated FSCA inhibition (52%) is markedly reduced upon selective stimulation of 5-HT\(_{2B}\) receptors by 100 nm BW723C86 (15%) or even totally abolished upon the addition of DOI (activating both 5-HT\(_{2B}\) and 5-HT\(_{2A}\) receptors) in combination with 100 nm MDL100.907 (a selective antagonist of 5-HT\(_{2A}\) receptors). The addition of PrP antibodies even potentiates the BW723C86-mediated inactivation of the G\(_i\)-coupled 5-HT\(_{1B/D}\) system, leading to a 14% (SAF61) increase in FScA versus 15% FScA inhibition (Fig. 3B).

Altogether, our data establish that PrPC interferes with the signaling activity of all three 5-HT receptors present on 1C11\(^{5-HT}\) serotonergic cells. They highlight specific impacts of PrPC ligation on the transduction pathways related to each receptor. Eventually, our findings demonstrate that PrPC acts as a modulator of the cross-talks between the three 5-HT autoreceptors that control the overall serotonergic functions of 1C11\(^{5-HT}\) cells.

The Modulation of 5-HT Receptor Couplings by PrPC Is Differentiation-dependent and Involves Caveolin—At day 2 of their differentiation program, 1C11\(^{5-HT}\) cells, which express identical amounts of PrPC as 1C11\(^{5-HT}\) d4 cells, implement functional 5-HT\(_{2B}\) and 5-HT\(_{1B/D}\) receptors. Thus we examined the effect of antibody-mediated PrPC ligation on the signaling activities of these receptors in 1C11\(^{5-HT}\) d2 cells. First, we verified that PrPC ligation itself did not trigger any NOS, PLA2, nor PLC activation (Fig. 4, A–C). The DOI-induced NOS activity was insensitive to PrPC antibodies as well (Fig. 4A). Noteworthy, in contrast with our observations obtained at day 4, the DOI-induced PLA2 and PLC responses, specifically associated to 5-HT\(_{2B}\) receptors at this stage of differentiation, were both unaffected upon PrPC ligation, whatever the antibody used (Fig. 4, B and C). These observations indicate that in 1C11\(^{5-HT}\) d2 cells, PrPC does not interfere with the signaling activity of the 5-HT\(_{2B}\) receptor, whatever the coupling (NOS, PLA2, PLC) considered.

As for the 5-HT\(_{1B/D}\) receptor, the level of FSCA inhibition recorded in 1C11\(^{5-HT}\) d2 cells exposed to the agonist 5-CT also did not vary upon PrPC ligation (Fig. 4D). At last, as shown in Fig. 4D, PrP antibodies did not impact on the inhibitory effect of the 5-HT\(_{2B}\) receptor on the 5-HT\(_{1B/D}\) receptor function, which can be revealed upon combined exposure of 1C11\(^{5-HT}\) d2 cells to DOI or BW723C86 (which activate the 5-HT\(_{2B}\) receptor) and to CT (which activates the 5-HT\(_{1B/D}\) receptor). This result confirms the lack of any functional interaction between PrP and the 5-HT\(_{2B}\) and 5-HT\(_{1B/D}\) receptors in 1C11\(^{5-HT}\) d2 cells and strengthens the specificity of PrPC\(_\alpha\)-related effects on 5-HT receptor signaling in fully differentiated 1C11\(^{5-HT}\) d4 cells.

Because in 1C11\(^{5-HT}\) d4 cells only PrPC associates with the membrane protein caveolin within a signaling complex that involves the tyrosine kinase Fyn and governs the activation of downstream effectors (11, 12), we wondered whether a functional interaction between PrPC\(_\alpha\) and caveolin could play a role in the PrPC\(_\alpha\)-dependent modulation of 5-HT receptors signaling, restricted to fully differentiated cells as well. This idea was challenged by using tungsten microprojectiles coated with antibodies to caveolin-1 bombarded onto 1C11\(^{5-HT}\) d4 cells prior to receptor activation and PrPC\(_\alpha\) ligation as in Ref. 12. As shown in Fig. 5, immunoprecipitation of caveolin in live 1C11\(^{5-HT}\) d4 cells cancelled the effect of PrP antibodies (SAF61, SAF32, and BAR221) on the DOI-induced PLA2 response. Caveolin immunoprecipitation by itself did not, however, alter the 5-HT\(_{2B}\)-associated PLA2 activation in the absence of PrPC\(_\alpha\) ligation. Finally, when 1C11\(^{5-HT}\) d4 cells were bombarded with uncoated bullets used as a control, PrP antibodies retained their ability to potentiate the 5-HT\(_{2B}\)-PLA2 coupling.

