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A New Mechanism of Receptor Targeting by Interaction between Two Classes of Ligand-Gated Ion Channels

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The 5-HT₃ receptors are serotonin-gated ion channels that physically couple with purinergic P2X2 receptors to trigger a functional cross-inhibition leading to reciprocal channel occlusion. Although this functional receptor–receptor coupling seems to serve a modulatory role on both channels, this might not be its main physiological purpose. Using primary cultures of rat hippocampal neurons as a quantitative model of polarized targeting, we show here a novel function for this interaction. In this model, 5-HT₃AR receptors did not exhibit by themselves the capability of distal targeting in dendrites and axons but required the presence of P2X2R for their proper subcellular localization. 5-HT₃AR distal targeting occurred with a delayed time course and exhibited a neuron phenotype dependency. In the subpopulation of neurons expressing endogenous P2X2R, 5-HT₃AR distal neuritic localization correlated with P2X2R expression and could be selectively inhibited by P2X2R RNA interference. Cotransfection of both receptors revealed a specific colocalization, cotrafficking in common surface clusters, and the axonal rerouting of 5-HT₃AR. The physical association between the two receptors was dependent on the second intracellular loop of the 5-HT₃A subunit, but not on the P2X2R C-terminal tail that triggers the functional cross-inhibition with the 5-HT₃AR. Together, these data establish that 5-HT₃AR distal targeting in axons and dendrites primarily depends on P2X2R expression. Because several P2XR have now been shown to functionally interact with several other members of the 4-TMD family of receptor channels, we propose to reconsider the real functional role for this receptor family, as trafficking partner proteins dynamically involved in other receptors targeting.

Key words: 5-HT₃ receptor; ligand-gated ion channels; P2X2 receptor; receptor trafficking; receptor–receptor interactions; serotonin

Significance Statement
So far, receptor targeting mechanisms were found to involve intracellular partner proteins or supramolecular complexes that couple receptors to cytoskeletal elements and recruit them into cargo vesicles. In this paper, we describe a new trafficking mechanism for the neuronal serotonin 5-HT₃AR ionotropic channel receptor, in which the role of routing partner is endowed by a functionally interacting purinergic receptor: the P2X2 receptor. This work not only unveils the mechanism by which 5-HT₃ receptors can reach their axonal localization required for the control of neurotransmitter release, but also suggests that, in addition to their modulatory role, the family of P2X receptors could have a previously undescribed functional role of trafficking partner proteins dynamically involved in the targeting of other receptors.

Introduction
Receptor–receptor interactions have become an increasing field of investigations, unveiling a new level of complexity in receptor regulation. However, recent studies suggest that such interactions might go beyond classical functional cross talk mechanisms and extend to the modulation of receptor trafficking, surface expression, and positioning within the plasma membrane (Jo et al., 2011; Shrivastava et al., 2011; Pougnet et al., 2014). 5-HT₃ and
P2X2 receptors are two functionally interacting receptors belonging to two different classes of ligand-gated ion channels, of the three major classes present in mammals: the cys-loop receptors, the ionotropic P2X receptors, and the glutamate receptors (Collinge et al., 2009). 5-HT3 receptors are serotonin-gated channels mediating fast excitatory transmission in the CNS and periphery (Sugita et al., 1992) and neurotransmitter release (van Hooft and Vijverberg, 2000). Since the cloning of the canonical 5-HT3A homomeric form (Mariq et al., 1991), their diversity of functions has found a molecular basis with the discovery of additional subunits (Davies et al., 1999; Niesler et al., 2003) that coassemble with the 3A subunit to modulate their electrophysiological properties (Davies et al., 1999; Jensen et al., 2008; Holbrook et al., 2009). In the CNS, 5-HT3 receptors are mainly neuronal and are present both postsynaptically on GABAAergic hippocampal and cortical interneurons (Tecott et al., 1993; Lee et al., 2010; Vuurovic et al., 2010) and presynaptically on nerve terminals projecting from various peripheral ganglia (Miquel et al., 2002; Morales and Wang, 2002; Doucet et al., 2007), but their role in peripheral functions is also widespread, particularly in the intestinal tract (Glatzle et al., 2002; Holbrook et al., 2009). 5-HT3A subunits have been found to depend on the protein RIC-3 for their surface targeting in clonal cells (Castillo et al., 2005; Cheng et al., 2005; Walstab et al., 2010). However, RIC-3 appears to act as a chaperone protein mainly involved in proper receptor folding, common to 5-HT3A and nicotinic receptors (Castillo et al., 2005; Alexander et al., 2010), and no data confirm so far a relevant role of RIC-3 in 5-HT3 trafficking within neurons. Thus, the molecular mechanisms of 5-HT3 receptor targeting in neurons remain largely unknown.

P2X2 receptors are one subtype of the seven known P2X receptors that can all form homomeric or heteromeric ATP-gated channels widely distributed in CNS and periphery (Saul et al., 2013). In the periphery, P2X2 mediates fast synaptic transmission in the myenteric plexus (Ren et al., 2003) and were originally expected to have a similar function in the brain. However, reports of ATP-mediated fast synaptic transmission in the brain are scarce, suggesting that P2X2 rather play a neuromodulatory role at central synapses (Khakh and North, 2012). Indeed, because P2X2 localize mainly in the periphery of excitatory glutamatergic synapses and seem excluded from the postsynaptic density (Rubio and Soto, 2001; Richler et al., 2011), and because they are present on the terminals of ganglionic (Vulchanova et al., 1996) and hippocampal (Khakh et al., 2003) neurons, other functional roles for these receptors were assumed (Khakh et al., 2003; Vavra et al., 2011; Khakh and North, 2012).

P2X2 receptors have been found to interact physically and functionally with several other receptors and ion channels, including nicotinic (Barajas-Lopez et al., 1998; Khakh et al., 2000; Khakh et al., 2005), GABA_A (Boué-Grabot et al., 2004b) GABA_A (Boué-Grabot et al., 2004a), and 5-HT3 receptors (Barajas-Lopez et al., 2002; Boué-Grabot et al., 2003). These subunit-specific interactions result in a cross-inhibition induced by reciprocal channel occlusion that involves direct protein–protein contact (Boué-Grabot et al., 2003; Khakh et al., 2005). However, more recent work has found that P2X receptors could also influence GABA_A receptor dynamics (Jo et al., 2011; Shrivastava et al., 2011) and surface expression of AMPA receptor (Pouget et al., 2014), opening new insights into P2X receptor functionality. In this study, we have addressed a completely new functional role for P2X2 receptors, as trafficking partners for the axonal and dendritic localizations of 5-HT3 receptors in hippocampal neurons.

Materials and Methods

Animals

Female gestating Sprague Dawley rats (Charles River Breeding Center) were maintained under controlled environmental conditions (21 ± 1°C, 60% relative humidity, 12 h/12 h light/dark cycle), with food and water available ad libitum until killed for embryo removal. Experiments were performed in agreement with the institutional guidelines for use of animals and their care, in compliance with national and international laws and policies (Council directives no. 87-848, October 19, 1987, Ministère de l’Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale, permissions nos. 75-976 to M.B.E., 75-805 to J.M., 75-974 to M.D.).

