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Neurotransmitter Receptor Complexes In The Brain: Biochemical Characterization And Functional Analysis Of Receptor-Receptor Interactions

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1 Introduction

Neurotransmitter receptors are integral membrane proteins expressed at the cell surface that allow communication between neuronal cells. Communication between neurons in the mammalian central nervous system, also called synaptic transmission, is achieved through the release of one or more neurotransmitters or modulators from the same pre-synaptic terminal that leads to the activation of specific neurotransmitter receptors localized at the post-synaptic membrane. Receptors are classified by their transduction mechanisms into two main families: G protein-coupled receptors (GPCRs) and ligand-gated ion channels (LGICs). Upon ligand's binding, GPCRs mediate their effect through the activation of G proteins which engage second messenger pathways; whereas LGICs induce the opening of a central pore permeable to selected ions. Neurotransmitter-gated channels play a crucial role in controlling neuronal activity and the modulation of their function has important consequences for neuronal excitability. It is well established that changes in the number, molecular composition, properties, interaction with receptor-associated proteins and/or localization of LGICs modulate receptor function. Recent findings demonstrated that physical interaction between two distinct ligand-gated channel types represent an additional mechanism to regulate specific receptor function and/or trafficking and consequently synaptic transmission. In this chapter, we provide an overview on such interaction between distinct receptors and describe in details reliable biochemical methods for assessing physical interaction between distinct LGICs. We also mention other complementary methods to examine the functional impact of receptor-receptor interactions.

1.1 Diversity of Ligand-Gated Ion Channels

Genome and cDNA sequencing analysis reveals that there are three major families of neurotransmitter-gated channels each with a unique architecture. These are the 'cys loop' receptors, which include the nicotinic receptor for acetylcholine (nAChR) the γ -aminobutyric acid (GABA)_A receptor, the 5-hydroxytryptamine (5-HT)₃ receptor, the glycine (GlyR) receptor and the zinc-activated channel (ZAC) (Davies et al., 2003; Karlin, 2002; Michels & Moss, 2007; Peters et al., 2005; Sine & Engel, 2006); the glutamate receptors, comprising the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), the kainate and the N-methyl-d-aspartic acid (NMDA) receptors (Mayer, 2005, 2006) and the P2X receptors gated by ATP (North, 2002).

Those three families are especially different regarding the number and the topology of the subunits required to form a functional receptor (Figure 1). Cys-loop receptors are pentamers, glutamate receptors are tetramers and P2X receptors are trimers (Figure 1). Cys-loop receptor subunit has a short extracellular C-terminus, a large N-terminal extracellular region including a highly conserved extracellular Cys-Cys loop, four α -helical transmembrane domains (TM1-TM4) and a large variable-sized cytoplasmic loop between TM3 and TM4. The intracellular loop contributes for most of the cytoplasmic domain of a cys-loop receptor and includes multiple protein-protein interaction sites for

putative trafficking and both postsynaptic scaffold proteins and phosphorylation sites for diverse kinases. The amphiphilic TM2 domain provides the lining of the ion pore within the pentameric structure and contributes to its ion selectivity. Thus GlyR and GABA_AR are selectively permeable to anion and mainly chloride, while 5-HT₃R and nAChR are cation permeable channels (Lemoine et al., 2012). Glutamate receptors are cation-selective channels divided into AMPA, NMDA and kainate receptors formed by a tetrameric association of subunits with three transmembrane domains and a reentrant loop between TM1 and TM2 (Mayer, 2005). ATP- and proton-gated-channels constitute a recently discovered receptor family. Although these are unrelated in terms of sequences, both channel families consist of trimeric homo- or heteromeric associations of subunits with two transmembrane domains, a large extracellular domain and both intracellular NH₂ and COOH termini (Gonzales et al., 2009; Kawate et al., 2009) (Figure 1).

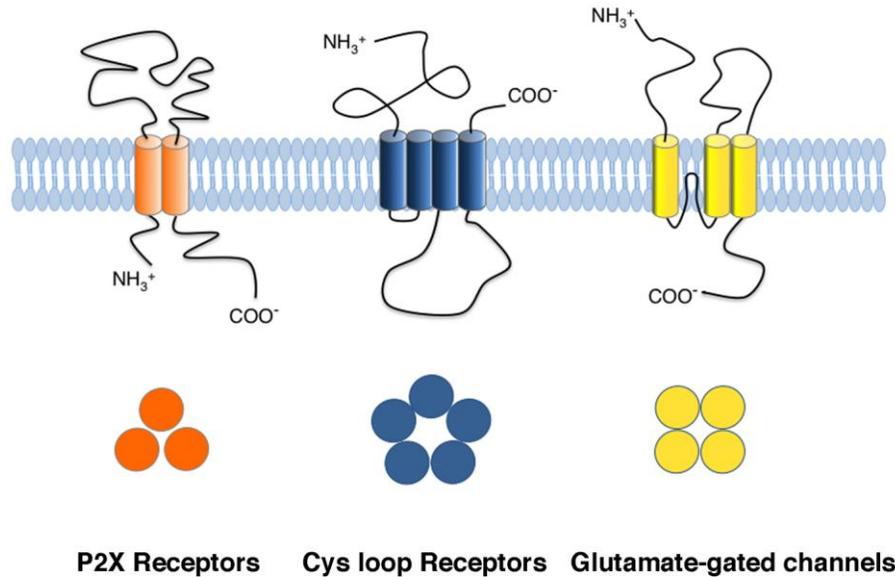


Figure 1: Membrane topology of the different ionotropic receptor subunits (top). Receptors are formed by association of several subunits delineating a central transmembrane pore (bottom, top view). Binding of a specific small molecule (Ligand) on the extracellular domains of these proteins lead to the opening of the intrinsic pore through which specific ions flow at high flux.

1.2 Functional and Biochemical Characterization of Interactions Between Distinct Ligand-Gated Ion Channels

In view of the clear structural differences between neurotransmitter-gated channels, it was assumed that each receptor type acts independently of the other. However the principle of independence has now been challenged by multiple studies that provide strong evidence for functional and physical interactions between several distinct ligand-gated channels.

Nakazawa *et al.* first observed in rat pheochromocytoma (PC12) cells, that the combined effect of ATP and nicotine was less than the linear sum of the individual ATP and nicotinic currents and initially proposed that ATP and nicotine activated the same channels formed by an association of P2X and the nicotinic subunit around the same pore (Nakazawa et al., 1991). Cross-inhibition between P2X and the nicotinic receptor was confirmed by several groups studying sympathetic and myenteric neurons but also in oocytes co-expressing P2X2 and $\alpha\beta4$ nicotinic receptors (Barajas-Lopez et al., 1998; Khakh et al., 2000; Searl et al., 1998; Zhou & Galligan, 1998). Together these studies clearly demonstrated that P2X2 and nicotinic receptors form separate channels and that the co-activation of both receptors results in non-additive responses owing to an inhibition of both channel types. In addition, decreasing the expression level of P2X2 and $\alpha\beta4$ nicotinic receptors resulted in additive responses suggesting that a close proximity

of the receptors is required for the inhibition (Khakh et al., 2000). Similar inhibitory cross-talk was also demonstrated between P2X2 and 5-HT₃, and between P2X2 and GABA_A receptor-channels through co-expression studies and in native myenteric neurons (Boue-Grabot et al., 2003; Boue-Grabot et al., 2004a; Boue-Grabot et al., 2004b). The investigation of P2X2 and GABA_A receptor co-activation revealed that the direction of the inhibition was dependent on a specific GABA_A subunit composition (Boue-Grabot et al., 2004b). In cells expressing GABA receptors containing α and β subunits, the channels showed reciprocal inhibition. In oocytes expressing α , β and γ GABA_A receptor subunits, GABA inhibited the response to ATP whereas ATP did not inhibit the response to GABA. Regardless of the GABA receptor composition, current occlusion between P2X2 and GABA_A receptors was independent of the ion flow direction, the calcium concentration or the voltage, thereby suggesting that a molecular process was involved.