These overall data highlight that in the 1C11 cell line the implementation of a complete phenotype is strictly required for PrPC\(_\alpha\) to modulate the couplings and the cross-talk of 5-HT receptors. They reassert the notion of a neuronal-specific function of PrPC\(_\alpha\) as well as the pivotal role of caveolin in this process.

DISCUSSION

Our present study provides evidence that the cellular prion protein PrPC interferes with the signaling activity of three serotonergic receptors belonging to the GPCR family. Our investigation exploits the properties of the 1C11 cell line, which expresses upon serotonergic differentiation a defined set of three 5-HT receptors within an integrated phenotype (4). The presence of other 5-HT receptor subtypes in 1C11\(^{5-HT}\) d4 cells could be firmly ruled out through binding and transductional experiments (18). As compared with transfected cells, this
clonal cell line enables us to get closer to in vivo physiological conditions in particular with respect to the spatial organization and stoichiometries of cell signaling protagonists. Having implemented a complete serotonergic differentiation program, 1C11-HT d4 cells transduce 5-HT inputs through 5-HT2B, 5-HT1B/D, and 5-HT2A receptor subtypes. These receptors act as autoreceptors with an essential role in regulating the intensities of 5-HT associated functions, i.e. 5-HT synthesis, storage, and transport (4). Following the elucidation of several signaling pathways coupled to these receptors, we established the occurrence of cross-talks between the three receptor subtypes (Fig. 6A). In brief, 5-HT2B receptors exert an inhibitory effect on the 5-HT1B/D-mediated Gi coupling, which is relieved upon concomitant stimulation of 5-HT2A receptors. We suspect that the antagonistic roles of the 5-HT2A and 5-HT2B receptors in regulating the function of 5-HT1B/D receptors have physiological implications, notably considering the expression of the three subtypes in raphe nuclei and meningeal and cardiovascular tissues (2, 6).

Fig. 4. Restriction of the PrPSc-induced modulation of 5-HT receptor couplings and cross-talk to fully differentiated 1C11-HT cells. The 5-HTmediated maximal NOS (A), PLA2 (B), and PLC (C) responses to DOI monitored in 1C11-HT cells at day 2 of their differentiation program do not vary upon concomitant PrPSc ligation using SAF61, SAF32, or BAR221 antibodies. D, in 1C11-HT day 2 cells, the inhibitory action exerted by 5-HT2B receptor stimulation (DOI or BW723C86) on the 5-HT1B/D receptor function (5-CT-induced FSCA inhibition) is not modified upon PrPSc ligation using SAF61, SAF32, or BAR221 antibodies. In all experiments, anti-SERT antibodies were used as a negative control. Data shown in each panel are the means ± S.E. of three independent experiments.
for the recruitment of both NOS isoforms. To date, there are several reported signaling pathways allowing for GPCR-mediated PLA2 activation through heterotrimeric G-proteins, although the accurate transduction mechanisms are still imperfectly understood (23). Preliminary data (not shown) indicate that the recruitment of the PLA2 pathway by 5-HT2B receptors involves Gαz, because antibody-mediated neutralization of the latter in permeabilized 1C115-HT d4 cells reduces by 70% the DOI-induced AA release. The coupling of the 5-HT2B receptor to PLA2 hence is driven by mobilization of a G-protein complex at the second and third intracellular loops of the receptor (24, 25). The simultaneous occurrence of G-protein-dependent (PLA2) and -independent (NO) couplings to the 5-HT2B receptor in 1C115-HT d4 cells is in keeping with the recent notion that GPCR may transduce signals through promiscuous couplings involving unrelated G-proteins or even G-protein-independent mechanisms (24).