Antibodies

The following primary antibodies were used: mouse monoclonal anti-HA antibody (Sigma; 1:1000), rabbit anti-HA antibody (Sigma, Abcam, Cell Signaling Technology, 1:1000), mouse monoclonal anti-Flag M2 antibody (Sigma, 1:2000), mouse monoclonal anti-myc (Roche, 1:500), rabbit anti-myc (Millipore, 1:500), mouse anti-GFP antibody (GE Healthcare, 1:1000), rabbit anti-GFP antibody (Millipore Bioscience Research Reagents, 1:1000), mouse monoclonal anti-α-tubulin antibody (Abcam, 1:2000), rabbit anti-tubulin antibody (Novus Biologicals, 1:1000), rabbit anti-MAP2 antibody (Millipore Bioscience Research Reagents, 1:1000), rabbit anti-P2X2R antibody (Alomone Labs, 1:300), guinea pig anti-P2X2R antibody (Millipore, 1:300), mouse monoclonal anti-dsRed antibody (Clontech, 1:1000), rabbit anti-5-HT3A antibody (1:1000) (Doucet et al., 2000), and goat anti-5-HT3A antibody (1:1000) (Doucet et al., 2007). The secondary antibodies used were AlexaFluor-488 and -594-conjugated antibodies from Invitrogen (1:1000) and HRP-conjugated anti-rabbit and anti-mouse antibodies (Sigma, 1:10,000).

Plasmid constructs and site-directed mutagenesis

5-HT3A-HA was generated from a pRC-CMV plasmid described previously (Emerit et al., 2002) by extraction of the mouse 5-HT3A sequence with HindIII and BamHI and insertion into the pcDNA3 vector (Invitrogen) between the HindIII and EcoRI sites in two steps, with a cassette containing the HA epitope (YPYDVPDYA) separated by a Gly3 arm in C-terminal position before the stop codon. The human HA-tagged 5-HT3A subunit (HA tag inserted between amino acids 5 and 6), subcloned into the pGWI plasmid (Boyd et al., 2003), was a generous gift of Dr. C. N. Connolly (Ninewells Medical School, University of Dundee, Dundee, Scotland). The mouse 5-HT3A-Flag plasmid was previously described (Emerit et al., 2002). The plasmids encoding rat 5-HT3A-eGFP (Carrel et al., 2008), sst2A-eGFP (Lelouvier et al., 2008), P2X2-YFP (Boué-Grabot et al., 2003), P2X2b-YFP (Koshimizu et al., 2006), PYP-5-HT3A (Graille et al., 2004), and P2X4-FlagN (Jo et al., 2011) have already been used and described. P2X2, P2X2Tr, P2X3-Flag, P2X3-YFP, myc-pl subcloned into pcDNA3, myc-tagged GluA1, or GluA2 subcloned into PrK5 vector were described previously (Boué-Grabot et al., 2000; 2004a; Pouget et al., 2014). Myc-NR2A was a gift from L. Groc (Interdisciplinary Institute for Neuroscience, Bordeaux, France). HA-tagged P2X2 and P2X2Tr were generated by insertion of a sequence encoding the YPYDVPDYA epitope between amino acids D78 and K79 within the extracellular domain of P2X2 subunits using the QuikChange site-directed mutagenesis method (Agilent) with specific oligonucleotides. P2X2Tr corresponded to a deletion of the 98 last amino acids of the C-terminal domain of P2X2 (Boué-Grabot et al., 2000). HA-tagged P2X2ΔC36 or P2X2ΔC57 was generated by insertion of a stop codon into the sequence of HA-P2X2 using the QuikChange mutagenesis method at specific position to delete the 36 or 57 last amino acids of HA-P2X2, respectively. 5-HT3A-IL23-HA and 5-HT3A-IL2y2-HA chimeras were generated by substitution of the second intracellular loop (IL2) of the 5-HT3A subunit with the homologous domain of GABA_A3, B3 or y2 subunit, respectively, using the QuikChange method and megaprimer strategy. The sequences corresponding to the IL2 of B3 or y2 subunits were first amplified by PCR from B3 or y2 constructs using pfu polymerase (Fermentas) and primers with flanking regions corresponding to adjacent 5-HT3A sequences. Each PCR product was then used as megaprimer on 5-HT3A-HA plasmid to generate by the QuikChange method
characterized above.

First and the fourth passage were used to prepare the conditioned media (DMEM GlutaMAXI [Invitrogen] supplemented with 1 g/L glucose, 1.25% basal medium without B27 supplement. After 15 min at room temperature, 0.5 μM of RNAiMAX (Invitrogen) in 25 μM of Neurobasal medium was added, and incubation continued for another 30 min. Simultaneously and separately, 2 μL of siRNA (160 nM final concentration) was mixed with 25 μM of Neurobasal medium without B27 supplement. After 15 min at room temperature, 0.5 μM of RNAiMAX (Invitrogen) in 25 μM of Neurobasal medium was added, and incubation continued for another 30 min. Plasmid and RNA complexes were mixed, and 150 μL of complete Neurobasal medium containing B27 supplement was added. The mix was then applied onto the neuronal culture, and transfection lasted for 3 h at 37°C. After transfection, the medium in which the neurons were grown was put back on to the neurons for the expression period (usually 48–72 h). Optimal results were obtained with siRNA(P2X2)-3.

**Immunofluorescence**

Cells on coverslips were washed with DPBS+ (DPBS containing 0.1 mM CaCl2 and 0.1 mM MgCl2) at 37°C, then fixed with 3% PFA containing 4% sucrose (PFA/sucrose) at 37°C in DPBS+, and permeabilized with 0.1% Triton X-100 in DPBS (without CaCl2, MgCl2). After two washes (10 min) in DPBS, cells were incubated for 30 min in antibody buffer (3% BSA, 2% normal donkey serum, 2% normal goat serum, except when using goat primary antibodies, in DPBS). Incubation with primary antibodies was then performed in antibody buffer at room temperature (1 h). After two washes in DPBS, incubation with secondary antibodies proceeded for 1 h. The coverslips were finally mounted in Fluoromount-G solution (Clinscience) for immunofluorescence visualization using confocal microscopy.

**Microscopy**

Immunofluorescence images were generated using a Leica TCS SP5 AOBS laser scanning confocal microscope (25, 40, 63, or 100× oil-immersion lenses). Contrast and brightness were chosen to ensure that all pixels were within the linear range. Images were the product of 12-fold line averages. In all cases, emission and excitation filters proper to each fluorophore were used sequentially, and the absence of cross talk between different channels was checked with selectively labeled preparations. Leica pictures (.lei) were saved as Tiff images, and plates were assembled using Adobe Photoshop CS2 (Adobe Systems). Background was lowered using Gaussian blur (radius 1 pixel), and contrast and brightness of images displayed in figures were modified using Adobe Photoshop CS2 only for illustrations.

**Quantification of dendrite fluorescence**

Fluorescence profiles along dendrites and axons were generated using the Luca 4.71 software (Nikon). For fluorescence profile comparisons, all neurons showing intact morphology with unambiguous visual identification of the axon were analyzed (one dendrite or axon per neuron). The variability of distribution in individual neurons was eliminated by using the cumulated fluorescence profiles obtained for 20–40 neurons in each group (Carrel et al., 2008). Usually, quantitative data were generated by extracting the area under each individual curve between the soma and 50–100 μm for the totality of neurons analyzed and then normalized to the mean value of the control condition. Data analysis was performed with Prism software (GraphPad). All results are reported as mean ± SEM. Statistical significance was assessed by using Student’s t test or one-way or two-way ANOVA with Bonferroni’s or Dunnett’s post hoc tests. The level of significance was set at p < 0.05.