Co-purification experiments indicated that P2X2 interacts physically with 5-HT₃ and ρ 1 GABA_C receptor channels (Boue-Grabot et al., 2003; Boue-Grabot et al., 2004a). In addition, the co-transfection of ρ 1 and P2X2 receptors revealed a co-clustering of these receptors in transfected hippocampal neurons and that P2X2 receptors modified the addressing of GABA_C receptors in a dominant way by inducing their translocation from an internal vesicular compartment to surface clusters shared by both receptors. The close spatial arrangement between P2X2 and α 4 β 2 nicotinic receptors in transfected hippocampal neurons was also demonstrated by fluorescence resonance energy transfer (FRET) analysis using fluorescent-tagged subunits (Khakh et al., 2005), suggesting that activity-dependent cross-inhibition between P2X2 and “cys loop” receptors including nicotinic, 5-HT₃ or GABA receptors may arise from molecular interactions.

Indeed, it was recently discovered that G-protein coupled receptors can interact directly with ligand-gated channels leading to a functional and reciprocal modulation of each receptor type. Such a direct interaction was first demonstrated between dopamine D5 and GABA_A receptors. Through a series of biochemical approaches, Liu et al. convincingly demonstrated that the intracellular COOH-terminal tail of the D5 receptor binds specifically and directly to the main intracellular loop of the GABA_A γ 2 subunit (Liu et al., 2000). D5 receptor activity was downregulated upon GABA receptor activation and the reciprocal activation of D5 receptors reduced the amplitude of GABA-induced currents. The reduction was abolished with the intracellular administration of peptides corresponding either to the D5 receptor or γ 2 interacting domains showing that their physical coupling underlies the functional cross-modulation. A direct interaction was also demonstrated between D1 and NMDA receptors (Lee et al., 2002; Pei et al., 2004). In this case, the authors identified not one but two distinct sites of interaction: one between the C-terminal domain of D1 and NR1 subunits and a second between the C-terminal domain of D1 and NR2A each one of them having different consequences for NMDA receptor function. By similar approaches, these authors have also shown that the interaction between D1 and NR2A leads to an inhibition of the NMDA-induced current by D1 stimulation, whereas the interaction between D1 and NR1 seems to suppress NMDA receptor-mediated cell death upon D1 activation. Reciprocally, NMDA receptor activation increased the number of D1 receptors on the plasma membrane surface, and consequently enhanced the response to dopamine.

The first evidence that molecular interactions lead to a functional cross-talk between ligand-gated channels came from experiments using a C-terminal truncated form of P2X2 subunits. Truncation of this domain abolishes the functional cross-talk between P2X2 and 5-HT₃, nicotinic or GABA receptor-channels (Boue-Grabot et al., 2003; Boue-Grabot et al., 2004a). Likewise, expression of a minigene encoding either the distal C-terminal portion of the P2X2 receptor or the main intracellular loop located between the third and fourth transmembrane domains of 5-HT₃ or ρ 1 subunits reduced the current inhibition between P2X2 and 5-HT₃ or ρ 1 GABA receptor-channels, respectively. These results indicated that an interaction between the intracellular domains of each receptor is required for the functional cross-talk. In addition using chimeric ρ 1-GABA_A receptors with α 2, β 3 or γ 2 sequences downstream from the third transmembrane domain (Boue-Grabot et al., 2004b), non-additive responses and co-clustering were observed between P2X2 and ρ 1- β 3 chimeras, but not ρ 1- α 2 or ρ 1- γ 2, indicating that P2X2 interacts mainly with β subunits of GABA_A receptors. This is consistent with FRET experiments between P2X2 and α 4 β 2 nicotinic receptors which showed a higher fluorescence transfer efficiency between P2X2 and β 2 subunits, rather than α 4 subunits (Khakh et al., 2005).

It is now clear that several P2X channels have the ability to interact functionally with distinct ion channels. A cross-inhibition between P2X3 or P2X2/3 and GABA_A was described in dorsal root ganglia (DRG) neurons but, in contrast to other studies, the authors suggested an ion-dependent mechanism (Sokolova et al., 2001). Toulme *et al.* (2007) provided convincing evidence for a molecular interaction between P2X3 and GABA_A receptors that was also responsible for the ion-independent cross-inhibition in DRG neurons and recombinant expression system (Toulme et al.,

2007). We identified a specific intracellular motif of three consecutive amino-acids, a QST motif at positions 386-388 in the C-terminal tail of P2X3 subunits that is required for the functional coupling with GABA_A receptors. The current occlusion between native P2X3 and GABA_A receptors in DRG neurons was abolished either by infusing a peptide containing the QST motif or by viral infection of the main intracellular loop of GABA_A β3 subunits.

Lately, it was clearly demonstrated a physical and functional interactions between excitatory ATP P2X4 and inhibitory GABA_A receptors at the cellular and molecular levels that appear to be critical for regulating synaptic strength at the synaptic level and, as a consequence, neuronal excitability (Jo et al., 2011). A series of biochemical approaches including co-immunoprecipitation, peptide-based pull-downs, mutagenesis and overexpression of peptides in a heterologous system provides converging evidence for a physical interaction between a specific intracellular motif (Tyr 374, Val 375) within the C-terminal tail of P2X4 subunits and GABA_A β subunits. Furthermore, a recent FRET study confirms the close proximity of the C-terminal tail of P2X2 subunits and the intracellular loop of GABA_A subunits (Shrivastava et al., 2011).

It is thus believed that the C-terminal tail of P2X subunits interacts directly with the main intracellular loop of cys loop receptor subunits, including GABA_A, nicotinic and 5-HT3 receptors. Importantly, the interaction motif identified in P2X4 subunits is different from the one identified in P2X3 subunits. It should also be noted that these identified motifs are absent in P2X2 subunits. Likewise, the interacting regions of cys loop receptors, including GABA, nicotinic, and 5-HT3 subunits show no primary sequence homology. It thus appears that the interaction between P2X and cys loop receptors is subunit-specific. In addition to the direct interaction between two receptors, undefined interacting proteins and/or regulatory factors may trigger or alter this negative interaction since these receptors appear to act independently in some neuronal populations (Khakh et al., 2005).

Together, these results strongly suggest that a molecular interaction between P2X and members of the cys loop receptor family leads to an activity-dependent cross-inhibition through a conformational spread mechanism in which the motion triggered by the gating of one channel type is communicated to the other channels and induces its closure (Bray & Duke, 2004).