How may PrPC ligation selectively potentiate the 5-HT2B receptor-dependent PLA2 activation? While itself acting as a cell surface receptor (11, 12), PrPC does not appear to be directly coupled to the PLA2 pathway (Fig. 1B). The possibility that PrPC may impact the pharmacological profile of the 5-HT2B receptor could be ruled out by binding experiments performed with or without antibody-mediated PrPC ligation (Fig. 2). Indeed, the pKᵦ values of agonists and antagonists remain unchanged under exposure to PrP antibodies, suggesting that the 5-HT2B receptor binds the various drugs identically irrespective of PrPC ligation. We may further exclude that PrPC ligation reinforces the binding of agonists to the 5-HT2B receptor because it has differential effects on the various 5-HT2B couplings and notably has no impact on the NO response. These binding data also argue against a direct interaction of PrPC with the 5-HT2B receptor. To account for these observations, one needs to bear in mind the concept of “agonist-specific trafficking of receptor signaling” which postulates that (depending on the agonist considered) a given GPCR may adopt multiple active states and show promiscuous coupling efficiencies for distinct signaling cascades (24). According to this paradigm, an impact of PrPC ligation on the conformational state of the 5-HT2B-receptor bound to a given agonist would be associated with a modification of the drug binding affinity for the receptor. It is thus unlikely that PrPC affects the coupling of the receptor by inducing local conformational changes in structural domains critical for the recruitment of G-proteins. Instead, our findings are consistent with a modulation of the intensities and/or the dynamics of G-protein activation by the receptor under PrPC ligation, which may in part depend on spatiotemporal parameters (see below).

This model would advantageously accommodate our experimental data related to the 5-HT1B/D receptor and particularly to the 5-HT2A receptor. Indeed, for the latter it is striking that PrPC ligation fully cancels the PLC coupling (Fig. 3A), whereas it does not change the maximal PLA2 response as reflected upon selective activation of the 5-HT2A receptor by MK212 (Fig. 1D). Of note, the couplings of the 5-HT2A receptor to the PLC and the PLA2 pathways appear to be independent in that they do not share a common signaling cascade (26, 27). Moreover, the capacity of 5-HT2A receptors to preferentially instigate PLC-IP3 accumulation or PLA2-mediated AA release differs according to the agonist used. Such results were presented as straightforward evidence for agonist-directed trafficking of receptor stimulus (26, 27). Finally, Kurrasch-Orbaugh et al. (27) proposed that the 5-HT3A receptor-mediated PLC and PLA2 signaling cascades mobilize two different receptor reserves associated to distinct G-protein ternary complexes. Although 5-HT2A receptors classically activate PLC through Gαq, including in 1C115-HT cells (18), it is still unclear which G-protein heterotrimer mediates the 5-HT2A-dependent PLA2 activation. In view of the selective impact of PrPC ligation on the PLC response and in agreement with Kurrasch-Orbaugh et al. (27), we may exclude an involvement of Gαq in the 5-HT2A-PLA2 coupling. PrPC thus appears to specifically alter the signaling activity of the 5-HT2A receptor reserve that couples to the PLC-IP3 pathway. Possibly, PrPC ligation switches off the agonist-induced recruitment of Gαq by 5-HT2A receptors.

Of note, although both 5-HT2A and 5-HT2B receptors couple to the PLA2 pathway, PrPC ligation modulates the PLA2 response associated to the 5-HT2B receptor only. This observation
again argues that the two receptors mobilize distinct signaling pathways to activate PLA2, not only in terms of G-protein subtypes. A participation of Gα in the recruitment of PLA2 by the 5-HT2A receptor hence may be ruled out because Gα heterotrimers mediate the 5-HT2B-dependent PLA2 stimulation.