**Colocalization analysis**

Receptor–receptor colocalization was measured by using the JACoP plugin of FIJI (ImageJ) software (Bolte and Cordelieres, 2006). The manual threshold method was adapted to the quantification of fluorescence overlap onto the clustered components of receptor distributions within the dendritic trees. ROI were chosen to avoid the cell bodies and the intense intracellular fluorescence found in the center of the larger dendrites. The threshold was chosen in each case to select the clustered component of receptor distribution and to avoid inclusion of the diffuse component that could lead to random overlap noise. Mander’s M1 coefficients (representing the proportions of clustered 5-HT3A receptors...
colocalized with clustered cotransfected receptors) were then used as the relevant parameters on which the statistical analyses were performed.

**Immunohistochemistry**

**Tissue preparation.** Rat pups (postnatal life 1 week) and adult mice were killed by decapitation. The brain of mice was rapidly removed and immediately frozen by immersion in isopentane cooled by dry ice (−40°C). The whole body of rat pups was also frozen by immersion in isopentane cooled by dry ice (−40°C). The frozen rat pups and brains of mice were conserved at −75°C until cryostat sectioning. Sagittal sections of whole body of pups and coronal sections of brains (12 μm thick) were prepared on a cryostat (JUNG3000, Leica), thaw-mounted onto slides SuperFrost-Plus (Menzel-Glaser), and kept at −75°C until use for immunohistochemistry.

**Immunohistochemistry: double immunofluorescent staining for 5-HT3 and P2X2 receptors.** Frozen sections were thawed to 4°C and fixed during 10 min with 2% PFA made up in 0.1 M PB, pH 7.4 at 4°C or fixed with methanol at −20°C for 15 min. After washing three times for 5 min each with 50 mM PBS, the sections were immersed in 50 mM Tris-buffered saline, pH 7.5 (TBS) and then blocked with the blocking buffer (10% normal donkey serum, 0.1% BSA, 0.1% gelatin, 0.1% Tween 20, and 0.1% Triton X-100 in TBS) for 1 h at 37°C. Sections were then incubated with the primary antibodies: rabbit anti-5-HT3A (1:500 dilution) and guinea-pig anti-P2X2 (Millipore, 1:500 dilution) or goat anti-5-HT3B and rabbit anti-P2X2 (Alomone, 1:500 dilution) in dilution buffer (50 mM TBS, pH 7.6; 0.1% BSA; 0.1% gelatin, 0.1% Tween 20, and 0.05% Triton X-100) overnight at 4°C. After washing three times with TBS (0.1% gelatin; 0.1% Tween 20), sections were incubated with the following secondary antibodies: Alexa488-donkey anti-rabbit and Cy3-donkey anti-guinea-pig or Alexa488-donkey anti-goat and Cy3-donkey anti-rabbit (Jackson ImmunoResearch Laboratories, 1:500 dilution for each in dilution buffer TBS (0.1% Tween 20) for 1 h at room temperature. After three rinses with TBS-Tween 20, sections were coverslipped with Fluoromount-G mounting media (Southern Biotechnology).

**Results**

**Native 5-HT3 and P2X2 receptors are coexpressed within the same neurons**

If 5-HT3 and P2X2 receptors undergo a common fate within a supramolecular partnership, then they should share an overlap distribution and be expressed within the same neurons. 5-HT3R tissue distributions are well documented by *in situ* hybridization (Tecott et al., 1993; Morales and Wang, 2002), radioligand binding (Laporte et al., 1992), and antibody labeling (Doucet et al., 2000; Miquel et al., 2002). In the CNS, 5-HT3R are present in scattered neurons in the cortex (Tecott et al., 1993; Lee et al., 2010), in hippocampus CA1 and CA3 layers, amygdala, nucleus of the solitary tract, Sp5, and dorsal horns of the spinal cord (Laporte et al., 1992; Doucet et al., 2000). In the periphery, 5-HT3R are mainly represented in several peripheral ganglia (Morales and Wang, 2002; Doucet et al., 2007) and in the intestinal tract, in neurons of the myenteric and submucosal plexus (Glatzle et al., 2002). P2X2R share a tissue distribution that is remarkably overlapping with 5-HT3R albeit wider, including many non-neuronal cell types (for an extensive review, see North, 2002). However, colocalization within the same neurons has only been described by electrophysiological recordings (Barajas-López et al., 2002; Boué-Grabot et al., 2003) and remained to be demonstrated by immunocytological methods. We have used previously described antibodies against the 5-HT3A (Doucet et al., 2000) and the 5-HT3B (Doucet et al., 2007) subunits to illustrate such a coexpression of native 5-HT3 and P2X2 receptors in neurons of the rat myenteric plexus using two independent couples of antibodies: rabbit anti-5-HT3A and guinea pig anti-P2X2

**Figure 1.** 5-HT3 and P2X2 receptors are coexpressed in neurons of the myenteric plexus. Immunohistochemical detection of 5-HT3A, 5-HT3B, and P2X2 subunits in the rat intestinal tract at 4 d postnatal. A, 5-HT3A (rabbit anti-5-HT3A antibody; green) and P2X2 (guinea pig anti-P2X2 antibody; red). B, 5-HT3B (goat anti-5-HT3B antibody; green) and P2X2 (rabbit anti-P2X2 antibody; red). Both 5-HT3 subunits colocalize with P2X2R in the same neurons (arrows). mp, Myenteric plexus; lm, longitudinal muscle; cm, circular muscle. Scale bar, 50 μm.
Delayed onset of 5-HT3A receptor trafficking in a subpopulation of cultured hippocampal neurons

We used primary cultures of hippocampal neurons to challenge a functional partnership between 5-HT3A homomeric and P2X2 receptors in regards to polarized trafficking. This model has proven to be fully adapted to the quantification of dendritic or axonal targeting of various receptors (Carrel et al., 2008, 2011; Al Awabdh et al., 2012). Because 5-HT3AR, although present in these cultures, are expressed at levels too low to be detected by specific antibodies, we used transfected tagged 5-HT3A subunits. We first tested various tags and their position within the 5-HT3 sequence and compared them with 5-HT3A-Wt (labeled with anti-5HT3A antibodies) to make sure that the tag itself did not interfere with trafficking mechanisms. 5-HT3A-GFP (GFP tag in C-terminal position) and YFP-5-HT3A (YFP in the N-extracellular domain after a signal peptide from the glycine receptor) (Grailhe et al., 2004) did not exhibit a subcellular distribution identical to 5-HT3A-Wt, the first one being retained within the endoplasmic reticulum, and the second one fully filling the dendritic tree in all transfected neurons. Instead, all tagged 5-HT3AR carrying a small reticulum, and the second one fully filling the dendritic tree in all functional partnership between 5-HT3A homomeric and P2X2 receptors in the entire dendritic and axonal networks, with a homogeneous distribution in almost 100% of neurons. Figure 2B shows cumulated fluorescence curves along the longest neurite for a sample of neurons expressing 5-HT3A-HA subunits in proximal dendrites only, and compares them with 5-HT3A-eGFP, sst2A-eGFP, and P2X2-YFP. Whereas cumulated fluorescence reached 800 μm for 5-HT3A-eGFP (dendrites only), and 900–1100 μm for sst2A-eGFP and P2X2-YFP (dendrites and axons), 5-HT3A-HA fluorescence did not exceed 100–200 μm from cell bodies. When we compared transfections performed from 5 DIV (Fig. 3A) to 12 DIV (Fig. 3B) (48 h expression), we observed an increasing proportion of neurons showing a distal targeting of 5-HT3A-HA receptors. 5-HT3A-R-HA showed a delayed distal localization (fluorescence detected at distances >100–200 μm from cell bodies) only in a subpopulation of transfected neurons representing 11.4 ± 1.2% at 5 DIV, and slowly increasing to 30%–40% after 7 DIV (Fig. 3E, blue area). The population of neurons that were not targeting 5-HT3A-R-HA distally could not be attributable to a degenerating process due to transfection, as shown by the integrity of their dendritic tubulin network (Fig. 3A). When transfected at 12 DIV, the discrepancy between neurons expressing 5-HT3A-R-HA proximally and neurons expressing the receptor distally increased, and surface labeling of the HA epitope revealed that only this latter population expressed the receptor at the surface (Fig. 3B). These results are consistent with the existence of two populations of neurons in regards to 5-HT3A expression: one devoid of a targeting ma-
chinery for these receptors and another one expressing a distally targeting machinery with a slow onset.