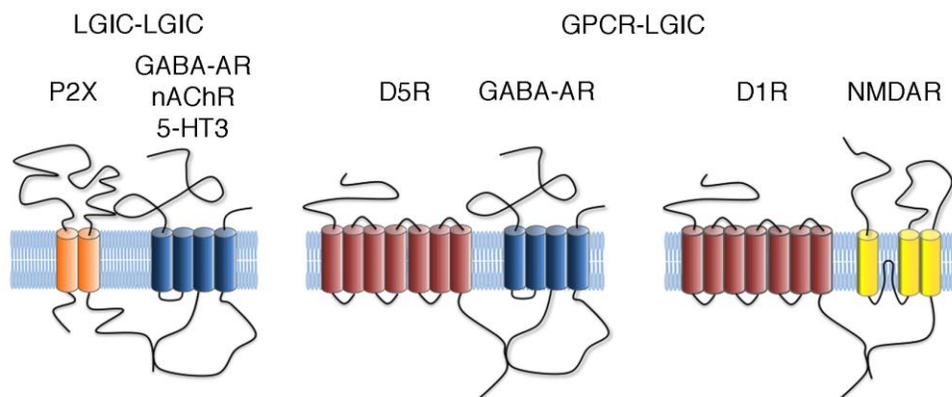


Figure 2: Schematic representation of interactions between P2X receptors and several members of the cys loop receptor family (left), between GABA and dopamine D5 receptors (middle) and between dopamine D1 receptors and NR1 and NR2A subunits (right).

Besides these physical interactions that modulate receptor function (Figure 2), studies have also provided evidence for activity-dependent cross-talk between two different members of the “cys loop” receptor family -GABA_A and glycine receptors- in spinal cord neurons or GABA_A and 5-HT3 receptors in myenteric neurons, and between two different ionotropic glutamate receptors -AMPA and NMDA receptors- in hippocampal neurons (Bai et al., 2002; Li et al., 2003; Miranda-Morales et al., 2007). The mechanism of the interaction between GABA and glycine receptors is

however different and involves the phosphorylation state of the GABA_A receptor and/or its mediator proteins. Cross-talk between distinct glutamate receptors has been found G-protein or protein kinase independent, so it would be interesting to determine if direct or indirect interactions occur between these channels.

Taken together, these data demonstrate that there is a complex interplay between fast neurotransmitter receptors in the nervous system and since fast neurotransmitters are co-released in the nervous system (Galligan & Bertrand, 1994; Jo & Role, 2002; Jo & Schlichter, 1999; Jonas et al., 1998; Redman & Silinsky, 1994), such interactions between their respective receptor-channels may exert powerful and rapid receptor modulation which is essential for the integration of synaptic signals.

In summary, direct interaction between two distinct LGIC was demonstrated between P2X receptors and several members of the cyst loop receptor family including GABA_AR, nAChR or 5-HT₃R as well as between a GPCR and a LGIC such as dopamine D1 or D5 receptors and GABA or NMDA receptors (Figure 2). We described (see section 2) several biochemical methods used to identify interaction between distinct receptors. These methods demonstrated that both receptors belong to the same protein complexes and were useful for the identification of interacting domains of each receptor. However, these methods are not sufficient to fully characterize interaction between distinct receptors and complementary approaches (see section 3) are required to discriminate between direct or indirect interaction or to address the functional impact of interaction between distinct receptors.

2 Biochemical Methods for Assessing Receptor-Receptor Interactions

We describe here classical *in vitro* methods such as co-immunoprecipitation, pull-down assays or overlay classically used to determine physical interactions between receptors from brain protein extracts. These methods are based on the use of specific antibodies (ideally from different species) directed against both receptor types. These protocols should be adapted depending on the quality of the primary antibodies, the frequency and strength of the interaction, the expression level of each receptor, and the distribution within the brain of both receptor types. Alternatively, these methods can be also performed with tagged-receptors heterologously expressed in recombinant expression systems using immuno- or affinity- purification methods. In this chapter, we describe several protocols to obtain protein lysates from total brain, specific brain structures or cells expressing receptors, multiple methods to purify of protein complexes as well as SDS-PAGE and Western blot analysis.

2.1 Material Setup

2.1.1 Collection of Brain Tissues

If both receptors are widely expressed within the brain, large pieces of brain such as cortex or cerebellum are collected with a scalpel from the whole brain that has been removed from the skull immediately after decapitation of deeply anesthetized animals. Tissues are immediately frozen at -80°C. Euthanasia methods must be adapted according to the ethical rules and the animal models (rat, mouse, monkeys).

If both receptors are localized in a specific region or structure of the brain, it is necessary to first isolate the structure of interest in order to avoid dilution of the receptors in the protein extracts. After removal, the whole brain is quickly frozen in a Snap Frost® 80 Specimen Freezing System (Alphelys) and placed in a cryostat to cut 200 µm-thick sections. In sections containing the structure of interest identified using a brain atlas, patches of tissue corresponding to the structure of interest are collected using a disposable tip of a P1000 pipette and conserved at -80°C before use (Porras et al., 2012).

2.1.2 Protein Expression in Various Heterologous Systems

Expression model 1: Xenopus laevis oocytes

Xenopus laevis oocytes are a powerful and widely used experimental system that can be exploited for expression of proteins. The large size and low maintenance of these cells make them easy to handle and to microinject with different molecules (Bossi et al., 2007). Among the different advantages linked to this method, we should point out that the

expression level can be easily controlled by varying the amount of nucleic acid injected, but also that different proteins can be co-expressed in the same cell.

Either plasmid constructs or RNAs encoding receptor subunits can be injected using a nanoinjector into the nucleus or cytoplasm of oocytes respectively. Injected oocytes are placed into Petri dishes containing oocyte culture medium and incubated at 19°C from 2 to 10 days to allow receptor expression.

Expression model 2: cell line culture

HEK293 or COS-7 cells grow in an incubator at 37°C with 5% CO₂ in 94 mm cell culture dishes with 10 mL of DMEM supplemented with 10% (v/v) foetal bovine serum, but no antibiotics. Split the cells 2 times per week. When the cells are 90% confluent, split them and inoculate 2x10⁵ cells per well in a 6-well plate.

The day after splitting the cells, the transfection can be performed using the Effectene reagent from Qiagen (kit #301425).

- Incubate the cells under their normal growth conditions (usually 37°C and 5% CO₂). The cells should be 40–80% confluent on the day of transfection. Warm the medium to 37°C prior its addition to the cells.
- The day of transfection, dilute 0.5 µg of DNA, with the DNA-condensation buffer (buffer EC in the Qiagen transfection kit), to a total volume of 90 µL. Add 10 µL of Enhancer and mix by vortexing for 1 s.
- Incubate at room temperature (15–25°C) for 2 min then spin down the mixture for a few seconds to remove drops from the top of the tube.
- Add 10 µL of Effectene transfection reagent to the DNA-Enhancer mixture. Mix by pipetting up and down 5 times.
- Incubate the samples for 10 min at room temperature (15–25°C) to allow transfection-complex formation.
- While complex formation takes place, gently aspirate the growth medium from the plate, and add 2 mL of fresh growth medium (that can contain serum and antibiotics) to the cells.
- Add 600 µL of growth medium (can contain serum and antibiotics) to the tube containing the transfection complexes. Mix by pipetting up and down twice, and immediately add the transfection complexes drop-wise onto the cells. Gently swirl the dish to ensure uniform distribution of the transfection complexes.
- Incubate the cells with the transfection complexes under their normal growth conditions allowing enough time for expression of the transfected gene. The incubation time is determined by the assay and the gene used.

Note: In many cases, removal of transfection complexes is not necessary. However if cytotoxicity is observed, remove the Effectene-DNA complexes after 6-18 h, wash the cells once with PBS, and add 2 mL of fresh growth medium.

