INTERACTION OF GABA<sub>A</sub> RECEPTORS WITH PURINERGIC P2X<sub>2</sub> RECEPTORS

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For Obtaining the Academic Degree

Doctor of Philosophy

Submitted by

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Dedicated to my parents and sisters
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1. ABSTRACT (ENGLISH)

GABA_ARs in the spinal cord are evolving as an important target for drug development against pain. Purinergic P2X_2Rs are also expressed in spinal cord neurons and are known to cross-talk with GABA_ARs. Here we investigated a possible “dynamic” interaction between GABA_ARs and P2X_2Rs using co-immunoprecipitation and FRET studies in HEK cells along with co-localization and single particle tracking studies in spinal cord neurons. Our results suggest that a significant proportion of P2X_2Rs forms a transient complex with GABA_ARs inside the cell, thus stabilizing these receptors and using them for co-trafficking to the cell surface. P2X_2Rs and GABA_ARs are then co-inserted into the cell membrane and are primarily located extra-synaptically. Furthermore, agonist induced activation of P2X_2Rs results in disassembly of the receptor complex and destabilization of GABA_ARs whereas P2X_2Rs are stabilized and form larger clusters. Antagonist-induced blocking of P2XR results in co-stabilization of this receptor complex at the cell surface. These results suggest a novel mechanism where association of P2XR with other receptors could be used for specific targeting to the neuronal membrane, thus providing an extrasynaptic receptor reserve that could regulate the excitability of neurons. We further conclude that blocking the excitatory activity of excessively released ATP under diseased state by P2XR antagonists could simultaneously enhance synaptic inhibition mediated by GABA_ARs.
2. ABSTRACT (GERMAN)

3. INTRODUCTION

3.1. GABA<sub>A</sub> RECEPTORS

GABA<sub>A</sub> receptors (GABA<sub>A</sub>Rs) are the major inhibitory transmitter receptors in the central nervous system and the site of action of benzodiazepines, barbiturates, neuroactive steroids, anesthetics and convulsants. They are ligand gated chloride channels composed of five subunits that can belong to different subunit classes. Each subunit has a large N-terminal extracellular domain and four transmembrane domains (TMs) and a large intracellular loop between TM3 and TM4. Though many possible subunit compositions have been demonstrated, the majority of these receptors is composed of one γ, 2α and 2β subunits (Fig. 1) (Sieghart, 1995; Olsen and Sieghart, 2008, 2009).

![Fig 1. Structure of GABA<sub>A</sub> receptors (Jacob et al., 2008)](image)

The diverse functions of GABA<sub>A</sub>Rs are not only mediated by the heterogeneity of the receptors but also by complex trafficking mechanism and protein-protein interactions that regulate and maintain receptor cell surface localization (Fig. 2) (Jacob et al., 2008). Several such proteins, such as gephyrin (Jacob et al., 2005), radixin (Loebrich et al., 2006), PLIC1 (Bedford et al., 2001), GABARAP (Wang et al., 1999) and many others, are now known to directly interact with GABA<sub>A</sub>Rs and to regulate their function. Of these proteins gephyrin is known to be primarily involved in regulating clustering of GABA<sub>A</sub>Rs at synapses. Gephyrin was first identified to be directly associated with glycine receptor’s
intracellular domain thus stabilizing them at inhibitory synapses in spinal cord (Pfeiffer et al., 1982; Meyer et al., 1995). Recent studies on GABA\(\text{A}\)Rs have

Fig 2. GABA\(\text{A}\) receptor trafficking and associated proteins (Jacob et al., 2008)
demonstrated that gephyrin is also one of the key molecules regulating clustering and synaptic stabilization of these receptors at synapses (Tretter et al., 2008).

The complex interactome of GABA\textsubscript{A}Rs suggests a highly dynamic nature of these receptors. The number of synaptic receptors are determined by exchange between synaptic and extrasynaptic receptors by lateral diffusion (Choquet and Triller, 2003; Triller and Choquet, 2005, 2008). The membrane dynamics of surface receptors is further dependent on interaction with scaffold proteins, adhesion molecules and other resident proteins. In contrast, the number of surface receptors is determined by their rate of recycling between surface and intracellular receptor pool as well as by receptor turnover and trafficking through the early secretory pathway (Fig. 2).

3.2. GABA\textsubscript{A} RECEPTOR INTERACTION WITH OTHER NEUROTRANSMITTER RECEPTORS

Recently, evidence has accumulated indicating that GABA\textsubscript{A}Rs are also able to complex selectively with other receptors by direct protein-protein interaction and that this physical association enables mutually inhibitory functional interaction between these receptor systems. Such interactions have been demonstrated for GABA\textsubscript{A}Rs and dopamine D5 receptors (Liu et al., 2000) as well as for rho1-GABARs and P2X\textsubscript{2} (Boué-Grabot et al., 2004a), GABA\textsubscript{A}Rs and P2X\textsubscript{2}Rs (Sokolova et al., 2001; Boué-Grabot et al., 2004b), GABA\textsubscript{A}Rs and GABA\textsubscript{B}Rs (Balasubramanian et al., 2004). Not only GABA\textsubscript{A}Rs, but also several different other neurotransmitter receptors seem to interact with other receptors. However, the mechanism behind such interactions has been studied only for a few interactions, namely between dopamine D1 and NMDA (Lee et al., 2002, Pei et al., 2004) and dopamine D\textsubscript{2} and AMPA receptors (Zou et al., 2004), but never investigated for pentameric channels such as GABA\textsubscript{A}Rs or nicotinic receptors. Endocytosis of the receptors, direct coupling of the receptors, and coupling possibly mediated through a protein complex are some of the proposed mechanisms governing such interactions.

3.3. P2X\textsubscript{2} RECEPTORS

P2X receptors are ATP gated ion channels that are permeable to Ca\textsuperscript{2+}, Na\textsuperscript{+}, and K\textsuperscript{+}. The receptors are trimeric in structure with each subunit having two transmembrane domains, separated by an extracellular domain (approximately 280 amino acids) (Fig. 3). The P2X receptor super family comprises seven different subunits (P2X\textsubscript{1}-P2X\textsubscript{7}) which are 40-50% identical in their amino acid
sequence (Burnstock and Knight, 2004). One of the important and abundant subunits of the P2X receptor family is P2X$_2$, that mainly forms homo-trimeric P2X$_2$Rs but also assembles with P2X$_3$ subunits to form hetero-trimeric P2X$_{2/3}$ receptors (P2X$_{2/3}$Rs) (Gever et al., 2006). Among the various subtypes, P2X$_2$R and P2X$_3$R are enriched in spinal cord (Collo G et al., 1996; Wirkner et al., 2007), where they play a role in sensory transmission and modulation of synaptic function.

Fig 3. P2X receptor subunit (Wikipedia)

P2X$_2$Rs have been demonstrated previously to be localized in excitatory synapses of the brain. They are especially localized at the periphery of postsynaptic densities where AMPA receptor subunits are present at low density (Rubio and Soto, 2001). It is presumed that the location of P2XRs to the periphery of postsynaptic densities arises from specific protein interactions: one such protein that co-localizes and interacts with P2X$_2$Rs in postsynaptic membranes is Fe65 (Masin et al., 2006), better known as a partner to ß-amyloid precursor protein. Possibly the number of synaptic P2X channels depends critically on the rates of insertion and removal by regulated trafficking (Khakh and North, 2006).

Even though the reported distribution of P2X$_2$Rs and GABA$_A$Rs suggests a non-overlapping function of these two receptor proteins in brain, these two receptors have been demonstrated to be co-localized and to functionally interact with each other in the spinal cord dorsal root ganglion (Labrakakis et al., 2003; Sokolova et al., 2001). Simultaneous activation of GABA$_A$Rs and P2X$_2$Rs resulted in non-additive current or “cross-talk” of the receptors (Sokolova et al., 2001; Boué-Grabot et al., 2004b;
Karanjia et al., 2006). Interestingly, a similar cross-talk has been observed between P2X$_2$Rs and other members of the cys-loop receptor family (Köles et al., 2008 and references therein).

In this study, we investigated a possible interaction of P2X$_2$Rs with GABA$_A$Rs. We demonstrate that these two receptors interact with each other and that the interaction regulates the trafficking and membrane dynamics of GABA$_A$Rs.
4. MATERIALS AND METHODS

4.1. CULTURE OF HEK CELLS FOR TRANSFECTION

Human Embryonic Kidney cells (also called HEK cells, HEK 293 or 293 cells) are an epithelial cell line generated by transformation of normal human embryonic kidney cell cultures with adenovirus 5 DNA in the laboratory of Frank Graham in the late 1970s. These cells are easy to work with, and thus are widely-used in cell biology research.