5-HT3A receptor targeting in distal dendrites and axons is neuron phenotype dependent

Primary cultures of hippocampal neurons contain two major neuronal populations: large pyramidal neurons that are mainly glutamatergic (Graves et al., 2012), and interneurons, some of them GABAergic (Benson et al., 1994). However, we could not find any correlation between the capability of neurons to distally address 5-HT3A-R and the size of the cell bodies, the shape of the neurons, or GAD65 immunolabeling (data not shown). Instead, we found a positive correlation between the distal localization of 5-HT3A-HA and the presence of endogenous P2X2R in these neurons. We labeled 5-HT3AR-HA-transfected hippocampal neurons at an intermediate differentiation time (7 DIV) with anti-P2X2 antibodies (Fig. 3C). The cumulated frequency distribution of P2X2R mean fluorescence intensity on somas (inset) revealed the existence of two populations of neurons (n = 51, bimodal distribution, median = 50); P2X2R-immunonegative (68.3%, Ṁ = 47, quartiles = 40) and P2X2R immunopositive (31.6%, Ṁ = 121, quartiles = 110). Only P2X2R-immunopositive neurons (example in C) expressed 5-HT3AR distally (linear regression, $r^2 = 0.71$, $p < 0.0001$).

![Figure 3](https://example.com/figure3.png)

Figure 3. 5-HT3A receptor distal targeting is dependent on endogenous P2X2 receptors. Hippocampal neurons were transfected at (A) 5 DIV, (B) 12 DIV, or (C) 7 DIV, with 5-HT3A-HA. Immunofluorescence detection was performed with the following: A, anti-HA (red) and anti-tubulin (green) antibodies; B, anti-HA antibodies without permeabilization (green), and then anti-HA antibodies after permeabilization (green), and then anti-HA antibodies after permeabilization (red); or C, anti-HA (red) and anti-P2X2 (green) antibodies. Scale bars, 50 μm. D, Endogenous P2X2R (arbitrary units) were quantified by Western blot (black line) in hippocampal cultures at the indicated times (DIV). Surviving neurons were counted for each condition (red line), and the amount of endogenous P2X2R was divided by the number of live neurons in the culture at each time (blue line, endogenous P2X2/surviving neurons). E, The intensity of mean endogenous anti-P2X2R immunofluorescence (arbitrary units) measured on neuron somas was plotted versus the distance of the 5-HT3A-HA detection limit (above background) along the longest immunolabeled neurite at 7 DIV (C). The cumulated frequency distribution of P2X2R mean fluorescence intensity on somas (inset) revealed the existence of two populations of neurons (n = 51, bimodal distribution, median = 50); P2X2R-immunonegative (68.3%, Ṁ = 47, quartiles = 40) and P2X2R immunopositive (31.6%, Ṁ = 121, quartiles = 110). Only P2X2R-immunopositive neurons (example in C) expressed 5-HT3AR distally (linear regression, $r^2 = 0.71$, $p < 0.0001$).
regression, $n = 51$, $r^2 = 0.71$, $p < 0.0001$), showing that the distal targeting of 5-HT$_{3A}$R was linked with the expression of native P2X$_2$R in the neurons. Moreover, the bimodal distribution corresponded to two neuronal populations: a low intensity (background, P2X$_2$R negative) and a threefold higher intensity (P2X$_2$R positive), representing 68.6% and 31.4%, respectively, of the total. These two populations could be defined by setting a fluorescence cutoff at the minimum value of the fitted curve (Fig. 3E, inset). Measurements of the lengths of detectable 5-HT$_{3A}$R-HA fluorescence yielded corresponding values of 58±9 μm and 312±29 μm (mean ± SEM, $n = 51$, $t$ test, significant difference, $p < 0.0001$), respectively, for the two populations (Fig. 3E, colored areas). The same results were obtained with human HA-tagged h5-HT$_{3A}$ subunits in which the HA tag was introduced in the N-terminal domain between amino acids 5 and 6 (Boyd et al., 2003) (data not shown), suggesting that this correlation was not rodent specific or induced by the tag position. Quantification of SDS-PAGE analysis followed by immunoblotting (Fig. 3D) confirmed the increasing presence of endogenous P2X$_2$R in cultured hippocampal neurons from 3 to 10–11 DIV, followed by a plateau from 12 to 21 DIV, an observation consistent with previous reports of P2X$_2$R expression in the hippocampus (North, 2002; Khakh et al., 2003). As a result, the slow onset of native P2X$_2$R expression in the culture explains the delayed capacity of the P2X$_2$-immunopositive subset of neurons to distally address 5-HT$_{3A}$R.

**5-HT$_{3A}$R receptor targeting to distal dendrites and axons is dependent on endogenous P2X$_2$ receptors**

The dependency of transfected 5-HT$_{3A}$R on native P2X$_2$R expression for their distal neuritic localization was confirmed by RNA interference. We found that simultaneous transfection of 5-HT$_{3A}$R-HA and siRNA(P2X$_2$) at 7 DIV (i.e., inhibition of de novo expressed P2X$_2$R) was optimal to significantly affect 5-HT$_{3A}$R localization. Hippocampal neurons were transfected with 5-HT$_{3A}$R-HA, with or without siRNA(P2X$_2$) or control siRNA at 7 DIV, and the neurons were simultaneously quantified for the following: (1) their anti-P2X$_2$ fluorescence intensity on cultured hippocampal neurons from 3 to 10–11 DIV, followed by a plateau from 12 to 21 DIV, an observation consistent with previous reports of P2X$_2$R expression in the hippocampus (North, 2002; Khakh et al., 2003). As a result, the slow onset of native P2X$_2$R expression in the culture explains the delayed capacity of the P2X$_2$-immunopositive subset of neurons to distally address 5-HT$_{3A}$R.