2.2 Protein Preparation

In order to analyze the interactions between membrane receptors, it is necessary to adapt the conditions of protein extraction and solubilization to the strength of the interaction between the receptors as well as receptor protein solubility. Nature and concentration of the detergent are the critical element to ensure a complete solubilization of the proteins without disrupting receptor-receptor interaction. In this chapter, we describe fast and reliable protocols used for extraction and solubilization of total proteins or enrichment of membrane proteins from brain tissue or receptor-expressing cells (Boue-Grabot et al., 2003; Boue-Grabot et al., 2004a; Coussen et al., 2002; Frugier et al., 2007; Jo et al., 2011; Porras et al., 2012; Toulme et al., 2010).

2.2.1 Total Protein Preparation from Brain or Transfected Cells

Lysis buffer for oocytes or brain tissue:

10 mM HEPES,
300 mM Sucrose, pH 7.5

Lysis buffer for HEK cells:

20 mM HEPES,
100 mM Sodium Chloride,
1 mM DTT,
0.8 - 1% Triton X-100, pH 8.0

For Xenopus oocytes or brain tissue:

- 48 to 72 hours after DNA injection, 20-25 oocytes are rinsed twice with 500 µL of PBS.

- Remove all traces of PBS and add 400-500 μL of homogenization buffer supplemented with a cocktail of protease inhibitors.
- Homogenize oocytes first by pipetting up and down using a P1000 pipette and then through a syringe with a 26g needle, 8 times.
- For brain tissue, homogenization will be performed using a tissue homogenizer (such as a Polytron®) or by sonication in the same buffer (5 volumes of buffer for one volume of tissue).
- Add 0.8-1.0 % of triton X-100 and place under agitation during 2 hours at 4C.
- Centrifuge the sample for 10 min at 13,000g at 4°C.
- Transfer the supernatant containing the solubilized proteins into a clean tube.

For HEK cells:

- 24 to 48 hours after transfection, cells are rinsed twice with of PBS (500 μL /well of a 6-well plate).
- Remove all traces of PBS and add 500 μL of lysis buffer supplemented with a cocktail of protease inhibitors.
- Use a cell scraper to remove all cellular material from the cell culture plate and transfer the extract to a clean microcentrifuge tube.
- Triturate the cells through a syringe with a 26g needle 4 times and incubate on ice in the lysis buffer or on a rotating wheel for 30 min to 1 hour at 4°C.
- Centrifuge the sample for 30 min at 13,000g at 4°C.
- Transfer the supernatant containing the solubilized proteins into a clean tube.

2.2.2 Total Protein Preparation from Patches of Tissue

RIPA buffer (Sigma):

50 mM Tris-HCl (pH 8.0),
 150 mM Sodium Chloride ,
 1.0% Igepal CA-630 (NP-40),
 0.5% Sodium Deoxycholate,
 0.1% Sodium Dodecylsulfate

- Add 10 μL of RIPA buffer containing a cocktail of protease inhibitors to one patch of tissue.
- Sonicate the sample for 3 sec.
- Leave on ice for 30 min.
- Centrifuge for 10 min at 20,000g
- Transfer the supernatant into a clean microcentrifuge tube.

2.2.3 Membrane Protein Preparation from Brain or Transfected Cells

Homogenization buffer:

20 mM HEPES,
 0.15 mM EDTA,
 10 mM Potassium Chloride, pH 7.5-8.0

Solubilization buffer:

20 mM HEPES,
 150 mM Sodium Chloride,
 0.15 mM EDTA,
 1 to 2% Triton X-100 with or without 0.5% of deoxycholate, pH 7.5-8.0

- Homogenize brain tissue (1 volume into 5 volumes of homogenization buffer supplemented with a cocktail of protease inhibitors) using a tissue homogenizer at 4°C.

- For transfected cells, remove carefully the medium from one well of a 6 well-plate. Add 500 μ L of homogenization buffer supplemented with a cocktail of protease inhibitors, pipet up and down 4 times and leave on ice for 30 min under agitation.
- Centrifuge for 10 min at 600g and remove the supernatant into a clean tube.
- Centrifuge the supernatant for 30 min at 15,700g.
- Discard the supernatant and homogenize the pellet with 15 strokes using a potter homogenizer in the homogenization buffer adjusted at 15% sucrose.
- Centrifuge the homogenate for 10 min at 600g to remove genomic DNA.
- Transfer the supernatant (containing the membrane) into a clean tube and centrifuge again for 30 min at 15,700g.
- Remove the supernatant and resuspend the pellet (containing brain membranes) in 3-4 mL of solubilization buffer supplemented with a cocktail of protease inhibitors.
- Homogenize using a Potter homogenizer 20 times and then incubated on ice for 1 hour.
- Centrifuge the samples for 45 min at 15,700g.
- Transfer the supernatant into a clean tube.

Note: All the steps of the procedures described in the section 2.2 should be performed at 4°C to limit protein degradation. Protein concentration should be determined using the method of your choice such as colorimetric methods (for review see (Simonian & Smith, 2006)). Methods described above result in protein concentration around 5 to 10 mg/mL.

2.3 Co-Immunoprecipitation Methods

Co-immunoprecipitation (Co-IP) experiments are designed to purify a bait protein using a specific antibody directed against the bait. An important characteristic of Co-IP reaction is their ability to precipitate the target protein as well as protein complexes associated to this protein from a lysate. Immunoprecipitated proteins and their binding partners are commonly detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. This method was extensively used to identify receptor-associated proteins as well as interaction between LGICs (Boue-Grabot et al., 2003; Boue-Grabot et al., 2004a; Buddle et al., 2003; Chen et al., 2000; Gardoni et al., 1998; Jo et al., 2011; Kneussel et al., 2000; Lainez et al., 2010; Le et al., 1999; Liu et al., 2000; Rumbaugh et al., 2003; Sans et al., 2001; Santos et al., 2012; Wang & Olsen, 2000). However, in classical Co-IP, the antibody is co-eluted with the target proteins and may create interfering bands during the Western blot gel analysis in particular when antibodies directed against both receptors types are from the same specie. For that matter, an alternative solution would be to use covalently coupled antibody to a resin. In those conditions, if the elution is done in mild-enough conditions, the covalently coupled antibody stays bounded to the resin whereas the target receptor and associated partners are eluted. We describe below both methods.

2.3.1 Classical Co-Immunoprecipitation

Classical Co-IP experiments involve the formation of antibody:antigen complexes in a sample solution and then the binding of that complex to an IgG-binding protein that is covalently bound to beaded sepharose resin (ProteinA/G Sepharose). Protein A Sepharose is generally used for mouse monoclonal antibodies and protein G Sepharose for polyclonal antibodies. After several washes to remove unbound components from the sample, the antigen and antibody are recovered from the beaded resin with an elution buffer.

Before beginning, it is important to keep a fraction of your protein samples to use as a starting material for Western blot analysis.

- Incubate remaining protein homogenate (600 μ L) with 30 μ L of Protein A/G sepharose under agitation on a wheel for one hour at 4°C to limit non-specific binding (clearing).
- Centrifuge at 2,500g for 3 minutes and keep the pre-cleared protein supernatant.
- Add 5-10 μ g of the appropriate antibody to the protein sample and place under rotation on a wheel for 1.5 hour at 4°C.
- Add 30 μ L of the protein A/G-Sepharose slurry and place under rotation overnight on a wheel at 4°C.
- Wash the protein A-Sepharose beads with 600 μ L of solubilization buffer and repeat this step five more times.
- Remove the liquid phase and elute retained proteins by adding 60 μ L of 2X SDS gel-loading buffer.