4.1.1. REQUIREMENTS

A. Culture Media

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Stock Solution</th>
<th>Working Solution</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM (Dulbecco’s Modified Eagle Medium)</td>
<td>--</td>
<td>--</td>
<td>500ml</td>
</tr>
<tr>
<td>FBS (Fetal Bovine Serum)</td>
<td>--</td>
<td>--</td>
<td>50ml</td>
</tr>
<tr>
<td>Penicillin-Streptomycin Solution</td>
<td>1:1</td>
<td>--</td>
<td>5ml</td>
</tr>
<tr>
<td>L-Glutamine, 200mM</td>
<td>100X</td>
<td>1X</td>
<td>5ml</td>
</tr>
<tr>
<td>MEM (Non-Essential Amino Acids)</td>
<td>100X</td>
<td>1X</td>
<td>5ml</td>
</tr>
</tbody>
</table>

B. Trypsin-EDTA

C. Phosphate-Buffered Saline (PBS), 1X

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>140mM</td>
</tr>
<tr>
<td>KCl</td>
<td>2.7mM</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1.5mM</td>
</tr>
<tr>
<td>Na₂HPO₄.2H₂O</td>
<td>4.3mM</td>
</tr>
</tbody>
</table>

Adjust pH to 7.3, Autoclave & store at RT

4.1.2. PROTOCOL

A. Start the laminar flow at least 15 minutes before working.
B. Thaw all the culture media components in a 37°C water bath.
C. Prepare the culture media as per the recipe and leave it inside the hood.
D. Take a culture dish with highly confluent cells.
E. Remove the medium and rapidly wash once with 10 ml 1X PBS.
F. Add 1ml trypsin and leave it for 3-4 min until cells detach.
G. Mix the cells/trypsin suspension using a sterilized Pasteur pipette.
H. Stop the trypsin reaction by diluting with 10 ml culture media.
I. Mix well but gently using a pipette.
J. Transfer the contents to a falcon.
K. Count the cells and plate as follows:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Biochemistry</td>
<td>$2.1 \times 10^6$ cells/9cm dish</td>
</tr>
<tr>
<td>FRET</td>
<td>$0.15 \times 10^6$ cells/well in 6 well dish on top of 24mm cover slip</td>
</tr>
<tr>
<td>Live Imaging</td>
<td>$0.10 \times 10^6$ cells/well in 12 well dish on top of 12mm cover slip</td>
</tr>
</tbody>
</table>

L. Transfer the dishes into the incubator at 37°C with 5% CO_2.
M. Perform transfection 24 hours after plating.

4.2. TRANSFECTION OF HEK CELLS

HEK cells are transfected using calcium phosphate transfection method (Chen and Okayama, 1988). The principle involves mixing of DNA in a phosphate buffer with calcium chloride. The resulting calcium-phosphate-DNA complexes adhere to the cell membrane and enter the cytoplasm by endocytosis. The critical factor in the transfection using Ca$_3$(PO$_4$)$_2$ co-precipitation is the pH of the solutions. High transfection efficiencies can be obtained in some cell lines by generating a glycerol shock. It may be performed 4-16 hours after transfection; but the cells should not be left in glycerol for more than 2 minutes.

4.2.1. REQUIREMENTS

A. 1X PBS *(refer section 3.1.1)*

B. 2.5 M CaCl$_2$
C. HEBS (HEPES Buffered Saline) Buffer

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>2mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>280mM</td>
</tr>
<tr>
<td>HEPES</td>
<td>50mM</td>
</tr>
<tr>
<td></td>
<td>Adjust pH to 7.08,</td>
</tr>
<tr>
<td></td>
<td>Filter and sterilize. Store at -20°C</td>
</tr>
</tbody>
</table>

D. 15% Glycerol in 1XPBS

4.2.2. PROTOCOL (TRANSFECTION FOR BIOCHEMISTRY)

A. Measure the cell density for transfection. 2.0-2.8 X 10$^6$ cells per plate are transfected with 20µg DNA in 40µl volume per dish.

B. Take 500µl HEBS buffer in one 15ml falcon, and in other mix 50µl CaCl$_2$ with 40µl DNA mix and 410µl H$_2$O. Using Pasteur pipette transfer drop wise DNA-CaCl$_2$-H$_2$O mix to HEBS buffer while constantly bubbling latter using a pipette man. This ensures efficient mixing. The final volume is now 1000µl.

C. Vortex for a few seconds.

D. Leave for ~6 min at room temperature.

E. Invert twice, and then transfer 950µl DNA-CaCl$_2$-H$_2$O-HEBS mix evenly over culture dish.

F. Swivel the culture dish gently for uniform mixing, transfer it back to incubator.

G. Perform **glycerol-shock** four hours after transfection.
   - Remove culture media from transfected dish.
   - Add 1ml 15% glycerol solution from side for maximum of 1 min.
   - Gently add 10ml 1X PBS from side to wash off glycerol.
   - Remove the glycerol-PBS solution from dish.
   - Add 10ml pre-warmed culture medium and transfer the dish back to the incubator.

H. Perform the experiments 48 hours post transfection.
4.2.3. PROTOCOL (TRANSFECTION FOR FRET AND LIVE-CELL IMAGING)

A. Transfection is performed 20-24 hours after plating when cells are 60-80% confluent.

B. Dilute plasmid DNA to 1µg/µl.

C. Take 100µl HEBS buffer in a microfuge tube. In another tube, mix 86µl autoclaved water, 12µl CaCl₂ and 2µg plasmids.

D. Transfer drop wise DNA-CaCl₂-H₂O mix to HEBS buffer while tapping the bottom of the tube gently. This ensures efficient mixing of the complex.

E. Leave the mix at room temperature for 6 min.

F. Mix the content again and transfer 180µl/6-well dish or 90µl/12-well dish.

G. After 4 hours of transfection, remove the medium, wash once with 1X PBS and then add 3ml pre-warmed culture medium.

H. Transfer the culture dish back to the incubator. Experiments can be typically performed 24-48 hours after transfection.

4.3. RAT PRIMARY HIPPOCAMPAL NEURONS: CULTURE AND TRANSFECTION

Primary cultures of hippocampal neurons are prepared from the hippocampus of embryonic day 18 (E18) rats. At this age, the generation of pyramidal neurons, which begins in the rat at about E15, is essentially complete, but the generation of dentate granule cells, which largely occurs postnatally, scarcely has begun. The tissue is still easy to dissociate, the meninges are removed readily, and the number of glial cells still is relatively modest. The protocol is adapted from the Goslin, Assmussen & Banker chapter on “Rat hippocamal neurons in low-density culture” (Culturing Nerve Cells, 2nd edition; edited by Garry Banker & Kimberly Goslin).

4.3.1. REQUIREMENTS

A. The list of materials and equipments and suppliers are tabulated below:

<table>
<thead>
<tr>
<th>MATERIAL</th>
<th>CATALOG</th>
<th>SUPPLIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dumont #5 Forceps, Straight 11cm</td>
<td>11254-020</td>
<td>FST (Fine Science Tools)</td>
</tr>
<tr>
<td>Dumont #7 Forceps, Curved 11.5cm</td>
<td>11274-020</td>
<td></td>
</tr>
<tr>
<td>Westcott Spring Scissors, 11 cm</td>
<td>15015-11</td>
<td></td>
</tr>
<tr>
<td>Mayo Scissors, Straight, 15 cm</td>
<td>14010-15</td>
<td></td>
</tr>
<tr>
<td>Item</td>
<td>Number</td>
<td>Supplier</td>
</tr>
<tr>
<td>----------------------------------------------------------------------</td>
<td>---------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>Standard Pattern Forceps, 12 cm</td>
<td>11000-12</td>
<td></td>
</tr>
<tr>
<td>MEM-EARLES &amp; L-Glut</td>
<td>31095-029</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Horse Serum, Heat Inactivated</td>
<td>26050-088</td>
<td></td>
</tr>
<tr>
<td>Sodium Pyruvate MEM, 100 mM</td>
<td>11360-039</td>
<td></td>
</tr>
<tr>
<td>Neurobasal medium (1X)</td>
<td>21103-049</td>
<td></td>
</tr>
<tr>
<td>B27 Supplement (50X)</td>
<td>17504-044</td>
<td>MEM- EARLES &amp; Glut</td>
</tr>
<tr>
<td>HBSS w/o Ca(^{2+}) &amp; Mg(^{2+}) (10X)</td>
<td>14180-046</td>
<td></td>
</tr>
<tr>
<td>HEPES Buffer (1M)</td>
<td>15630-056</td>
<td></td>
</tr>
<tr>
<td>Trypsin (2.5%), 10X</td>
<td>15090-046</td>
<td></td>
</tr>
<tr>
<td>L-Glutamine, 200 mM (100X)</td>
<td>25030-024</td>
<td></td>
</tr>
<tr>
<td>Penicillin/Streptomycin</td>
<td>15140-122</td>
<td></td>
</tr>
<tr>
<td>Trypan Blue</td>
<td>15250-061</td>
<td></td>
</tr>
<tr>
<td>D(-) Glucose, 45% liquid</td>
<td>G8769</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Deoxyribonuclease I (Bovine)</td>
<td>DN25</td>
<td></td>
</tr>
<tr>
<td>Poly-L-Lysine Hydrobromide</td>
<td>P2636</td>
<td></td>
</tr>
<tr>
<td>Rat Neuron Nucleofector kit</td>
<td>VPG-1003</td>
<td>Amaxa</td>
</tr>
<tr>
<td>Cover slips</td>
<td>1943-10012</td>
<td>BELCO</td>
</tr>
<tr>
<td>Double Spatula Type Chattaway</td>
<td>3141</td>
<td>BOCHEM® LABORBEDARF</td>
</tr>
<tr>
<td>6cm Petri-dishes Dishes Nunclon™ Polystyrene with sterile lid</td>
<td>150288</td>
<td>Nunc</td>
</tr>
<tr>
<td>Thick glass dishes</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>(for dry heating cover slips)</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>Boric Acid</td>
<td>1.00165.1000</td>
<td>Merck</td>
</tr>
</tbody>
</table>