Figure 4. P2X$_2$ receptor depletion inhibits 5-HT$_{3A}$ receptor targeting toward distal neurites. Hippocampal neurons were transfected at 7 DIV with the 5-HT$_{3A}$R-HA alone or cotransfected with the 5-HT$_{3A}$R-HA plus siRNA against endogenous P2X$_2$R or control siRNA for 48 h. A, Immunofluorescence detection of 5-HT$_{3A}$R-HA in representative neurons belonging to the P2X$_2$-immunopositive subpopulation. Scale bar, 50 μm. B, 5-HT$_{3A}$R-HA immunofluorescence detection limits along the longest neurite (one neurite per neuron) were measured for each transfected neuron, and the neurons belonging to the P2X$_2$-immunonegative and P2X$_2$-immunopositive groups were represented separately. After RNA interference of endogenous P2X$_2$R, the 5-HT$_{3A}$R-HA immunofluorescence detection limit was significantly lower in neurites of P2X$_2$-immunopositive neurons only. Bars indicate mean ± SEM; $n = 40$–50. **** $p < 0.0001$ (two-way ANOVA with Bonferroni’s post hoc test). C, Cumulated fluorescence intensity profiles of 5-HT$_{3A}$R-HA (arbitrary units) along the longest neurite of P2X$_2$-immunopositive monitored neurons (μm) in control conditions (blue) compared with siRNA(P2X$_2$) (red) and siRNA(Cont) (green). D, Areas under individual curves (cumulated in C, for distances of 0–500 μm, were averaged. The 5-HT$_{3A}$R-HA immunofluorescence intensity is significantly lower in neurites after RNA interference of endogenous P2X$_2$R. Bars represent the mean ± SEM ($n = 20$–30). *** $p < 0.001$ (one-way ANOVA with Dunnett’s Multiple Comparison post hoc test).
the soma, (2) their anti-HA (5-HT3A-R) fluorescence intensity along their longest neurite, and (3) the anti-HA (5-HT3A-R) fluorescence detection limit for their longest labeled neurite (Fig. 4). Figure 4A shows characteristic neurons for the three conditions, in the population of P2X2R-immunopositive neurons. Quantification of 5-HT3A-R-HA detection limit (above background) revealed that siRNA(P2X2) reduced significantly the average distance of anti-HA detection by 47% (53.4 ± 5.2% of control, two-way ANOVA, n = 46, p < 0.0001) in P2X2R-immunopositive neurons (Fig. 4B), whereas control siRNA had no effect (106.6 ± 10.9% of control, two-way ANOVA, n = 49, p < 0.0001). There was no significant effect of siRNAs on P2X2R-immunonegative neurons (Fig. 4B). When cumulated fluorescence intensity profiles along the longest neurite (one neurite per neuron) for the P2X2R-immunonegative subset of 5-HT3A-R-HA transfected neurons were plotted, the depletion of 5-HT3A-R-HA receptor expression in the neurites was clear cut for siRNA(P2X2)-treated neurons but was indistinguishable from the control for siRNA(Control) (Fig. 4C). Quantification of the areas under the curves for each P2X2R-positive neuron (0–500 μm from soma) yielded an average inhibition of 53% (46.6 ± 7.2% of control, two-way ANOVA, n = 46, p < 0.0001) for siRNA(P2X2) and no effect of control siRNA (104.5 ± 8.5% of control, two-way ANOVA, n = 49, p < 0.0001).

Because this protocol of siRNA interference only inhibited de novo expression of endogenous P2X2R (i.e., simultaneously expressed with transfected 5-HT3A-HA) and did not affect the receptors already present at 7 DIV, no clear decrease of P2X2 signal could be detected in immunofluorescence at the level of cell somas or on immunoblots (data not shown). Nevertheless, these results demonstrate the dependency of 5-HT3A-R on P2X2R for their localization in distal neurites and suggest that both receptors probably undergo a common trafficking fate at an early stage.

5-HT3A and P2X2 colocalize in common surface clusters

We then cotransfected hippocampal neurons with both tagged 5-HT3A and tagged P2X2 subunits to visualize how the presence of additional P2X2R would influence the neuronal distribution and topology of 5-HT3A-R. Figure 5A shows a typical neuron transfected with 5-HT3A-R-HA alone and one cotransfected with 5-HT3A-R-HA plus P2X2-YFP subunits. The main effect of the presence of both transfected receptors in the same neurons was a redistribution of 5-HT3A-R subcellular localization in the neuritic processes. 5-HT3A-R were “boosted” to fulfill the entire dendritic tree and also the axonal arborization up to the very end. Magnification of an axonal section revealed that 5-HT3A-R-HA and P2X2-YFP were both expressed under a diffuse and a clustered component. Colocalization of the two receptors within these surface clusters was largely observed in the majority of cotransfected neurons as shown by the correspondence between fluorescence profiles (Fig. 5B, C), indicating physical proximity between the two proteins during the trafficking process.

The P2X2 receptor is a specific routing partner of the 5-HT3A receptor for axonal targeting

The rigorous quantification of 5-HT3A-R subcellular redistribution by P2X2R cotransfection within hippocampal neurons required to first solve two technically interfering drawbacks: (1) the reduction of the endogenous P2X2R component contribution to 5-HT3A-R redistribution; and (2) the isolation of the axonal and dendritic components of fluorescence profile measurements. Both problems were solved by transfecting early plated neurons (4–5 DIV) because, at that time, (1) native P2X2R are still expressed at low levels (Fig. 3D) and contribute to distal targeting of 5-HT3A-R for <10% of neurons (Fig. 6D); and (2) the dendritic trees of neurons are still poorly developed, whereas axons are in rapid growing phase and are unambiguously characterized from dendrites on the basis of their shapes and sizes (Fig. 6A) without the use of axonal or dendritic specific markers that are not selective of their respective compartments in neurons that are not completely differentiated (4–10 DIV). Thus, 5-HT3A-R-HA and P2X2-YFP cotransfection was performed on 4–5 DIV neurons,
and quantitative fluorescence analysis was performed on the total number of transfected neurons for each condition (Fig. 6). Typical neurons expressing either 5-HT3A-R-HA and eGFP or 5-HT3A-R-HA and P2X2R-YFP are shown on Figure 6A. Cotransfection of P2X2R-YFP changed completely the targeting of 5-HT3A-R-HA from (proximal) dendritic only to (distal) dendritic and axonal, whereas eGFP did not induce 5-HT3A-R-HA immunofluorescence at distances above (distal) or below (proximal) 100 μm from somas for 5-HT3A-R-HA plus eGFP or 5-HT3A-R-HA plus P2X2-YFP. Bars represent the relative proportions of the two groups (mean ± SEM) for each cotransfection condition (two-way ANOVA, n = 324 and n = 399, respectively, p < 0.0001). E, Areas under individual curves (cumulated in B) for distances of 0–1000 μm were averaged. Bars represent the mean ± SEM; n = 30. ****p < 0.0001 (two-way ANOVA with Bonferroni’s Multiple Comparison post hoc test).