- Run the eluted proteins on a SDS-PAGE gel and analyze by Western blot.

2.3.2 Covalent Co-Immunoprecipitation

Traditional Co-IP methods using Protein A or G result in co-elution of the antibody that co-migrates on SDS-PAGE gels. If antibodies directed against the bait and the prey receptors are from the same species, revelation of the light and heavy chains of the antibody results in contaminating signals at 25 kDa and 50 kDa that can mask the detection of the prey receptors such as cys loop receptor subunits that migrate at 50kDa. This issue can be circumvented by covalently coupling antibodies onto a resin. The protocol described below is adapted from Jo and coworkers (2011) using an agarose-beads from Pierce (Co-Immunoprecipitation Kit #23600). Technology based on activated magnetic kit from various companies such as Invitrogen are also available and were successfully used to reveal interaction with receptors (Mikasova et al., 2012).

Coupling buffer:

140 mM Sodium Chloride

8 mM Sodium Phosphate

2 mM Potassium Phosphate

10 mM Potassium Chloride, pH 7.4

Coupling step:

- Equilibrate the Antibody Coupling Resin and reagents at room temperature.
- Prepare a 1X coupling buffer.
- Gently swirl the bottle of AminoLink Plus Coupling Resin (Pierce #26149) to obtain an even suspension. Prepare enough columns for your number of samples. Follow step (a) if you only have one sample or (3b) if you have several samples:
 - (a) Add the 50% resin slurry into a Spin Cup Column. Place the column inside a microcentrifuge tube and centrifuge for 30 sec at 1,500g. To facilitate the transfer of the slurry mixture, cut off 2-3 mm from the narrow tip of a standard 200 μ L disposable pipette tip.
 - (b) Take the necessary quantity of resin for X columns and put into a dolphin tube. Centrifuge for 1 min at 1,000g.
- Discard the flow through (3a) or the supernatant (3b).
- Wash 2 times with 200 μ L of coupling buffer 1X (3a) or 500 μ L of coupling buffer 1X (3b).
- Remove the excess liquid (3a) or distribute 50 μ L of resin in clean microcentrifuge tubes (3b).
- Fix the screw cap on the bottom of each column.
- Prepare your antibody: add 20 μ L of monoclonal antibody (0.25 μ g/ μ L per immunoprecipitation) and adjust the final volume to 200 μ L with coupling buffer.
- Add 3 μ L of Sodium Cyanoborohydride per 200 μ L of diluted antibody.
- Incubate 2 to 3 hours at room temperature.
- Keep a fraction of diluted antibody to measure the optic density (OD).
- Put spin columns into a 2 mL collection tube. Remove the plug and centrifuge for 1min at 1000g.
- Keep an aliquot of flow through to measure the OD corresponding to the antibody binding efficacy.
- Add 200 μ L of coupling buffer and centrifuge for 1 min at 1000g.
- Repeat step 14 one more time.
- Add 200 μ L of quenching buffer and centrifuge for 1 min at 1000g.
- Add 200 μ L of quenching buffer supplemented with 3 μ L of sodium cyanoborohydride. Incubate 15 min on a rotator.
- Put each column on a collection microcentrifuge tube, spin for 1min at 1000g and then remove the flow through.
- Wash 2 times the resin with 200 μ L of coupling buffer and centrifuge for 1min at 1000g.
- Wash 6 times the resin with 150 μ L of wash solution.

Co-IP step:

- Dilute the protein sample with one volume of homogenization buffer with no detergent. Final volume should be between 100 to 500 μ L.

- Wash the resin twice by adding 200 μ L of homogenization buffer to the spin column containing the antibody-coupled resin, centrifuge and discard the flow-through.
- Add 0.5-2 mg protein extract to 50 μ L of antibody-coupled resin.
- Incubate overnight on a rotator at 4°C.
- Spin for 1min at 1000g.
- Add 200 μ L of coupling buffer and centrifuge.
- Wash 2 times with coupling buffer.
- Elute proteins with 35 μ L with 2X SDS loading buffer.
- Run the eluted proteins on a SDS-PAGE gel and analyze by Western blot.

Co-immunoprecipitation was successfully used to demonstrate constitutive interaction between distinct LGICs or between LGIC and GPCR (Boue-Grabot et al., 2003; Boue-Grabot et al., 2004a; Jo et al., 2011; Lee et al., 2002; Liu et al., 2000) as well as for transient interactions such as agonist-dependent interactions between GABA_A and Dopamine D1 receptors. In this latter case, Co-IP was observed from brain hippocampus lysates, whereas in transfected HEK293 cells co-precipitation was only observed when both agonists were added to the lysate indicating that physical coupling required activation of both receptor types (Liu et al., 2000).

2.4 Affinity Purification using Tagged-Protein Methods

When specific antibodies directed against the receptors are not available, a very useful strategy consists in using epitope tagged-receptors expressed in heterologous systems and performing co-affinity purification using an immobilized affinity ligand specific for the corresponding tag. Popular tags such as hexa-His (6His), Flag, HA or c-Myc were often used to perform Co-IP experiments. 6His tag displays a high affinity for Nickel and Nickel-coupled resin were extensively used to purify 6His tagged-proteins and to detect interactions between receptors (Boue-Grabot et al., 2003; Le et al., 1999; Torres et al., 1999). Moreover, anti-6His antibodies enhance the system by providing sensitive detection of His-tagged receptors. The Flag, c-Myc or HA epitopes were also extensively used since monoclonal antibodies can be covalently immobilized agarose resin. Anti-Flag antibodies coupled to a resin for purification are commercially available (Sigma). In addition, such short tag fused to the NH₂ or COOH termini of a receptor subunit generally do not affect its functional properties or interaction with partners.

2.4.1 Affinity Purification Using 6His-Tagged Receptor and Nickel Resin.

Elution buffer:

Imidazole 500 mM

10 mM Hepes

0.3M sucrose, pH 7.4

Nickel Resin preparation:

- Shake the bottle containing the resin and pipet 50 μ L of resin per condition (i.e 25 μ L of dry resin).
- Centrifuge for 4 min at 2,000g. Discard the supernatant.
- Wash with 250 μ L of distilled water.
- Centrifuge for 1 min at 2,000g. Discard the supernatant and repeat step 4 one more time.
- Add 250 μ L of equilibrium buffer.
- Centrifuge for 4 min at 2,000g. Discard the supernatant and repeat step 6 one more time.
- Add 1 volume of equilibrium buffer and divide the resin beads in clean microcentrifuge tubes.

Affinity purification step:

- Add solubilized proteins (150-400 μ L) with one volume of homogenization buffer without detergent to the resin beads and place under agitation overnight at 4°C.
- Centrifuge for 4 min at 2,000g and discard the supernatant.
- Wash the resin beads with 500 μ L of equilibrium buffer supplemented with 20 to 50 mM Imidazole (starting at 20 mM) to reduce non specific binding.
- Centrifuge for 4 min at 2,000g and discard the supernatant.

- Repeat wash step three times.
- Elute proteins with 30 μL of elution buffer by flicking the tubes for 10 min at room temperature.
- Run the eluted proteins on a SDS-PAGE gel and analyze by Western blot.