**B. Neuronal Plating Medium**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEM-EARLES &amp; L-Glut</td>
<td>500 ml</td>
</tr>
<tr>
<td>Horse Serum, Heat Inactivated</td>
<td>50 ml</td>
</tr>
<tr>
<td>Sodium Pyruvate MEM, 100 mM</td>
<td>5 ml</td>
</tr>
</tbody>
</table>
(Pyruvic Acid)

D-(+) Glucose, 45% liquid 6.6 ml

C. Neuronal Maintenance Medium

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurobasal medium (1X)</td>
<td>500 ml</td>
</tr>
<tr>
<td>L-Glutamine, 200mM (100X)</td>
<td>5 ml</td>
</tr>
<tr>
<td>Penicillin/Streptomycin</td>
<td>5 ml</td>
</tr>
<tr>
<td>D-(+) Glucose, 45% liquid</td>
<td>6.6 ml</td>
</tr>
<tr>
<td>B27 Supplement (50X)</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

D. Dissection Medium [1X CMF-HBSS (Ca$^{2+}$ and Mg$^{2+}$ free-HBSS)]

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X HBSS w/o Ca$^{2+}$ &amp; Mg$^{2+}$(10X)</td>
<td>50ml</td>
</tr>
<tr>
<td>HEPES Buffer (1M)</td>
<td>5ml</td>
</tr>
<tr>
<td>FINAL VOLUME with ddH$_2$O</td>
<td>500ml</td>
</tr>
</tbody>
</table>

E. Borate Buffer (0.1M) (for dissolving poly-L-lysine)

- Dissolve 0.62 g of Boric Acid (MW. 61.14) in 90 ml water.
- Adjust the pH to 8.0 with 5M NaOH.
- Add water to make it 100 ml.
- Autoclave before use.

F. Trypsin (2.5%)

- Aliquot 5ml in falcon and store at -20°C.

G. Poly-L-Lysine solution (PLL)

- Dissolve 100 mg of poly-L-lysine powder in 100 ml borate buffer.
- Filter in falcon using 0.20 µm syringe filter.
- Aliquot 5 ml in falcons and store at 4°C.
4.3.2. PROTOCOL

A. Sterilize the cover slips by heating at 160°C for 4 hours in a thick glass dish.
B. Keep the cover slips inside the laminar air flow (once sterilized, can be stored for few weeks).
C. Transfer sterilized cover slips on to a culture dish (6 cm dish/12-well dish).
D. Prepare working solution (0.5µg/ml) of PLL by diluting the stock (1µg/ml) with autoclaved water.
E. Coat the cover slips with PLL by covering the top with few drops of the working solution.
F. Leave it at room temperature inside the hood overnight.
G. Following day, wash the cover slips twice with autoclaved water; let them remain in water until the hippocampus is dissected and trypsinized.
H. Thaw one aliquot of the 2.5% trypsin on ice before beginning the preparation.
I. Dissect out hippocampus:
   (The whole process should ideally take ~2.5 hours for best preparation)
   - Kill the rat as per the guidelines.
   - Sterilize the abdomen with ethanol.
   - Cut out the upper skin.
   - Carefully cut the abdomen with a sterilized scissor.
   - Take out the embryos on a sterile dish placed over ice.
   - Carefully take out the small pubs and place them on ice.
   - Dissect the head and place over ice-cold 1X HBSS buffer.
   - Carefully remove the upper skin starting from nose, and take out the brain in fresh 1X HBSS buffer.
   - Now use the brains to dissect out hippocampus carefully in 1X HBSS buffer.
   - Transfer the dissected hippocampus to a falcon using a pipette.
J. Centrifuge at 1500 rpm for 2 minutes.
K. Discard supernatant and then add another 4.5 ml fresh cold 1X HBSS & 500µl 2.5 % Trypsin.
L. Incubate the cells in 37°C, CO₂ incubator for 15 minutes. This dissociates the cells.
M. Wash 3 times with cold buffer, re-suspend in 1 ml HBSS.
N. Use flame to reduce the diameter of a Pasteur pipette. Use this pipette to homogenize the trypsinized hippocampus. Avoid air bubbles.
O. Reduce the diameter of 2nd Pasteur pipette such that it just allows the flow of the suspension. Homogenize few more times.

P. Count the cells using a haemocytometer by diluting 5µl cell suspension with 40µl 1X HBSS and 5µl trypan-blue.

Q. Plating without Transfection:
   - Drain off the water from the dish having cover slips.
   - Add 5 ml of pre-warmed Neuronal plating medium per dish.
   - Plate 250,000 cells per 6 cm dish or 60,000 cells per 12-well dish.

R. Plating after transfection:
   - Transfection was performed using AMAXA nucleofactor kit.
   - Mix 2.5 X 10^6 cells with 3-4 µg plasmids DNA.
   - Add 100 µl of the transfection buffer.
   - Run Program 3 *(Suitable for rat neurons)*.
   - Transfer the transfected cell-suspension onto a 6 cm dish.

4.4. CULTURE AND TRANSFECTION OF MOUSE PRIMARY SPINAL CORD NEURONS

Spinal motor neurons are highly differentiated post-mitotic cells that normally do not enter mitosis in adults. The culture was performed using embryonic day 15 pubs from mRFP-gephyrin knock-in mice (Machado and Triller, unpublished). Whole spinal cord is dissected and dissociated out from the pups, so there is no separation between dorsal and ventral part.

4.4.1. REQUIREMENTS

A. Poly-L-Ornithine solution

B. Coating Medium

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEM</td>
<td>50ml</td>
</tr>
<tr>
<td>Fetal Calf Serum</td>
<td>2.5ml</td>
</tr>
<tr>
<td>Glutamine (200mM)</td>
<td>0.5ml</td>
</tr>
<tr>
<td>Sodium Pyruvate (100mM)</td>
<td>0.5ml</td>
</tr>
</tbody>
</table>
### C. HBSS-Glucose Medium

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autoclaved water</td>
<td>30ml</td>
</tr>
<tr>
<td>Glucose (20X)</td>
<td>1.5ml</td>
</tr>
<tr>
<td>HBSS (10X)</td>
<td>3ml</td>
</tr>
<tr>
<td>HEPES (1M)</td>
<td>0.6ml</td>
</tr>
</tbody>
</table>

### D. Neurobasal Complete Medium

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurobasal</td>
<td>50ml</td>
</tr>
<tr>
<td>B27 (50X)</td>
<td>1ml</td>
</tr>
<tr>
<td>Glutamine (200mM)</td>
<td>0.5ml</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>25μl</td>
</tr>
</tbody>
</table>

### E. 20X PBS-Glucose

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>6g</td>
</tr>
<tr>
<td>1X PBS</td>
<td>50ml</td>
</tr>
</tbody>
</table>

Filter and store at 4°C

### F. 20X H₂O-Glucose

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>6g</td>
</tr>
<tr>
<td>Autoclaved water</td>
<td>50ml</td>
</tr>
</tbody>
</table>