Figure 6. P2X2 receptors induce 5-HT3A receptor targeting in axons. Hippocampal neurons were cotransfected at 4–5 DIV with 5-HT3A-R-HA plus eGFP or 5-HT3A-R-HA plus P2X2-YFP. A, Immunofluorescence detection (red represents anti-HA; green represents anti-eGFP) was performed 48 h after transfection. Arrows indicate the axons. Scale bar, 50 μm. B, Cumulated fluorescence intensity profiles of 5-HT3A-R-HA along the axons (longest path, n = 30) for 5-HT3A-R-HA plus eGFP (blue) or 5-HT3A-R-HA plus P2X2-YFP (red). C, Cumulated fluorescence intensity profiles of eGFP and P2X2-YFP along the same axons (n = 30) for 5-HT3A-R-HA plus eGFP (green) or 5-HT3A-R-HA plus P2X2-YFP (orange). D, Quantification of the number of neurons exhibiting 5-HT3A-R-HA immunofluorescence at distances above (distal) or below (proximal) 100 μm from somas for 5-HT3A-R-HA plus eGFP or 5-HT3A-R-HA plus P2X2-YFP. Bars represent the relative proportions of the two groups (mean ± SEM) for each cotransfection condition (two-way ANOVA, n = 324 and n = 399, respectively, p < 0.0001).

The specificity of the physical partnership between 5-HT3A and P2X2 receptors was evaluated by quantification of the colocalization of the clustered components of receptor fluorescence...
in the dendritic tree, and comparison with other noninteracting receptors (Fig. 7). We chose this method rather than the quantification of the axonal distribution because it was appropriate for all receptors tested, all of them having a dendritic representation, whereas most of them were not axonal. Neurons were cotransfected with 5-HT3AR-HA and either P2X2R-YFP or seven other tagged receptors that were not described to functionally interact with 5-HT3AR. ROI were chosen within the dendritic trees, and colocalization was monitored with the JACoP plugin of ImageJ (Bolte and Cordelieres, 2006), using manual thresholding to restrict the quantification to the clustered component of 5-HT3AR-HA immunostaining. Although colocalization of immunofluorescence signals cannot be considered as proof of physical interaction between proteins, we used this method to show that the absence of colocalization between noninteracting receptors could evidence the specificity of the 5-HT3AR/P2X2 interaction. Indeed, this method proved to distinguish between physically interacting receptors exhibiting coclustered dendritic distributions (Mander’s M1 coefficient close to 0.8, characteristic plume-shaped fluorograms) and noninteracting receptors characterized either by nonoverlapping clusters or by one receptor being clustered and the other exhibiting a diffuse component only (random overlap, Mander’s M1 coefficient close to 0.4, diffuse or bimodal fluorograms) (Figs. 7B, 8C, 9B). Mander’s M1 coefficients (percentage of clustered 5-HT3AR-HA overlapping with the clustered component, when relevant, of the cotransfected receptor) are presented in Figure 7B. Moreover, none of the receptors yielding Mander’s M1 coefficients ~0.4 changed significantly 5-HT3AR-HA distribution in either neuritic compartment (Fig. 7A), showing that 5-HT3AR retargeting by P2X2R was related to their specific physical receptor–receptor interaction.

The 5-HT3A and P2X2 physical interaction depends on 5-HT3A IL2, but not on the P2X2 C-tail

We have demonstrated previously that the functional cross-inhibition between 5-HT3A and P2X2 receptors involved the C-terminal tail of the P2X2R and the IL2 domains of the cys-loop ionotropic receptors (Boué-Grabot et al., 2003), and that channel occlusion was subunit dependent in the case of GABAAR (Boué-Grabot et al., 2004a). To challenge whether the functional and physical couplings of 5-HT3A and P2X2 receptors depended on the same peptidic domains, we prepared truncated forms of the P2X2R (Fig. 8A) and chimeras of the 5-HT3AR in which the IL2 domain was replaced by the corresponding loops of δ or γ2 subunits of the GABAA receptor (Fig. 8E). HA-tagged P2X2ΔC36 and P2X2ΔC57 (lacking the C-terminal 36 and 57 amino acids, respectively) appeared to be fully functional and responded to ATP application in Xenopus oocytes (Fig. 8A) as expected by their surface expression in neuron somas and dendrites (Fig. 8B). However, because we found their axonal targeting partially impaired, their physical association with 5-HT3AR-Flag was monitored by measuring Mander’s M1 coefficients for their co-clustering in the dendritic tree (Fig. 8B,C) and was found to be statistically not different from HA-P2X2Wt (Fig. 8C), thereby

Figure 7. Specificity of the 5-HT3A and P2X2 receptor interaction. Hippocampal neurons were cotransfected at 7 DIV with 5-HT3A-HA and P2X2-YFP, 5-HT1A-eGFP, sst2A-eGFP, myc-GABAc (γ1), myc-MT2, myc-GluA1, myc-GluA2, or myc-NR2A subunits. A, Immunofluorescence detection was performed after 48 h (red represents anti-HA; green represents anti-eGFP or anti-myc). Scale bars, 20 μm. ROI were chosen within the dendritic trees to select cluster-rich areas and avoid the center of large neurites, and colocalization was monitored with the JACoP plugin of ImageJ (manual thresholding). Corresponding fluorograms for each cotransfected couple of receptors are represented on the right panels. B, Bars represent mean ± SEM values of Mander’s M1 coefficients (percentage of 5-HT3AR-HA fluorescence overlapping with cotransfected receptor’s fluorescence). Significant differences appeared only between interacting (P2X2) and noninteracting subunits (one-way ANOVA with Dunnett’s Multiple Comparison post hoc test); n = 4 – 8. ***,*** p < 0.0001.
Figure 8. The physical interaction between 5-HT\textsubscript{3}A and P2X2 receptors depends on 5-HT\textsubscript{3}A IL2, but not on the P2X2 C-tail. A, Schematic representation of P2X2Wt and the C-terminal truncated forms: P2X2\textsubscript{C36}, P2X2\textsubscript{C57}, and P2X2Tr. Bottom, Current traces evoked with 100 $\mu$M ATP by voltage-clamp recordings from *Xenopus* oocytes expressing each form, showing that they are all functional. B, Hippocampal neurons were cotransfected with 5-HT\textsubscript{3}A-Flag and HA-P2X2, HA-P2X2\textsubscript{C36}, HA-P2X2\textsubscript{C57}, HA-P2X2Tr (top) or 5-HT\textsubscript{3}A-HA and P2X2b-YFP (bottom). Immunofluorescence detection was performed at 48 h (red represents anti-Flag; green represents anti-HA [top]; or red represents anti-HA; green represents anti-GFP [bottom]). Right panels, Fluorograms as in Figure 7A. Scale bars, 20 $\mu$m. N/A, Not applicable. C, Bars indicate mean ± SEM values of Mander’s M1 coefficients. No significant differences appeared (Figure legend continues.)
demonstrating that the physical association between 5-HT 3A and P2X2 receptors did not require any of the motifs present in the C-terminal tail. We also tested a naturally occurring splice variant of neurons (M 5-HT in oocytes expressing each chimera show their nonfunctionality (Simon et al., 1997). However, we did not find any differences between P2X2–YFP and P2X2b–YFP, in their cocolocalization with 5-HT 3A–HA in the dendritic trees of neurons (Fig. 8B, bottom, C), in their axonal distributions, or in their capability of retargeting 5-HT 3AR in the axons (data not shown). Surprisingly, the complete removal of the C-terminal tail of the P2X2R had a dramatic effect on 5-HT 3AR localization. P2X2Tr is a truncated form of the P2X2R lacking the 98 C-terminal amino acids, and devoid of functional interaction with 5-HT 3A receptors (Boué-Grabot et al., 2003) but in which the proximal 20 amino acids containing the YXXXK motif controlling the surface expression are conserved (Chaumont et al., 2004). Figure 8B shows that this truncated form lacked completely the capability to be targeted further than 50 μm from cell somas, indicating that the C-tail of the P2X2R contains both axonal and dendritic trafficking motifs. Interestingly, the loss of these P2X2R trafficking motifs induced a concomitant sequestration of cotransfected 5-HT 3AR within the neuron soma where both receptors colocalized completely (Fig. 8D). Further controls were designed to verify that (1) the sequestration of both receptors within the soma was not the consequence of a nonspecific blockade of the trafficking machinery by P2X2Tr overexpression and (2) the sequestration of the 5-HT 3AR was specific and did not occur for a noninteracting receptor. Figure 8D shows that cotransfection of HA–P2X2Tr completely impaired 5-HT 3AR–Flag trafficking, even in proximal dendrites, but was without consequence on eGFP expression in dendrites and axons, thereby demonstrating the selectivity of this effect for 5-HT 3A receptors. Moreover, the cotransfection of P2X2R–YFP with HA–P2X2Tr could rescue the latter from sequestration within cell somas (Fig. 8D), as expected if the two subunits were coassembled and if the trafficking machinery was not altered. Although these results could be interpreted either by a tight association between 5-HT 3A and P2X2Tr subunits despite the absence of the C-terminal domain, or by a complete loss of 5-HT 3AR trafficking due to competition of P2X2Tr with endogenous P2X2R, they clearly demonstrate the complete dependency of 5-HT 3AR for their P2X2R targeting partner.