2.4.2 Affinity Purification Using Flag-Tagged Receptor

Flag affinity Resin preparation:

- Shake the bottle containing the resin to make a uniform suspension and immediately transfer the total volume of resin for all conditions to a clean microcentrifuge tube.
- Transfer the required Flag-antibody-coupled agarose beads into a microcentrifuge tube (25 μL per immunopurification) and pellet the beads by centrifugation for 10 seconds at 13,000g.
- Remove the supernatant and wash the beads twice with the cell homogenization buffer.
- Resuspend the beads in 10 volumes of the homogenization buffer and dispense 250 μL (containing 25 μL of beads) into microcentrifuge tubes. Centrifuge briefly the tubes to pellet the beads and remove excess buffer.
- Add 200 μL to 500 μL of cell lysate with one volume of homogenization buffer without detergent to the Flag antibody-agarose beads and incubate on a rotating wheel for 1 hour or overnight at 4°C.
- Pellet the beads by centrifugation for 10 seconds at 13,000g and remove the supernatant.
- Wash the beads five times with 1 mL of freshly made homogenization buffer and after the final wash, carefully remove all traces of the supernatant.
- Resuspend the beads in 40 μL of 100 mM Glycine (pH 3.0) to elute the proteins.
- Transfer the eluate to a clean microcentrifuge tube and neutralize the final eluate with 4 μL of 1 M Tris-HCl (pH 8.0).
- Alternatively, elution can be performed Elute proteins with 40 μL of SDS loading buffer 2X.
- Run the eluted proteins on a SDS-PAGE gel and analyze by Western blot.

2.5 Pull-Down Assay

Pull-down assay is an *in vitro* method used to determine a physical interaction between two or more proteins. Pull-down assays are useful for both confirming the existence of a receptor-receptor interaction predicted by other research techniques (e.g., co-immunoprecipitation) and for identifying receptor domains of interaction (Jo et al., 2011; Lee et al., 2002; Liu et al., 2000). Pull-down assays are a form of affinity purification and are very similar to immunoprecipitation except that a "bait" protein is used instead of an antibody. In short, a tagged bait polypeptide is captured on an immobilized affinity ligand specific for the tag, thereby generating a support for purifying proteins that interact with the bait protein.

2.5.1 GST Fusion Protein Pull-Down Technique

GST-fusion proteins are used as probes for the identification of unknown receptor-receptor interactions or to confirm such suspected interactions. The GST-pull down technique is based on affinity capture of the receptor complex in solution by its interaction with the GST-fusion protein previously immobilized on glutathione-agarose beads. The "target" proteins are subsequently isolated using reducing buffers or competitive elution before being analyzed by Western blots.

Glutathione-S-transferase (GST) is a 26 kDa protein with a relatively high affinity for reduced glutathione. It can be easily fused to a probe protein such as it becomes a GST fusion protein used as a bait to identify unknown interacting proteins. The production of GST fused proteins starts with the cloning of the appropriate gene or gene fragment into an isopropyl- β -D-thiogalactoside (IPTG)-inducible expression vector which contains a coding GST sequence. *Escherichia Coli* host cells are transformed with recombinant DNA and GST fusion proteins are purified by affinity chromatography on glutathione-agarose beads. The cell or brain lysates usually containing the unidentified putative interacting receptor are incubated with the immobilized GST fusion protein as well as the GST alone for a negative control.

- Incubate cell lysate or whole brain extracts with previously immobilized GST-fusion protein or immobilized GST alone as a negative control for 2 hours at 4°C in a mixing device.
- Centrifuge the tubes and discard the supernatant (or save it for further analysis of the non-binding component).

- Wash the beads with ice-cold PBS containing 1% Triton X-100 for unbound proteins, and discard supernatants.
- For the elution step, using a reducing agent such as the Sodium-Dodecyl Sulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer will allow the elution of the entire protein-protein complex. However, the denaturing agent will also allow the collection of proteins that are non-specifically bound to the glutathione-agarose beads and thereof interfere with the latter analysis. A much more specific technique would be to use reduced glutathione as a competitive agent which should not elute any proteins that are non-specifically bound to the glutathione-agarose beads. Moreover, this method is non-denaturing, allowing the elution of a whole functional protein complex.
- Run the eluted proteins on a SDS-PAGE gel and analyze by Western blot

2.5.2 Pull-Down Technique Using a Biotinylated Peptide

In such a pull-down assay, a biotinylated bait peptide is captured on an immobilized streptavidin thereby generating an affinity support for purifying proteins interacting with the bait peptide. The main advantage of this method is the use of a synthetic peptide that eliminate time-consuming steps such as plasmid construction and production of fusion proteins in bacteria. In a typical pull-down assay, the immobilized bait peptide is incubated with a cell lysate, and after the prescribed washing steps, the complexes are selectively eluted using reducing buffers for Western blot analysis. The protocol described below using a kit Pull-down biotinylated protein-protein interaction from Pierce was adapted to identify interaction domains involved in the physical coupling between P2X₄ and GABA_A receptors (Jo et al., 2011).

TBS buffer:

50 mM Tris.HCl,
150 mM NaCl, pH 7.4

Immobilized Streptavidin preparation:

- Label enough spin columns to include sample(s), a non-treated gel control and an immobilized bait control.
- Thoroughly resuspend the Immobilized Streptavidin by inverting the tube several times. Pipette 40 µL of the gel slurry per sample. To facilitate slurry transfer, cut off 2-3 mm from the narrow tip of a standard 200 µL disposable pipette tip, and put in a dolphin tube.
- Centrifuge for 30 sec at 1,000g. Take the supernatant and keep a measure of the volume that will be useful for the last step of the protocol.
- Wash the resin 4 times with 200 µL per sample.
- Centrifuge for 30 sec at 1,250g.
- Dilute biotinylated peptide at 1 µg/µL in distilled water.
- Add 10 µL of the diluted peptide per condition and 190 µL of TBS on the resin. Incubate the mix for 3 hours on a gentle rocking platform.
- Centrifuge 30 sec at 1,250g.
- Add the volume measured in step 3, mix the resin and put 40 µL on each column provided in the kit.

Biotin blocking:

- Add 250 µL of Biotin Blocking Solution to each spin column. Secure the top screw caps onto the columns and mix by gently inverting the columns 3-5 times.
- Incubate the spin columns at room temperature for 5 min. Remove the top screw caps from the columns, place columns in collection tubes and centrifuge at 1,250g for 30-60 sec.
- Repeat steps 1 and 2 once.
- Add 250 µL of the TBS to each spin cup. Place the top screw caps on the columns and mix by gently inverting the column 3-5 times.

Note: The TBS may be replaced for column rinsing and pre-equilibration if an alternate Bait/Prey binding buffer is formulated for a particular interaction.

- Remove top screw caps from columns. Place columns in collection tubes and centrifuge at 1,250g for 30-60 seconds.
- Repeat steps 3 and 4 two additional times. Insert bottom plugs to the columns.

Pull-down steps:

- Protein extracts (500 µg) are incubated with immobilized streptavidin bound to the indicated biotinylated peptides overnight at 4 °C.
- After incubation, remove top cap from the spin columns, then remove the bottom plug from the columns and place in the pre-labeled tubes.

Note: If subsequent analysis indicates excessive flow through of lysate protein, the protein can be diluted in the TBS or a pre-determined binding buffer for the next time the assay is performed.