Filter and store at 4°C

### G. DNAse

### H. 4% BSA
4.4.2. PROTOCOL
A. Coat 18mm cover slips with ~200µl poly-L-ornithine (15-80µg/ml) in 12-well dish.
B. Transfer the cover slips to an incubator maintained at 37°C overnight.
C. Next day, aspirate completely the poly-L-ornithine.
D. Let it dry in the hood.
E. Add coating medium in each well and incubate it in a CO₂ incubator maintained at 37°C for 2-3 hours before plating.
F. Prepare 80ml 1X PBS-Glucose solution in 1X PBS.
G. Dissect out spinal cord and make several pieces (8-9) of the tissue.
H. Transfer the tissue from a maximum of four spinal cords to a 15ml falcon.
I. Wash tissue thrice using 6ml 1X PBS-Glucose.
J. Discard the solution leaving tissue in 1ml solution.
K. Add 20µl trypsin and incubate at 37°C for 10 min (longer incubation damages the cell).
L. Completely remove the trypsin solution and wash thrice with 6ml HBSS-Glucose medium.
M. Dissociate the tissue using a solution of 900µl HBSS-Glucose medium, 100µl 4%BSA and 50µl DNAse. Triturate 6 times using 1000µl tip to get single cell suspension.
N. Repeat the step but with 20µl DNAse. Triturate 9 times using 1000µl tip.
O. Transfer the dissociated cells to a 15ml falcon containing 6ml HBSS-Glucose.
P. Centrifuge for 5s at 900 rpm. Transfer the supernatant in another falcon.
Q. Add 4% BSA cushion (between 1.5-2ml) to the bottom of the tube containing dissociated cells (very gently with a Pasteur pipette; without mixing cell & BSA).
R. Centrifuge at 1000 rpm for 9 mins. Discard supernatant.
S. Re-suspend cell pellet in 1 ml Neurobasal complete medium and 15 µl Dnase.
T. Count the cells.
U. Aspirate off coating medium from the top of cover slips leaving 100-200µl in the well.
V. Plate 160,000 cells/12-well dish.
W. Change medium every 4-5 days.

4.4.3. TRANSFECTION OF SPINAL CORD NEURONS USING LIPOFECTAMINE-2000 REAGENT
A. Pre-heat the heating plate to 37°C inside a laminar flow.
B. Pre-warm neurobasal medium in a water bath maintained at 37°C.
C. Dilute plasmid DNA to 1µg/µl.
D. Mix 0.5µg DNA in 50µl neurobasal medium (Invitrogen) in a microfuge tube.
E. In another tube mix 2µl lipofectamine-2000 (Invitrogen) in 50µl neurobasal medium.
F. Incubate both solutions separately for 10 min at room temperature.
G. Gently mix DNA-neurobasal solution to lipofectamine-neurobasal while tapping at the bottom of the tube.
H. Incubate the mix at room temperature for 15 min.

(During incubation period take out the culture dish out of the incubator and keep it on top of the heating plate maintained at 37°C. Immediately replace (and collect in a falcon) the conditioned medium (in which cells are growing) and add 500µl pre-warmed neurobasal medium in each well. Transfer the cells back to the incubator until next step and keep the conditioned medium in a water bath (37°C)).
I. After the 15 min incubation is over, take the cells out of incubator again and then add 100µl lipofectamine-DNA mix on to the cells (600µl total volume). Transfer the cells back to the incubator for another 30 min.
J. After 30 min, remove the medium and wash cells twice with 1ml pre-warmed neurobasal medium.
K. Add 2 ml conditioned medium (from step H) on the cells and transfer the cells to the incubator.
L. Experiments can be performed 24-48 hours after transfection.

4.5. CO-IMMUNOPRECIPITATION OF RECEPTORS FROM HEK293 CELLS

Immunoprecipitation is a technique for extracting an antigen (protein of interest) from a protein solution. A specific antibody is added to the protein mixture containing the antigen allowing the antibody to bind to the antigen. Following this, the antibody-antigen complex is pulled down using Protein A or Protein G. Protein A is a 40-60 kD surface protein originally found in the cell wall of the bacteria *Staphylococcus aureus*. It has found use in biochemical research because of its ability to bind immunoglobulins. It binds proteins from many mammalian species, most notably IgG’s. It binds with the Fc region of immunoglobulins through interaction with the heavy chain.
N.B. Our lab uses PANSORBIN® Cells to pull down the antibody-antigen complex. These cells are heat-killed, formalin-fixed *Staphylococcus aureus* cells that have a coat of protein A.

### 4.5.1. REQUIREMENTS

**A. Extraction Buffer**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Conc.</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyoxyethylene-10 lauryl ether</td>
<td>1 %</td>
<td>100µl</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>0.18 %</td>
<td>18mg</td>
</tr>
<tr>
<td>NaCl, 3M</td>
<td>150 mM</td>
<td>500µl</td>
</tr>
<tr>
<td>EDTA, 0.5M</td>
<td>5 mM</td>
<td>100µl</td>
</tr>
<tr>
<td>Tris-HCl, 1M (pH 7.4)</td>
<td>50 mM</td>
<td>500µl</td>
</tr>
<tr>
<td>Autoclaved water</td>
<td>--</td>
<td>8.8ml</td>
</tr>
</tbody>
</table>

Dissolve for few hours at 4°C. Add protease inhibitor before use.

**B. IP-Low Buffer**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>50mM</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>0.2%</td>
</tr>
<tr>
<td>NaCl</td>
<td>150mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>1mM</td>
</tr>
</tbody>
</table>

Dissolve at RT, then cool at 4°C. When cold (4°C), adjust the pH to 8.0 with HCl.

**C. Immunoprecipitin**

- Centrifuge 1ml formalin fixed *Staphylococcus aureus* cells (Immunoprecipitin; Life Technologies Inc.) at 9000g at 4 °C.
- Re-suspend the pellet in 900µl of buffer (3% SDS & 10% β-mercaptoethanol in PBS).
- Heat the suspension at 95°C for 30 min.
- Centrifuge at 8000g. Wash the pellet thrice with IP Low Buffer.
• Re-suspend the pellet finally in 900 µl IP low buffer containing 100 mg/l bacitracine, 1 mM Benzamidine, & 0.3 mM PMSF.

D. 5 % Non-Fat Dry Milk Powder (in IP Low Buffer)

E. NuPAGE Sample Buffer

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDS Sample Buffer (4X)</td>
<td>1X</td>
</tr>
<tr>
<td>Reducing Agent (10X)</td>
<td>1X</td>
</tr>
<tr>
<td>Dissolve in autoclaved water</td>
<td></td>
</tr>
</tbody>
</table>

4.5.2. PROTOCOL

A. Harvest the cells using a cell scraper and transfer to a 15ml tube. The cells can be stored at -80°C for later use. It is recommended to use 4 dishes per sample to compensate for heterogeneity in transfection efficiency.

B. Thaw the harvested cells stored at -80 °C at room temperature for 5 min or take the freshly harvested cells.

C. Add 1ml extraction buffer to the cell pellet and leave it shaking vigorously overnight at 4°C.

D. Transfer the suspension into ultra-centrifuge tubes and spin at 45,000 rpm, 4°C for 20 min.

E. Transfer the supernatant in 2ml tubes.

F. Incubate the samples with appropriate antibody for 4 hrs shaking at 4°C.

G. Add 20µl immunoprecipitin and 40µl 5% milk powder in IP-Low buffer.

H. Incubate for 2 hrs shaking at 4°C.

I. Centrifuge at 7000 rpm, 4°C for 5 min; discard the supernatant and re-suspend the pellet in 1 ml IP-low buffer. Repeat the washing step thrice.

J. Dry the pellet and then re-suspend in 20-40 µl NuPAGE sample buffer.

K. Denature the proteins by heating for 15 min at 65°C.

L. Spin down the pellet for 1 min at 13,000 rpm using a bench centrifuge.

M. Collect the supernatant in a fresh microfuge tube. Sample can be used immediately for western blotting or can be stored at -20°C for later use.
4.6. CO-PRECIPITATION OF CELL SURFACE RECEPTORS

The underlying principle of cell surface precipitation is similar to immunoprecipitation apart from the fact that only cell surface receptors are precipitated in this case. The cell surface proteins are first labeled with saturating concentration of antibodies followed by removing the free antibodies by washing, extraction of proteins, and precipitation of antibody-bound receptors by protein A. The initial antibody binding step ensures that only surface proteins are pulled down.

4.6.1. REQUIREMENTS

A. 1X PBS (refer section 4.1.1.)
B. 5mM EDTA in PBS solution
C. DMEM Culture medium (refer section 4.1.1.)
D. IP-Low buffer (refer section 4.5.1.)
E. 1% Triton-X100 in IP-Low buffer
F. Immunoprecipitin (refer section 4.5.1.)
G. 5% Non-fat dry milk powder in IP-Low buffer (refer section 4.5.1.)
H. Sample buffer (refer section 4.5.1.)