5-HT 3A,IL2β3–HA and 5-HT 3A,IL2γ2–HA chimeras were found to be nonfunctional in Xenopus oocytes (Fig. 8E). However, this lack of response to 5-HT could largely be attributed to their lack of surface expression, as we observed in neurons (data not shown). Cotransfection of both chimeras with P2X2R–YFP in neurons at 4–5 DIV revealed that neither their surface expression nor their dendritic or axonal distributions were affected by the presence of the P2X2R within the same neurons (Fig. 8F). Cumulated fluorescence analysis in axons (Fig. 8F, right panels) and comparison with 5-HT 3AR WT curves (in gray) showed no effect, and consequently confirmed that 5-HT 3A wild-type IL2 integrity was necessary for its physical coupling with the P2X2R.

From all these observations, we can conclude that the molecular determinants of the physical and functional couplings of 5-HT 3A,R and P2X2R are different. As a result, even if the functional coupling of the two receptors leading to cross-inhibition of the channels has been shown to involve a direct protein–protein contact between subunits, such a physical interaction between subunits is only one of the possible mechanisms explaining the 5-HT 3AR/P2X2 trafficking, and we cannot totally exclude here the participation of a third-party scaffolding protein partner.

5-HT 3A receptors interact with other members of the P2X family

To determine whether the receptor–receptor partnership between 5-HT 3A,R and P2X2R concerned other members of the P2X family, we chose the P2X4 receptor because P2X2 and P2X4 are the two predominant purinoceptors present in brain neurons, and the P2X3 receptor because it is largely expressed in the periphery with the P2X2R to form heteromers (North, 2002). P2X3R have been de-
scribed to undergo a rapid constitutive internalization that results in only a modest fraction being present at the plasma membrane in HEK293 cells (Vaccia et al., 2009). We confirmed such a distribution in neurons cotransfected with 5-HT$_{3A}$-HA and P2X3-Flag. In 100% of immunopositive neurons, P2X3R were only found in proximal dendrites with a distribution typical of a predominantly intracellular localization (Fig. 9A), and the same results were obtained with P2X3-YFP (data not shown). As expected from a strong interaction with P2X3 subunits, 5-HT$_{3A}$R were also found with the same distribution in 100% of the neurons cotransfected with both subunits. No immunolabeled axons could be detected, showing that transfected P2X3R could override the lower expression of endogenous P2X2R, with a dominant effect on 5-HT$_{3A}$R distribution. Colocalization of 5-HT$_{3A}$ and P2X3 subunits in the proximal dendritic trees was quantified by the JACoP method (Fig. 9B), which confirmed the overlap, although the clustered component of the labels was in this case mainly intracellular. Because P2X4R also undergo a rapid constitutive internalization triggered by a YXXGL motif located in the C-terminal moiety (Royle et al., 2002), we used here P2X4FlagIN, a chimera in which the internalization motif was replaced by a Flag epitope (Jo et al., 2011). This mutant exhibits a considerable increase of surface expression and thus behaves as the P2X2R in regards to trafficking within neurons, allowing to challenge its effect on 5-HT$_{3A}$R distribution without the bias of a different targeting. Cotransfection of 5-HT$_{3A}$-R-Flag with P2X4FlagIN (Fig. 9A, bottom) revealed extensive cluster colocalization, leading to Manders' M1 coefficients similar to the ones obtained with P2X2R controls (Fig. 9B). Analysis of 5-HT$_{3A}$R retargeting in axons yielded results similar to those obtained with P2X2R (data not shown). These results show that 5-HT$_{3A}$R can interact with other members of the P2X family of receptors, and thereby suggest that their trafficking fate depends on the type of P2X partner present in the neuron, confirming the idea that, in vivo, 5-HT$_{3A}$R subcellular localization in neurons is phenotype dependent.

**Discussion**

We demonstrated here that the main occurrence determining 5-HT$_{3A}$R subcellular localization within neurons relied on the presence of one of its functionally interacting partners: the P2X2R. We first showed that 5-HT$_{3A}$R and P2X2R are coexpressed in the same neurons in situ, and then described a differential targeting of 5-HT$_{3A}$R in a subpopulation of P2X2R-expressing hippocampal neurons in culture. We then showed specific colocalization of the two receptors in surface clusters and quantitatively measured the influence of P2X2R on 5-HT$_{3A}$R distribution in the distal parts of axons and dendrites in cotransfected neurons. We finally identified the molecular determinants involved in this physical interaction. Our results provide evidence of a novel pathway for receptor trafficking by means of a direct receptor–receptor functional interaction, and thereby unveil the mechanism by which 5-HT$_{3A}$R can reach their axonal localization required for the control of neurotransmitter release (Laporte et al., 1992; Doucet et al., 2000).

**P2X2 receptors control the subcellular localization of 5-HT$_{3A}$ receptors**

Our results are consistent with an asymmetrical dominant role of the P2X2R as a specific targeting protein partner for the 5-HT$_{3A}$ homomeric receptor in neurons. Six lines of evidence support this hypothesis: (1) 5-HT$_{3A}$R and P2X2R share a remarkably overlapping regional distribution, and we presented here evidence for their coexpression in the same neurons in myenteric ganglia (Fig. 1); (2) in hippocampal neurons in culture, 5-HT$_{3A}$R were unable to reach axonal or dendritic distal localization, or even surface representation, in neurons devoid of endogenous P2X2R (Fig. 3); (3) inhibition of native P2X2R by RNA interference led to a concomitant inhibition of the distal neuritic localization of 5-HT$_{3A}$R in the population of P2X2-expressing neurons (Fig. 4); (4) cotransfection of both receptors “boosted” the distal trafficking of 5-HT$_{3A}$R in neurites (Fig. 5) and particularly in axons (Fig. 6); (5) 5-HT$_{3A}$R and P2X2R cotransfected into hippocampal neurons were found to colocalize within surface clusters (Fig. 5); and (6) when the P2X2R was replaced by a truncated mutant devoid of neuritic trafficking, the 5-HT$_{3A}$R was also completely sequestered within cell somas (Fig. 8B).