- Spin columns at 1,250g for 60 sec.
- Remove spin columns from the collection tubes (from step B), secure the caps and set the tubes aside for later evaluation. Place the spin columns in separate collection tubes for perform washing steps.
- Add 250 µL of TBS to each spin column. Secure the top screw caps onto the columns and mix by gently inverting the columns 5-7 times.
- Incubate spin columns for 1 min at room temperature. Remove the top screw caps from the columns, place columns in “waste” collection tubes and centrifuge at 1,250g for 30-60 sec.
- Repeat steps 1 and 2 three additional times.
- After the washing steps, place spin columns in separate collection tubes.
- Add 40 µL of 2X SDS loading buffer and leave at room temperature for 10 min.
- Centrifuge for 30 sec at 1,250g.
- Run the eluted proteins on a SDS-PAGE gel and analyze by Western blot.

The GST-fusion protein or peptide-based purification has been a powerful tool not only to identify unknown receptor-receptor physical interactions but also to precisely narrow it to their interacting domains and subunit specificity (Jo et al., 2011; Lee et al., 2002; Liu et al., 2000; Toulme et al., 2007). However, such a method implies a strong physical interaction between both partners. Weak or transient interactions can be identified using these methods by first covalently crosslinking the receptors to freeze the interaction during the Co-IP or pull-down.

2.6 SDS-PAGE and Western Blot Analysis

Immuno- or affinity-precipitated receptors (or domains) and their binding receptors are commonly detected by denaturing polyacrylamide gel SDS-PAGE and Western blot analysis. After gel separation and protein transfer from the gel to a membrane, associated receptor subunits are identified by Western blot using specific antibodies. It is critical to confirm that the detection of an interactor is a true physiological interaction and do not result from non-specific binding. Thus, all control experiments should be included to verify that i) the starting lysate contains both bait and prey ii) the bait protein is immunoprecipitated, iii) the uncoupled resin or irrelevant antibodies do not show signal, iv) the cells or tissue non-expressing the bait do not give signal; v) the identified partner can reciprocally precipitate the bait protein.

SDS-PAGE:

Lower buffer:

1.5 M Tris-HCl (pH 8.8)

0.4% SDS

Upper buffer:

1M Tris-HCl (pH 6.8)

0.4% SDS

Running buffer:

25mM Trizma base

192mM Glycine

0.1% SDS, pH8.3

Transfer Buffer:

25mM Trizma base
192 mM Glycine, pH 8.3

4X SDS loading buffer:
100 mM Tris-HCl pH 6.8
30% Glycerol
4% SDS
0.2% Bromophenol Blue

PBS buffer:
137 mM NaCl
2.7 mM KCl
10 mM Na₂HPO₄
2 mM KH₂PO₄

The percentage of polyacrylamide used in the resolving gel will be defined according to the size of the proteins of interest. Usually, 8- 10% polyacrylamide gels are used for the detection of LGIC subunits on SDS-PAGE.

- Assemble the vertical polyacrylamide gel casting chamber. Use 1 mm spacers between clean glass plates.
 - To cast two 10% (w/v) polyacrylamide resolving gels, mix 5.3 mL of 30% (w/v) acrylamide/bis-acrylamide, 4 mL of lower buffer solution and 6.7 mL of distilled water. Add 100 µL of a 10% APS solution and 25 µL TEMED. Pour immediately in the casting chamber leaving 2 cm of the upper part for the stacking gel. Overlay with a solution of 2-propanol to obtain an even gel surface. Allow the gel to polymerize for approximately 30 min and decant or aspirate the 2-propanol phase. Wash two times with distilled water.
 - To cast two stacking gels, mix 1.5 mL of 30% (w/v) acrylamide/bis-acrylamide, 2.5 mL of upper buffer solution and 6 mL of distilled water. Add 50 µL of a 10% APS solution and 20 µL TEMED. Pour immediately and place in position a ten-tooth (each tooth creating a well easily able to accommodate a sample volume of 30 µL) 1.5 mm comb. Allow the gel to polymerize for approximately 30 min. After polymerization, carefully pull the comb up and rinse the well with 1X running buffer.
- Use the gel immediately or store it moistened for 2-3 days at 4°C.
- Assemble the gel running chamber. Fill the inner and the outer chamber with 1X running buffer.
 - Load 20 µL of previous sample into one well, and ensure that one well is filled with a molecular weight protein marker for immunoblotting.
 - Perform electrophoresis at a current of 150V for 1hour until the Bromophenol Blue front has reached the bottom of the gel.
 - Disassemble the running chamber, carefully lift the gel from the glass plate and place it into a plastic dish.

Western Blotting:

- Cut a piece of nitrocellulose membrane at the dimensions of the polyacrylamide gel.
- Incubate the membrane and the gel in blotting buffer for 10 min.
- Place the gel on the membrane between two pieces of blotting paper soaked with blotting buffer and blot for 30 min à 100V.
- Wash one time in PBS-Tween 0.1% (PBST).
- Incubate in 5 % (w/v) skim milk solution for 1hour at room temperature to block non specific sites.
- Prepare solutions of primary antibody (diluted as recommended by the supplier, usually 1:100-1:10000) in PBST.
- Discard the skim milk and add the primary antibody solution. Gently shake overnight at 4°C.
- Discard the primary antibody solution and rinse two times quickly follow by three washes for 10 min each using PBST.

- Add the secondary antibody in PBST supplemented with 5% (w/v) skim milk powder and gently shake for 1 hour at room temperature. Discard the secondary antibody solution and rinse two times quickly followed by three washes for 10 min each using PBST.
- Reveal the membranes either by chemiluminescence and autoradiographic films or directly from the membranes using a scanner or imager such as Odyssey (Stratagene) when Horseradish peroxidase (HRP) coupled or infra-red dye-coupled secondary antibodies are used, respectively.

3 Fluorescence-based and Functional Methods for Assessing Receptor-Receptor Interactions

3.1 Fluorescence Resonance Energy Transfer

Unlike conventional biochemical methods, the use of differentially labeled receptors in Fluorescence Resonance Energy Transfer (FRET) analysis allows the elucidation of cell surface receptor interaction in living cells (Khakh et al., 2005; Shrivastava et al., 2011). FRET is based on a principle in which a fluorophore, called donor, in an excited state transfers energy to a neighboring fluorophore, namely acceptor, through dipole-dipole interaction.

Changes in FRET efficiency depend on the proximity of the donor and acceptor fluorophores, in fact energy transfer occurs over distances of 1 to 10 nm. During FRET, the donor signal will be quenched, meanwhile the acceptor signal will be sensitized or increased.

In order to proceed to FRET measurements, the proteins of interest are fused in frame respectively to two different compatible fluorescent proteins such as Cyan Fluorescent protein (CFP) and Yellow Fluorescent protein (YFP) (see (Sekar & Periasamy, 2003) for several of the different fluorophore combinations) and transfected into the heterologous system of interest. FRET microscopy system requires first of all a stable excitation light source (Mercury or Xenon lamp, UV, visible or infrared lasers) as well as some appropriate filters sets (excitation, emission and dichroic) for the selected fluorophore pair. The dichroic filter will allow simultaneous monitoring of spectral emissions at two wavelengths. It must reflect the excitation wavelength to excite the donor and transmit the donor and acceptor emission bands.