4.6.2. PROTOCOL

A. Remove the medium from 4 culture dishes.
B. Wash once with 2ml 1X PBS.
C. Add 2.5ml 5mM EDTA/PBS solution to detach the cells from the dish. Leave the cells in EDTA for maximum 5 min.
D. Immediately add 5ml pre-chilled DMEM and make a cell suspension by gently pipetting without damaging the cells.
E. Transfer the cell suspension to a falcon. Centrifuge for 5 min at 1500 rpm, 4°C.
F. Discard the supernatant, and re-suspend the pellet in 3ml chilled DMEM.
G. Transfer the cell suspension on to a 6 cm dish.
H. Incubate with saturating concentration of appropriate antibodies for 45 min at 4°C.
I. Gently shake the dish every 15 min to ensure efficient binding of the antibodies.
J. Collect the suspension into a 15 ml falcon tube.
K. Centrifuge at 1500 rpm, 5 min, and 4°C.
L. Discard the supernatant and wash thrice with 6ml 1X PBS.
M. Extract the proteins using 1ml Triton-X-100 solution for 1 hour shaking vigorously at 4°C.
N. Transfer the contents to an ultracentrifuge tube and spin at 45,000 rpm, 4°C for 20 min; collect the supernatant.
O. Add 20µl immunoprecipitin and 40µl 5% milk powder blocking buffer.
P. Incubate for 2 hrs at 4°C shaking gently.
Q. Centrifuge for 5 min at 7000 rpm at 4°C. Discard the supernatant and re-suspend the pellet in 1 ml IP-low buffer. Repeat washing with IP-low thrice.
R. Dry the pellet as good as possible and re-suspend in 20-40 µl NuPAGE sample buffer.
S. Denature by heating at 65°C for 15 min.
T. Spin at 13,000 rpm for 1 minute and collect the supernatant. Sample can be used immediately for western blotting or can be stored at -20°C for later use.

### 4.7. PROTEIN ELECTROPHORESIS AND WESTERN BLOTTING

The basic principle of SDS-PAGE (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis) is the separation of proteins according to their molecular masses by electrophoretic migration through a polyacrylamide gel.

Polyacrylamide gels are formed through the polymerization of acrylamide monomers into long polymers that are cross-linked together by bis-acrylamide. This gives rise to a gel matrix, the porosity of which is directly related to acrylamide concentration. Therefore, as acrylamide concentration increases the pore size decreases. Polyacrylamide gels are used as medium through which proteins are allowed to migrate according to charge. Proteins are denatured and dissociated by boiling in SDS and a reducing agent. The SDS gives the protein a uniform negative charge and binds in proportion to the size of the protein. Treated proteins are loaded into wells and current is applied. This results in migration of proteins towards anode. The relative mobility of each protein is determined by the
porosity of the gel that acts as molecular sieve, and therefore low molecular mass proteins migrate more quickly than high molecular mass proteins.

After the protein components have been sufficiently separated by electrophoresis, they are transferred to a PVDF (polyvinylidene difluoride) or nitrocellulose membrane.

4.7.1. REQUIREMENTS

A. 4X Separating Gel Buffer
   • Dissolve 156.9g bis-tris in 500ml Milli-Q water.
   • Adjust the pH to 8.4 with HCl.

B. 4X Stacking Gel Buffer
   • Dissolve 26.2g bis-tris in 250ml Milli-Q water.
   • Adjust the pH to 6.4 with HCl.

C. 30% Acrylamide/Bis

D. 10% SDS

E. 10% APS (Ammonium Persulfate)
   • Dissolve 100mg APS per ml Milli-Q water.
   • Store as aliquots at -20°C.

F. TEMED (Tetramethylethylenediamine)

G. Gel Cassettes (Invitrogen)

H. 0.1% SDS

I. 20X MOPS Buffer

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Conc.</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOPS</td>
<td>1M</td>
<td>418.5g</td>
</tr>
<tr>
<td>Tris</td>
<td>1M</td>
<td>242.3g</td>
</tr>
<tr>
<td>EDTA</td>
<td>20mM</td>
<td>14.9g</td>
</tr>
<tr>
<td>SDS</td>
<td>2%</td>
<td>40g</td>
</tr>
<tr>
<td>--------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>Milli-Q water</td>
<td>--</td>
<td>Up to 2l</td>
</tr>
</tbody>
</table>

Solution should reach a pH of 7.7 automatically

**J. 10X Transfer Buffer**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Conc.</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>479mM</td>
<td>116g</td>
</tr>
<tr>
<td>Glycin</td>
<td>386mM</td>
<td>242.3g</td>
</tr>
<tr>
<td>SDS</td>
<td>0.5%</td>
<td>10g</td>
</tr>
<tr>
<td>Milli-Q water</td>
<td>--</td>
<td>Up to 2l</td>
</tr>
</tbody>
</table>

**K. 1X Transfer Buffer**

<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Transfer Buffer</td>
<td>200ml</td>
</tr>
<tr>
<td>Methanol</td>
<td>200ml</td>
</tr>
<tr>
<td>Milli-Q water</td>
<td>1600ml</td>
</tr>
</tbody>
</table>

**L. 10X PBS**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Conc.</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>1369mM</td>
<td>160g</td>
</tr>
<tr>
<td>KCl</td>
<td>26.8mM</td>
<td>4g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>18mM</td>
<td>4.9g</td>
</tr>
<tr>
<td>Na₂HPO₄.2H₂O</td>
<td>100mM</td>
<td>35.6g</td>
</tr>
<tr>
<td>Milli-Q water</td>
<td>--</td>
<td>Up to 2l</td>
</tr>
</tbody>
</table>

Solution should reach a pH of 7.4 automatically. Autoclave

**M. Anti-oxidant (Invitrogen)**

**N. Blocking Buffer:**

- Dissolve 15g not fat dry milk powder in 1L 1X PBS.
- Add 1ml tween-20 to the solution.
O. **Assay Buffer**
   - Dissolve 250µl 1M MgCl$_2$ and 2.4 ml diethanolamine in around 200ml Milli-Q water.
   - Adjust the pH to 10. Adjust volume to 10ml.

4.7.2. **PROTOCOL: CASTING OF SEPARATING GELS**
   A. Mix all the ingredients, except 10% APS as per the table below.

<table>
<thead>
<tr>
<th>10% Separating Gel</th>
<th>20 ml</th>
<th>40 ml</th>
<th>50 ml</th>
<th>80 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>4x Separating-Buffer</td>
<td>5 ml</td>
<td>10 ml</td>
<td>12.5 ml</td>
<td>20 ml</td>
</tr>
<tr>
<td>30% Acrylamide/Bis</td>
<td>6.6 ml</td>
<td>13.2 ml</td>
<td>16.5 ml</td>
<td>26.4 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>200 µl</td>
<td>400 µl</td>
<td>500 µl</td>
<td>800 µl</td>
</tr>
<tr>
<td>Milli-Q H$_2$O</td>
<td>8.0 ml</td>
<td>16.0 ml</td>
<td>20.0 ml</td>
<td>32.0 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>20 µl</td>
<td>40 µl</td>
<td>50 µl</td>
<td>80 µl</td>
</tr>
<tr>
<td>10% APS</td>
<td>200 µl</td>
<td>400 µl</td>
<td>500 µl</td>
<td>800 µl</td>
</tr>
</tbody>
</table>

   B. Prepare empty gel casting.
   C. Add 10% APS to the solution, mix well, and cast the gel.
   D. Cover the gel by spraying 0.1% SDS. This ensures formation of smooth surface.
   E. Polymerize for nearly 4 hours.
   F. Fill with 0.1% SDS, and store at 4°C in a vacuum bag.
   G. Gels can be stored air-tight for up to 3 weeks. If the gel top gets dried, it should be discarded.

4.7.3. **PROTOCOL: CASTING OF STACKING GELS**
   A. This is the top gel layer and is casted on top of separating layer (1/4 gel volume, ~2 ml solution).
   B. Discard the SDS over pre-cast separating gel.
   C. Wash the cassette thrice with 1X Stacking buffer.
   D. Drain out remaining liquid using vacuum pump.
   E. Mix all the ingredients, as in table. Add APS to the mixture just before casting.
### 5% Stacking Gel

<table>
<thead>
<tr>
<th></th>
<th>5 ml</th>
<th>10 ml</th>
<th>15 ml</th>
<th>20 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>4x Stacking-Buffer</td>
<td>1,25 ml</td>
<td>2,5 ml</td>
<td>3,75 ml</td>
<td>5 ml</td>
</tr>
<tr>
<td>30% Acrylamide/Bis</td>
<td>850 µl</td>
<td>1,7 ml</td>
<td>2,55 ml</td>
<td>3,4 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>50 µl</td>
<td>100 µl</td>
<td>150 µl</td>
<td>200 µl</td>
</tr>
<tr>
<td>Milli-Q H₂O</td>
<td>2,8 ml</td>
<td>5,6 ml</td>
<td>8,4 ml</td>
<td>11,2 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>5 µl</td>
<td>10 µl</td>
<td>15 µl</td>
<td>20 µl</td>
</tr>
<tr>
<td>10% APS</td>
<td>75 µl</td>
<td>150 µl</td>
<td>225 µl</td>
<td>300</td>
</tr>
</tbody>
</table>

F. Cast the stacking gel on top of separating gel.

G. Insert the comb immediately. Prevent bubbles below the comb.

H. After 20-30 min, gently remove the comb from the stacking gel.

I. The gel is ready for loading.

### 4.7.4. PROTOCOL: PERFORMING ELECTROPHORESIS

A. Remove the white strip at the back bottom of the gel cassette.

B. Place the cassettes in the electrophoresis unit, and set the unit.

C. Prepare 1X MOPS running buffer (600 ml per chamber).

D. To 200ml 1X MOPS, add 500 µl anti-oxidant. It prevents re-oxidation of proteins and maintains them in a reduced state.