The slow onset and neuron phenotype dependency of 5-HT$_{3A}$R distal trafficking in hippocampal neurons, compared with other receptors (Fig. 2), already suggested that the 5-HT$_{3A}$R trafficking mechanism was not driven by relatively ubiquitous proteins expressed at early times in vitro, as it is the case for the dendritic targeting of 5-HT$_{3A}$R by Yif1B (Al Awabdh et al., 2012), but rather by the cell-dependent availability of cargo or interacting proteins expressed upon neuron differentiation. Thus, both the kinetics of onset and the phenotype dependency fitted well with a P2X2R-dependent process (Fig. 3). The “boosting” of 5-HT$_{3A}$R axonal targeting by cotransferion of P2X2R at 4–5 DIV confirmed that differentiation of P2X2R-expressing neurons could be mimicked by ectopic P2X2R expression, which resulted in a shift of neurons expressing axonal 5-HT$_{3A}$R targeting from 11% to 79% (Fig. 6). This phenomenon could not be attributed to a nonspecific driving of one protein by the other due to overexpression because it was not observed with GFP controls or when P2X2R were replaced by other receptors that were not interacting functionally with the 5-HT$_{3A}$R, and was asymmetrical because the subcellular distribution of P2X2R was not modified by cotransfection of 5-HT$_{3A}$R. Conversely, 5-HT$_{3A}$R trafficking observed in the subpopulation of neurons expressing endogenous P2X2R could be inhibited either by P2X2 RNA interference (Fig. 4) or by cotransfection of a P2X2R trafficking-deficient mutant: the P2X2TrR (Fig. 8B). Interestingly, this last result could have two different interpretations: (1) P2X2TrR could have retained its capability of interaction with the 5-HT$_{3A}$R and could induce a dominant sequestration of the latter in cell somas, without affecting the trafficking of other receptors (Fig. 8D); or (2) P2X2TrR could have lost its interaction with the 5-HT$_{3A}$R but competed with endogenous P2X2R to override their normal trafficking by coassembly. Thus, both hypotheses led to the same conclusion: the 5-HT$_{3A}$R is dependent on its P2X2R partner for trafficking. The 5-HT$_{3A}$R and P2X2R physical association was further supported by the colocalization of both receptors in surface clusters along axonal and dendritic membranes, suggesting a common surface trafficking mechanism (Fig. 5). This colocalization was specific, as demonstrated by the cotransfection with other receptors noninteracting functionally with the 5-HT$_{3A}$R (Fig. 7), and was robust, suggesting that the physical association between 5-HT$_{3A}$R and P2X2R is probably a long-lasting interaction involving a high percentage of receptors, unlike the one between GABA$_A$ and P2X2 that seems more transient (Shrivastava et al., 2011) and exhibits subunit dependency (Boué-Grabot et al., 2004a).

Our results do not exclude that other proteins, not ubiquitously expressed in all neurons, could be involved in the fate of 5-HT$_{3A}$R because ~20% of neurons transfected by both receptors still did not address 5-HT$_{3A}$R distally (Fig. 6D). For instance, RIC-3, a protein that has been shown to participate to 5-HT$_{R}$ and a$_7$R surface expression (Cheng et al., 2005), was found in only 17% of hippocampal neurons in culture at 18–24 DIV and promoted the folding and the trafficking of native a$_7$R in dendrites, but not axons (Alexander et al., 2010). However, this percentage does not account for the 30% of neurons exhibiting distal
5-HT₃R targeting capacity after 7 DIV, whereas 97% of neurons expressing native α7R were found immunonegative for RIC-3 (Alexander et al., 2010). This makes the participation of RIC-3 in P2X2R-induced distal trafficking of 5-HT₃R unlikely, unless the presence of RIC-3 in neurons actually has the opposite effect and prevents 5-HT₃R to couple to P2X2R, which would be more consistent with the preferential sequestration of α7R in the dendritic endoplasmic reticulum compartment induced by the high expression of RIC-3 in neurons.

The physical and functional couplings of 5-HT₃A and P2X2 receptors involve different molecular determinants

Convergent data demonstrated that the physical coupling between P2X2R and other receptor classes is a direct protein–protein interaction involving anstrom scale distances consistent with dimer formation between subunits (Khakh et al., 2005). Thus, we have previously shown that the molecular determinants of the functional cross-inhibition between 5-HT₃A and P2X2 receptors are located within the IL2 of the 5-HT₃AR and the intracellular C-terminal tail of the P2X2R (Boué-Grabot et al., 2003). However, because competition experiments by P2X2-CT mini-genes could uncouple 5-HT₃A and P2X2 functionally but not physically in Xenopus oocytes, as shown by coimmunoprecipitation, it was concluded that the C terminus of the P2X2R was not implicated in the formation of the 5-HT₃A/P2X2 physical complex (Boué-Grabot et al., 2003). The results obtained here confirm this hypothesis because all the C-terminal truncated forms of the P2X2R tested still colocalized with 5-HT₃AR (Fig. 8B) or influenced 5-HT₃AR axonal targeting. Conversely, replacing the IL2 of the 5-HT₃A by the corresponding sequences of GAPA β3 or γ2 subunits resulted in the uncoupling of both chimeras from the P2X2R and led to the independent trafficking of both receptors (Fig. 8F). This result shows that IL2 is necessary for the cotrafficking of 5-HT₃R and P2X2R. However, its replacement, even by a functionally interacting loop as the β3, is not sufficient to restore the physical coupling, whereas in the case of the homomeric GABAergic (ρ1) receptor, partial restoration of the physical coupling could be obtained with the same strategy (Boué-Grabot et al., 2004b). From these results, we can conclude that (1) the molecular determinants of the physical and functional couplings of 5-HT₃AR and P2X2R are different, and (2) the mechanisms of physical coupling between P2X2R and the other interacting 4-TMD channel receptors are probably not identical.

5-HT₃A receptors interact with other members of the P2XR family

The results obtained with the P2X3R and the internalization-defective mutant P2X4FlagIN (Jo et al., 2011) suggest that the P2X2R is probably not the only partner of the 5-HT₃AR in vivo (Fig. 9) and that this receptor–receptor physical coupling probably results from intrinsic properties common to several P2XRs. Thus, a new level of complexity in 5-HT₃R trafficking could be inferred, depending on the P2X subunits coexpressed with the 5-HT₃R, some of them being targeted distally and some others being internalized. Moreover, as many P2X subunits coassemble to form heterotrimers, another level of complexity might result from intermediate trafficking fates of heteromers (Saul et al., 2013). Our results also disclose a possible physiological role for the natural splice variants of P2X receptors that are devoid of electrophysiological activity, as the P2X2c and P2X2d (Simon et al., 1997), as they might just be trafficking proteins for other receptors. Moreover, the existence of four additional human 5-HT₃ subunits that can coassemble with the 5-HT₃A (Niesler et al., 2003) and the possibility that they could all differentially interact with more than one P2X subunits open an intriguing number of possible trafficking schemes.

In conclusion, we describe here that the receptor–receptor interaction between 5-HT₃R and P2X2R receptors is not restricted to a functional coupling but extends to an intimate physical interaction that leads to a dominant influence of P2X2R on 5-HT₃R subcellular localization. Hence, we propose to assign, to this family of ATP receptors, a new role of trafficking partners for other classes of receptors.

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