Most of FRET measurement analyses are based on the “three-filter method” (Xia & Liu, 2001) that allows a reduced spectral bleed-through to improve signal-to-noise ratio. In brief, a set of 3 images of a same field are taken from the donor, the acceptor and the FRET filter sets. Each image is then subtracted for background before its fluorescence intensity being analyzed using an appropriate software. Image analysis can be performed for example with Image J (NIH, <http://rsb.info.nih.gov/ij/>). The normalized FRET signal (N_{FRET}) is calculated using the following formula: $N_{FRET} = (I_{FRET} - BT_{donor} \times I_{donor} - BT_{acceptor} \times I_{acceptor}) / (I_{donor} \times I_{acceptor})^{1/2}$, where BT indicates bleed-through and I indicates intensity (Bunt & Wouters, 2004).

The advantages of such a method are multiple since most of the time, proteins of interest fused to a fluorescent protein retain their physiological functions and subcellular targeting, FRET is a powerful tool for investigating receptor-receptor interactions in living cells or fixed cells (Khakh et al., 2005; Shrivastava et al., 2011). Moreover, it has a nanometer depth resolution and is a non-intrusive technique as opposed to the previous biochemical methods described in this chapter. However, it should be noted that FRET measurements are affected by a number of disadvantages since cross-talks between donor fluorescence and acceptor fluorescence can occur as well as between FRET and non-FRET fluorescence emitted from donor and/or acceptor proteins. Since FRET is based on fluorescence proteins, it is also dependent on photobleaching/quenching properties of fluorescent proteins, as well as on molecular position/orientation of the proteins which could add a bias to the measurement. It should also be noted that irreversible modifications of the sample follows its excitation (Michalet et al., 2005).

3.2 Functional Analysis of Receptor-Receptor Interactions *In Vitro*

The function of ligand-gated ion channels is commonly analyzed by electrophysiological voltage-clamp recordings in the whole cell configuration from cells expressing both receptors. From a functional point of view, when two ligand-gated ion channels are independent, the co-application of their respective agonist evoke an inward current corresponding to the sum of the individual currents evoked by application of each agonist. On the contrary, two receptors do not

function independently when co-application of both agonists evoke a current different from the sum of the individual responses. Cross-talk between LGIC reported to date lead to an instantaneous and transient current inhibition of one or both receptors only during their co-activation. (Barajas-Lopez et al., 1998; Barajas-Lopez et al., 2002; Boue-Grabot et al., 2003; Boue-Grabot et al., 2004a; Boue-Grabot et al., 2004b; Jo et al., 2011; Khakh et al., 2000; Nakazawa, 1994; Searl et al., 1998; Sokolova et al., 2001; Toulme et al., 2007; Zhou & Galligan, 1998). Such physical interaction may have also important consequences on the trafficking of both receptors as demonstrated for P2X and GABA_A receptors or between NMDA and D1 receptors (Boue-Grabot et al., 2004a; Jo et al., 2011; Lee et al., 2002; Shrivastava et al., 2011)

Once the two receptors have been shown to function not independently and that a physical coupling is confirmed using biochemical or non-biochemical methods, the next step is to narrow down the interplay between the two proteins to the molecular level. Therefore, the identification of crucial and sufficient domains and/or amino acids involved in the crosstalk allows for a more precise way of modulating the interaction.

To determine which domains of the receptors are involved in the interaction, the strategy consist on suppressing or modifying domains of receptors using molecular biology techniques and then analyze its impact on either the function by electrophysiology techniques, the trafficking by imaging approaches or the physical coupling by biochemical approaches from cells co-expressing a modified receptor with the wild-type interacting receptor and *vice versa*.

Using molecular biology techniques derived from the Quickchange mutagenesis technique (Stratagene), it is now fast and easy to modify plasmid encoding receptor subunits and to perform deletion, substitution of a specific domain or to mutate specific aminoacids. Such constructs are then analyzed for their functionality in a heterologous expression system that can either be injected *Xenopus* oocytes or transfected mammalian cells by whole-cell patch-clamp electrophysiological techniques. Using these approaches, we identified specific intracellular domains and/or residues required for the interaction between several P2X receptors and 5-HT₃ or GABA receptors (Boue-Grabot et al., 2003; Boue-Grabot et al., 2004a; Boue-Grabot et al., 2004b; Jo et al., 2011; Toulme et al., 2007) by showing that modification or mutation of these domains lead to additive responses during co-activation of both receptor types.

3.3 *In Situ* or *In Vivo* analysis of Receptor-Receptor Interactions

To assess the functional impact of receptor-receptor interaction *in situ*, the strategy consists on expressing one or the other identified interacting domain between both receptors. This polypeptide by mimicking the interaction domain will compete with endogenous receptors and thus, act as a competitive inhibitor preventing the physical association between targeted receptors in native systems such as primary culture of neurons or brain slices.

The interacting domain can be generated as a GST-fusion protein or a synthetic peptide. In both cases, the polypeptide need to be directly delivered into the cell. Since LGIC function is mainly analyzed by electrophysiological techniques, the polypeptide can be included into the patch pipette and diffuses into the cytoplasm of the cell through the dialysis of the patch pipette internal solution under whole cell patch clamp configuration.

Competitors can be also delivered into cultures of neurons by viral infection of construction encoding the polypeptide of interest such as recombinant Semliki Forest virus or adenovirus. Using these approaches, physical interaction between receptors in native neurons was demonstrated as well as their role in the regulation of the synaptic transmission. (Boue-Grabot et al., 2003; Lee et al., 2002; Liu et al., 2000; Toulme et al., 2007).

This method can be also adapted to analyze consequences of the loss of interaction between receptors in a specific structure of the brain *in vivo* by behavioral studies. In this case, either a synthetic peptide fused to the cell membrane transduction domain of the human immunodeficiency virus 1 (i.e. the TAT signal) to gain cell permeability or a viral construction encoding the interacting domain will be stereotactically injected using a canulae implented directly into the structure of interest of anesthetized animals. Injection of a TAT-peptide encoding the domain of interaction between dopamine D1 receptor and PSD-95 in the striatum of rat model of L-dopa induced dyskinesia (LID), demonstrated that disrupting the interaction between D1 receptor and PSD-95 reduces dyskinesia development and severity (Porrás et al., 2012).

4 Conclusion Remarks

While biochemical methodology described above is straightforward, identifying receptor-receptor interaction can be difficult and methods should be chosen or adapted depending on the nature of the interaction, non-specific binding or antibodies contamination. The critical steps to maintain physiological interactions are the one involving lysis and washing buffers. We described several lysis and wash buffer composition with relatively low ionic strength (100-150 mM NaCl) and non-ionic detergent such as Triton X-100 that has been successfully used to reveal receptor-receptor interactions, however several parameters such as the salt concentration, the percentage of detergent as well as the amount of primary antibody should be optimized for specific interactions.

It is also important to note that even if these biochemical methods show that both receptors belong to the same protein complexes, such methods do not discriminate between direct or indirect interactions. Indeed, interacting or accessory proteins that are in the lysate may contribute to the receptor-receptor interplay. Overlay or Far Western techniques using a labeled or antibody-detectable "bait" domain to probe and detect a target "prey" domain on a membrane could theoretically answer this question as shown between GABA_A and D5 receptors (Liu et al., 2000). However, these methods are performed in denaturing conditions and limit the detection of interactions insensitive to the loss of the native conformation.

It is now obvious that a combination of biochemical and functional approaches is required to demonstrate the existence of receptor-receptor interactions. In addition to these classical methods, the emergence of new technologies such as surface plasmon resonance (SPR) or super-resolution imaging represents sensitive and dynamic methods that should facilitate in the close future the characterization and visualization of the interactions between distinct membrane receptors.

Appendix

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