E. Fill the inner chamber with anti-oxidant containing 1X MOPS buffer.

F. Check for any possible leakage.

G. Pour remaining buffer in the outer chamber.

H. Load the samples and then run the gel at fixed voltage (200V) and current being 130mA/gel for 40-45 min.

I. When the run is over, turn off the current, break open the cassette and keep the gel in transfer buffer for 15 min. Proceed with blotting.

### 4.7.5. WESTERN BLOTTING

A. Equilibrate the PVDF membrane in methanol for 15 sec followed by washing for 2 min in milli-Q water and finally in transfer buffer for 10 min.
B. Wet filter paper to be used for transfer.

C. Setup the blot:
   a. Wet the surface of blotter with transfer buffer.
   b. Put one filter paper over it gently.
   c. Remove any air bubbles by rolling a glass pipette on top of it.
   d. Add few drops of transfer buffer (this should be done in every step to prevent drying).
   e. Then gently transfer the membrane on top of the filter paper preventing any air bubble.
   f. Add a few drops of buffer on top to prevent drying of membrane.
   g. Now place the gel over the membrane and then finally cover it with another filter paper.
   h. Remove air bubbles by gently rolling a glass pipette over it.

D. Run the blot for a maximum of 20 V, 58 mA/gel for 1 hour.

   \[
   \frac{(\text{Blot Area}) \times 1.2 \text{ mA}}{\text{gel}}; \text{ optimum for most of our proteins}
   \]

   \[
   \frac{(\text{Blot Area}) \times 1.5 \text{ mA}}{\text{gel}}; \text{ optimum for high molecular weight protein}
   \]

E. After transfer, place the membrane in blocking buffer shaking gently for 1 hr.

F. Incubate the membrane overnight with primary antibody in blocking buffer.

G. Next morning, wash the membrane three times 15 min with blocking buffer.

H. Incubate with secondary antibody conjugated to alkaline-phosphatase (AP) for 1 hour.

I. Wash three times 15 min with blocking buffer.

J. Wash three times 5 min with assay buffer.

K. Prepare substrate for detection of alkaline phosphatase by mixing 10 µl CDP-Star reagents in 1ml assay buffer (per membrane). CDP-Star Reagent is a 1,2-dioxetane compound utilized in Phototope® alkaline phosphatase-based chemiluminescent detection assays. Light production resulting from chemical decomposition exhibits an initial delay followed by a persistent glow that lasts as long as free substrate is available.

L. Add assay buffer over membranes and develop the image.

4.8. FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET)

FRET also called as Förster Resonance Energy Transfer, is an microscopy technique used to measure the proximity of two fluorophores. Resonance energy transfer occurs only over very short distances, typically within 10nm, and involves the direct transfer of excited state energy from the donor fluorophore to an acceptor fluorophore. Upon transfer of energy, the acceptor molecule enters an
excited state from which it decays emissively (always of a longer wavelength than that of the acceptor emission). Thus, by exciting the donor and then monitoring the relative donor and acceptor emissions, one can determine when FRET has occurred and at what efficiency. FRET is often used to determine when and where two or more biomolecules, often proteins, interact within their physiological surroundings. Since energy transfer occurs over distances of 1-10nm, a FRET signal provides distance accuracy surpassing the optical resolution (~0.25 mm) of the light microscope.

4.8.1. REQUIREMENTS

A. Krebs-Ringer-HEPES buffer (pH 7.4)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
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<td>HEPES</td>
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</tr>
<tr>
<td>NaCl</td>
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</tr>
<tr>
<td>KCl</td>
<td>3mM</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>2mM</td>
</tr>
<tr>
<td>Glucose</td>
<td>20M</td>
</tr>
</tbody>
</table>

4.8.2. PROTOCOL

A. Plating and transfection of HEK cells for FRET measurement has been described in section 1.2 and 2.3 respectively. FRET was performed for CFP and YFP tagged proteins.

B. 24 hour after transfection, transfer a cover slip to the imaging chamber and seal the chamber.

C. Wash the cells once with Krebs´s buffer.

D. Add 500µl additional pre-warmed Krebs´s buffer (37°C) and transfer the chamber on to the microscope.

- Inverted fluorescence microscope (Zeiss/Nikon)
- Mercury arc lamp (Zeiss HBO 100W intensity)

<table>
<thead>
<tr>
<th></th>
<th>ECFP Filter</th>
<th>EYFP Filter</th>
<th>FRET Filter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excitation</td>
<td>440nm</td>
<td>500nm</td>
<td>440nm</td>
</tr>
<tr>
<td>Beam Splitter</td>
<td>455nm</td>
<td>525nm</td>
<td>455nm</td>
</tr>
<tr>
<td>Emission</td>
<td>480nm</td>
<td>535nm</td>
<td>535nm</td>
</tr>
</tbody>
</table>
E. Manually select the cells and then capture the images using Metamorph software.

4.8.3. QUANTIFICATION

FRET with the three-filter set system was quantified according to the method introduced by Xia and Liu (2001) to normalize the FRET intensity to ECFP and EYFP concentrations in each region of interest (ROI). ImageJ plugin PixFRET was used to generate FRET images after bleedthrough correction according to the following formula:

\[
N_{\text{FRET}} = \frac{[I_{\text{FRET}} - (a)I_{\text{CFP}} - (b)I_{\text{YFP}}]}{(I_{\text{CFP}} \times I_{\text{YFP}})^{1/2}}
\]

- \(N_{\text{FRET}}\): is normalized FRET intensity value
- \(a\): Percentage of CFP contribution to FRET intensity
- \(b\): Percentage of YFP contribution to FRET intensity.
- \(I_{\text{FRET}}\): Intensity of FRET channel
- \(I_{\text{CFP}}\): Intensity of CFP channel
- \(I_{\text{YFP}}\): Intensity of YFP channel

4.9. IMMUNOCYTOCHEMISTRY

Immunocytochemistry is a technique used to detecting the presence of proteins in cell by the use of a specific antibody. The bound antibodies are then detected using fluorescently labeled secondary antibodies. Cells are stained after fixation to a solid support to allow easy handling in subsequent procedures. This is primarily achieved by using paraformaldehyde (PFA). PFA treatment leads to the establishment of chemical cross-links between free amino groups. When the cross-links join different molecules, a latticework of interactions occurs that holds the overall architecture of the cell together. For intracellular staining, permeabilization is done which permits the immunoglobulin to bind to intracellularly localized epitopes.

4.9.1. REQUIREMENTS

A. 1X PBS (Refer section 4.1.1.)
B. 16% (w/v) Paraformaldehyde (PFA)
- Dilute to 4% with 1X PBS before use.

C. 1% (v/v) Triton-X-100 in 1X PBS

D. Blocking Solution
- Dissolve 2.5g bovine serum albumin (BSA) in cold 1X PBS to make 5% BSA blocking buffer.
- Store at 4°C.

E. 0.1% Triton Blocking Solution
- Dissolve 1 ml 1% triton-100 in 9 ml blocking buffer.

F. Mowiol mounting medium

Mowiol is a high quality anti-fade medium. It hardens and has the same refractive index as immersion oil. The preparation of mowiol mounting medium is described below:

Requirements:
- Mowiol 4-88
- Glycerol
- Tris-Cl (0.2 M, pH 8.5)
- 1,4-Diazabicyclo-[2,2,2]-octane (DABCO)

Day 1:
1. Add 2.4 g of Mowiol 4-88 to 6 g of glycerol in a clean sterilized glass beaker.
2. Stir to mix for 10 minutes.
3. Add 6 ml of H₂O and leave for several hours at room temperature (overnight).

Day 2:
4. Add 12 ml of 0.2 M Tris-Cl (pH 8.5).
   (DABCO works best at this pH of Tris)
5. Heat to 50°C for 10 minutes with occasional mixing.
6. After the Mowiol dissolves, clarify by centrifugation at 5000g for 15 minutes.
7. For fluorescence detection, add 1, 4-diazabicyclo-[2,2,2]-octane (DABCO) to 2.5% to reduce fading at 37°C.
8. Aliquot in airtight containers and store at -20°C. Stock is stable at room temperature for several weeks after thawing.