Altogether, our data lend support to the idea that PrPC may take part in the overall process of agonist-directed effector activation associated to the 5-HT2A, 5-HT2B, and 5-HT1B/1D receptors by interfering (either positively or negatively) with the recruitment of G-proteins. As mentioned above, it is unlikely that the modulation of G-protein couplings be caused by subtle conformational changes within the intracellular domains of the receptors. A scenario that we may instead consider is that PrPC alters the stoichiometry of G heterotrimeric available for downstream signaling by interacting with Gα species. This hypothesis would be in line with the observation that in lymphocytes the glycosylphosphatidylinositol-anchored proteins CD59, CD48, and Thy-1 can associate with heterotrimeric G-protein α subunits (28). Alternatively, PrPC ligation may affect 5-HT receptor activities by interfering with some other signaling partners of the receptors notably involved in desensitization and/or internalization processes such as GPCR kinases or arrestins, even in the inactivation of Gα subunits, e.g. regulators of G-protein signaling. Besides, we cannot exclude that the recruitment of intracellular targets by PrPC upon ligation as recently reported (Fyn, NADPH oxidase, and ERK1/2; see Refs. 11 and 12) causes an imbalance of G-proteins coupling.

Some of the complexity of cell signaling relates to the spatialtemporal context in which the signal transduction process occurs. Indeed, the ability of a given receptor to initiate a defined signaling cascade directly depends on the availability of the appropriate signaling partners in the vicinity of the receptor (24, 29). The stoichiometry of receptors and partners may vary in the first place according to the tissue or cell type examined. It may also depend on the cell state considered, for instance with respect to cell cycle or differentiation. At the subcellular level, cell-signaling protagonists are not randomly distributed but rather compartmentalize in microdomains. The present study was carried out using experimental approaches that ensure the maintenance of cell integrity in terms of level of expression and localization of signaling molecules. In the 1C11 cell line, the differentiation stage appears as a key parameter regarding signal transduction. Noticeably, we previously highlighted that the implementation of a complete neuronal differentiation program is strictly required in the 1C11 cell line for the onset of a functional PrPC-caveolin-Fyn signaling complex (12). Furthermore, this complex involves PrPC molecules located on cell processes and most likely on the varicosities of the neurites (12, 13). Beyond this neuronal specificity of PrPC coupling, we assigned a more ubiquitous signaling activity to PrPC, notably in 1C11 progenitor cells, independent from caveolin (11). It is striking that in the present study the impact of PrPC ligation on the 5-HT receptors is again restricted to fully differentiated 1C115-HT d4 cells. Indeed, whereas at day 2 of differentiation 1C115-HT cells already express 5-HT2B and 5-HT1B/1D receptors, the ligation of PrPC had no effect on the couplings of the receptors nor on their cross-talk (Fig. 4). Because the PrPC-related impact on 5-HT receptor signaling is specifically observed in cells having implemented the overall functions of serotonergic neurons, we may infer that the proper structural organization of the various partners within subcellular microdomains is mandatory to allow functional interactions between the protagonists. In line with this idea, our present findings also outline the prominent role of caveolin in the PrPC-mediated imbalance of G-proteins coupling (Fig. 5). It should be noted, however, that caveolin immunosequenation does not affect the 5-HT2B-mediated PLA2 activation in the absence of PrPC ligation, indicating that the 5-HT2B-PLA2 coupling and most likely other 5-HT receptor signaling cascades are not transduced through caveolin. Hence we may speculate that caveolin acts as a bridging molecule which, upon PrP ligation, interferes with the recruitment and/or dynamics of activation of G-protein heterotrimers. Such a scheme would be in keeping with the reported regulation of GPCR kinases and G-proteins α subunits by caveolin (30, 31).

In summary, our study emphasizes that PrPC acts as a modulator of 5-HT receptor couplings and cross-talks in 1C115-HT serotonergic cells and provides the first demonstration of a regulation of GPCR signaling by a glycosylphosphatidylinositol-anchored molecule. We thus identify PrPC as a novel player that contributes to the homeostasis of 5-HT related functions. As a consequence, the involvement of PrPC in the fine tuning of serotonergic functions could account for the alterations of 5-HT metabolism observed in scrapie-infected rodents (32–35) or in fatal familial insomnia patients (36). It may also confer a specific vulnerability to transmissible spongiform encephalopathies agents to serotonergic neurons. Assessing the impact of prion infection on the serotonin-associated signals and functions constitutes a priority to illuminate this issue.

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