4.9.2. PROTOCOL

A. Transfer the cover slips from culture medium on to a parafilm base. All steps are performed at room temperature.
B. Wash rapidly by adding 1X PBS rapidly.
C. Fix the cells in 4% PFA for 10 min.
D. Wash three times 5 min each with 1X PBS.
E. Permeabilize the cells with 0.1% triton-X-100 blocking solution for 3 min. Skip this step if not required.
F. Block the cells in 5% BSA blocking solution for 30 min.
G. Prepare primary antibodies in 5% BSA blocking solution. 100µl/cover slip is sufficient.
H. Add antibodies on cells and incubate for 2 hours.
I. Wash three times 5 min with 1X PBS. Prepare secondary antibody in 5% BSA in the meantime.
J. Add secondary antibodies and incubate for 45 min.
K. Wash three times 10 min each using 1X PBS. Thaw an aliquot of mowiol mounting medium meanwhile.
L. Put 5-7µl/cover slip on an ethanol wiped glass slide. Take a cover slip, dip it once in Milli-Q water to remove salts from PBS, dry the edges using a soft tissue and then mount the cover slip on the slides with cells facing the slide.
M. Leave the slides at 4°C in dark for overnight.

4.10. CONFOCAL MICROSCOPY AND IMAGE ANALYSIS

4.10.1. LIVE CELL CONFOCAL IMAGING

Confocal microscopy allows controllable depth of field image acquisition, elimination of out-of-focus information, and collection of serial optical sections. These are achieved by the use of spatial filtering to eliminate out-of-focus light in specimens that are thicker than the plane of focus.
A. Live Cells
- Prepare HEK cells (Refer section 4.1.2.)
- Transfect the cells (Refer section 4.2.3.)
- 24 hours after transfection, perform imaging. In our case cells expressing ECFP and EYFP tagged receptors were imaged

B. Microscope
- Zeiss Axiovert 200-LSM 510 confocal microscope
- Laser: Argon laser, 30mW; Helium/Neon laser, 1mW
- Objective: Zeiss Plan-Neofluar ×63/1.3
- ECFP excitation: 458nm
- EYFP excitation: 488nm

C. Software
- Zeiss LSM Image Browser

4.10.2. SPINNING DISK CONFOCAL MICROSCOPY
A spinning disk confocal scanner uses a pair of rotating disks with thousands of pinholes in a spiral Archimedes. As light is projected on to the disk the holes trace concentric arcs of excitation light across the sample. Fluorescent light from the specimen returns along the same path through the objective lens and the pinhole, and reflected by the dichroic mirror through a relay lens to the detector. As the whole field of view is scanned in this way during a single camera exposure a high quality confocal image is formed very quickly, allowing the capture of quality confocal images at incredibly high speed. The spinning disk confocal microscope collects multiple points simultaneously rather than scanning a single point at a time, which means that the technology is both faster and hits the sample with a lower dose of laser light. A high sensitivity CCD camera leads to the possibility of incredibly fast frame rates.

A. Microscope
- Leica DM5000B Spinning Disk Microscope
- Lasers: Cobolt calypso, 50mW; Cobolt jive, 50mW; Coherent cube, 25mW
B. Image Acquisition and Analysis
Images were captured, processed and analyzed using Metamorph software (Meta Imaging, Downington, PA). For quantitative analysis, fluorescent images were processed with multidimensional image analysis (MIA) interface which employs 2D object segmentation by wavelet transformation (Racine et al., 2007). Objects composed of ≥ 3 pixels are defined as clusters. Receptor clusters were considered synaptic when at least 1 pixel overlaps with mRFP-gephyrin clusters.

4.11. LIVE CELL STAINING AND QUANTUM DOT IMAGING
Quantum dot (QD) nanocrystals are fluorophores that exhibit some important differences as compared to traditional fluorophores such as organic fluorescent dyes and naturally fluorescent proteins. QDs are nanometer-scale (10-20 nm, roughly protein-sized) atom clusters, containing from a few hundred to a few thousand atoms of a semiconductor material (cadmium mixed with selenium or tellurium), which has been coated with an additional semiconductor shell (zinc sulfide) to improve the optical properties of the material. These are extremely efficient machines for generating fluorescence; their intrinsic brightness and photostability is often many times that observed for other classes of fluorophores.

4.11.1. REQUIREMENTS

A. Borate Buffer Stock
- Dissolve 0.5g Na₂B₄O₇·10H₂O in 100 ml sterile (culture) water.
- Add 1.221g H₂BO₃ and dissolve for 20 min.
- Adjust pH to 8 with 5M NaOH.
- Add up water to a final volume of 125 ml.
- Check pH again, adjust it to 8 with HCl.
- The solution can be stored for up to a year.

B. Sucrose (1.43M):
- Dissolve 4.9 g sucrose in 10 ml sterile water. Store at 4°C.

C. QDot Binding Buffer
- Dissolve 400mg BSA (SIGMA A2153) in 5 ml borate buffer stock solution for 10 min at 37°C.
- Add 50 µl 20% NaN₃ (Sodium Azide).
- Add 15 ml sterile water.
- Filter using 0.22 µm filter and store at 4°C for up to 6 months.

D. Imaging Medium (MEMair)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Conc.</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M HEPES, pH 7.4</td>
<td>20mM</td>
<td>1 ml</td>
</tr>
<tr>
<td>200mM glutamine</td>
<td>2mM</td>
<td>500 µl</td>
</tr>
<tr>
<td>Sodium Pyruvate</td>
<td>1mM</td>
<td>500 µl</td>
</tr>
<tr>
<td>B27</td>
<td>1X</td>
<td>1 ml</td>
</tr>
<tr>
<td>20X Glucose-MEM (6gm in 100 ml)</td>
<td>33mM</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>MEM + Eagle</td>
<td>--</td>
<td>44.5 ml</td>
</tr>
<tr>
<td><strong>FINAL VOLUME</strong></td>
<td></td>
<td>50 ml</td>
</tr>
</tbody>
</table>

E. Quantum Dots

- Qdot®655.

4.11.2. PROTOCOL

A. Turn on the 37° heating plate and place a parafilm over it.
B. Prepare the primary antibody in 100µl MEMair.
C. Transfer a cover slip on to the parafilm.
D. Wash once with MEMair (~250µl).
E. Incubate the cover slip with primary antibody for 3-5 min. The incubation time needs to be optimized for the antibodies.
F. Prepare biotinylated secondary antibody in 100µl meanwhile. Use 1:2000 concentrations for most of antibodies from Jackson Immunoresearch.
G. Wash primary antibody four times with MEMair.
H. Incubate with secondary antibody for 5 minutes.
I. Prepare quantum dot for labeling as:
   - Dilute stock quantum dot solution 1:10 in quantum dot binding buffer to prepare working solution (1µl QD + 9µl QBB).
   - In another tube, mix 85µl QBB with 15µl 1.43M Sucrose and 2µl QD working solution.

J. Wash another four times with MEMair.

K. Incubate with quantum dots for 1 min.

L. Wash for at least 10-12 times to ensure all unbound quantum dots are washed away.

M. Optional (In case of non-gephyrin-knock-in mice):
   - For synapse labeling, incubate the cells with 2µM FM4-64 in MEMair + KCl for 25 sec. (FM4-64 does not cross the cell membrane. Upon depolarization using KCl, the dye enters the vesicles that have fused transiently with the membrane. Vesicles that are loaded with this dye will then release the dye when they next fuse with the membrane; i.e. when the membrane is next depolarized.)
   - Wash four times with MEMair.

N. Carefully place the coverslip in recording chamber and add 500µl MEMair with/without drugs.

O. Perform the recording. The movement of QDs on the dendrites was recorded with an integration time of 75ms with 500 consecutive frames (37.5 s). The recording was done up to 20 min following drug addition.

4.11.3. Microscope
   - Olympus inverted microscope, IX71.
   - Objective: Olympus, 60x, NA 1.45
   - Xenon lamp
   - Cooled CCD camera Cascade+128 (Roper Scientific)
   - Heating chamber maintained at 37°C.
   - Filter sets:
     - QD: D455/70x and HQ655/20
     - GFP: HQ500/20 and HQ535/30
     - mRFP: D535/50 and E590lpv2
4.12. SINGLE-PARTICLE TRACKING AND ANALYSIS

Tracking and analysis of QDs has been well described recently (Renner et al. 2009; Bannai et al. 2009). Briefly, QDs were detected by cross-correlating the image with a Gaussian model of the point spread function, and the diffusion parameters were calculated using custom software (Bonneau et al. 2005, Charrier et al., 2006, Ehrensperger et al., 2007) using Matlab (The Mathworks Inc., Natick, MA). Single QDs were identified by intermittent fluorescence (i.e. blinking). The spots in a given frame were connected with the maximum likely trajectories estimated on previous frames of the image sequence. Only trajectories with at least 15 consecutive trajectories were used for further analysis. Synaptic area was defined by processing mRFP-gephyrin images with the MIA interface. GABA_A receptor QDs were classified as “synaptic” when the trajectories overlapped with synaptic area. The trajectories were considered “extrasynaptic” when they are ≥ 2 pixels away from the synapse. P2X_2 receptor QDs were rarely observed at/near mRFP-gephyrin clusters, so the analysis was performed independent of inhibitory-synapse localization. The mean square displacement (MSD) was calculated using MSD(ndt) = (N – n)^{-1} \sum_{i=1}^{N-n} ((x_{i+n} - x_i)^2 + ((y_{i+n} - y_i)^2), where x_i and y_i are the coordinates of an object on frame i, N is the total number of steps in the trajectory, dt is the time interval between two successive frames, and ndt is the time interval over which displacement is averaged. The diffusion coefficient D was calculated by fitting the first two to five points of the MSD plot versus time with the equation MSD(t) = 4D t + 4\sigma_x^2, where \sigma_x is the spot localization accuracy in one direction (Ehrensperger et al., 2007). Given the resolution, trajectories with D < 10^4 \mu m^2/s for QDs were classified as immobile. The size of the average confinement area was calculated fitting the average MSD plot with the equation proposed by Kusumi et al., 1993. Dwell time was calculated as in the study by Charrier et al., 2006.
4.13. ANTIBODIES

4.13.1. ANTIBODIES USED

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Host</th>
<th>Supplier</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1N, α1L</td>
<td>Rabbit polyclonal</td>
<td></td>
<td>IP, WB, ICC</td>
</tr>
<tr>
<td>β2N, β2L</td>
<td>Rabbit polyclonal</td>
<td></td>
<td>IP, WB</td>
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<tr>
<td>γ2N, γ2L</td>
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</tr>
<tr>
<td>EGFP</td>
<td>Rabbit polyclonal</td>
<td></td>
<td>WB</td>
</tr>
<tr>
<td>P2X$_2$R (extracellular)</td>
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<td></td>
<td>WB, ICC</td>
</tr>
<tr>
<td>GFP</td>
<td>Mouse monoclonal</td>
<td>Roche</td>
<td>IP</td>
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<td>SIGMA</td>
<td>IP, WB, ICC, SPT</td>
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<td>Mouse monoclonal</td>
<td>Covance</td>
<td>WB</td>
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<tr>
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<td>Alomone</td>
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</tr>
<tr>
<td>GABA$_A$R (β2/β3)</td>
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<td>Millipore</td>
<td>ICC</td>
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<tr>
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<td>Rabbit polyclonal</td>
<td>AbCam</td>
<td>WB, IP</td>
</tr>
<tr>
<td>Gephyrin</td>
<td>Mouse monoclonal</td>
<td>Synaptic Systems</td>
<td>ICC</td>
</tr>
</tbody>
</table>

4.13.2. GENERATION OF P2X$_2$R ANTIBODIES

Surface exposed residues were selected based on a P2X$_2$R homology model (kindly provided by T. Grutter, Université Louis Pasteur, Illkirch, France) (Guerlet et al., 2008). A peptide corresponding to amino acid sequence 205-214 (SQKSDYLKH) of the mature receptor subunit was selected and custom-synthesized (piCHEM, Graz, Austria) with an additional C-terminal cysteine and was coupled to keyhole limpet hemocyanin (KLH). Rabbits were immunized with this adduct and antibodies were purified from the serum of the rabbits by affinity chromatography on a column consisting of the respective peptide coupled to thiopropyl-Sepharose (Zezula and Sieghart, 1991).
4.14. LIST OF PLASMIDS

<table>
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<tr>
<th>Plasmid</th>
<th>Comment</th>
<th>Generated in Lab</th>
</tr>
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<tbody>
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<td>Lab</td>
<td></td>
</tr>
<tr>
<td>β2-pCI</td>
<td>Lab</td>
<td></td>
</tr>
<tr>
<td>γ2-pCI</td>
<td>Lab</td>
<td></td>
</tr>
<tr>
<td>α1-(A160C)-pCI</td>
<td>Point mutation in α1-subunit</td>
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</tr>
<tr>
<td>α1-pEYFP-C1</td>
<td>EYFP in intracellular loop</td>
<td></td>
</tr>
<tr>
<td>α1- pECFP-C1</td>
<td>ECFP in intracellular loop</td>
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</tr>
<tr>
<td>β2-pEYFP-C1</td>
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<td>ECFP in intracellular loop</td>
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<tr>
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<tr>
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<tr>
<td>P2X1-EGFP-N1</td>
<td>Wolfgang Junger, Harvard Medical School, USA</td>
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</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>P2X3-EGFP-N1</td>
<td>Wolfgang Junger, Harvard Medical School, USA</td>
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</tr>
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</table>
5. RESULTS

5.1. AGONIST-INDEPENDENT INTRACELLULAR OLIGOMERIZATION AND CO-TRAFFICKING OF GABA_A RS AND P2X_2 RS

We first investigated whether GABA_A Rs and P2X_2 Rs are able to interact directly and in an agonist-independent way. For that, receptors were extracted from HEK cells transfected as indicated in Fig. 4A and were subjected to immunoprecipitation using antibodies against the α1 subunit of GABA_A Rs. Precipitated receptors were subjected to SDS-PAGE and Western blot analysis. Since the α1 antibodies were not able to directly precipitate β2 or γ2 subunits (experiments not shown), co-precipitation of β2 and γ2 subunits indicated their assembly with α1 subunits. (Fig. 4A, lane 1). The α1 antibodies did not directly precipitate EYFP-labeled P2X_2 Rs (Fig. 4A, lane 2). Their precipitation from cells co-transfected with GABA_A Rs and EYFP-tagged P2X_2 Rs (Fig. 4A, lane 3) thus indicates an association of these receptors with GABA_A Rs. Interestingly, however, the total amount of GABA_A Rs precipitated by α1-antibodies was increased on co-expression with P2X_2 Rs (Fig. 4A, lanes 1 and 3).

In parallel experiments, the extracted receptors were immunoprecipitated with mouse monoclonal anti-GFP antibodies. These antibodies did not directly precipitate GABA_A R subunits (Fig. 4A, lane 4). Co-precipitation of α1, β2 and γ2 subunits (Fig. 4A, lane 6), thus again indicates association of GABA_A Rs with EYFP-tagged P2X_2 Rs. Interestingly, the amount of P2X_2 Rs precipitated was comparable in the absence or presence of GABA_A Rs (Fig. 4A, lanes 5 and 6). It is also important to note that only a very small fraction of GABA_A Rs was associated with P2X_2 Rs and vice versa (Fig. 4A, lane 3 and 6). To investigate whether the two receptors also interact at the cell surface, receptors were first labeled with antibodies directed against the extracellular N-terminus of the GABA_A R α1 subunit or against the FLAG-tag in the extracellular loop of P2X_2 Rs (P2X_2-FLAG-EGFP) followed by protein extraction and precipitation of the antibody-labeled receptors by immunoprecipitin. Results from a typical experiment are shown in Fig. 4B. Western blotting indicated that GABA_A Rs expression were reduced at the cell surface by 25.6 ± 0.7% (mean ± SE, p<0.0001, n=4 independent experiments; Fig. 4B, lane 1 and 2) when P2X_2 Rs were co-expressed even though we observed an increase in total GABA_A Rs under these conditions (Fig. 4A). P2X_2 Rs and GABA_A Rs could also be co-precipitated at the cell surface when antibodies against the extracellular FLAG-tag were used (Fig. 4B, lane 5). In contrast to GABA_A Rs, we observed no significant change in the surface expression of P2X_2 Rs on co-expression of GABA_A Rs (Surface level reduced by 5.3 ± 3.3%, mean ± SE, p=0.14, n= 4 independent experiments; Fig. 4B,
lane 4 and 5). As observed for total receptors, only a small fraction of GABA_\text{A}Rs and P2X_2Rs associate with each other.

**Figure 4.** Co-immunoprecipitation and co-trafficking of GABA_\text{A}Rs and P2X_2Rs. HEK cells were co-transfected with GABA_\text{A}R \alpha_1, \beta_2, and \gamma_2 subunits or/and EYFP-tagged P2X_2R subunits (P2X_2-EYFP) or a P2X_2-FLAG-EGFP receptors having the FLAG-tag in the extracellular region and EGFP at the C-terminus, as indicated. GABA_\text{A}Rs or P2X_2Rs were immunoprecipitated with rabbit anti-\alpha_1-subunit specific antibodies or mouse anti-GFP antibodies respectively. This was followed by SDS-PAGE and Western blot analysis using digoxigenin-labelled \alpha_1-Dig, \beta_2-Dig, \gamma_2-Dig and EGFP-Dig antibodies. (A) Results indicate co-immunoprecipitation of GABA_\text{A}Rs and P2X_2Rs from total protein extract. The total expression of GABA_\text{A}Rs increased while that of P2X_2Rs was not changed on co-expression of these receptors. Results are from a typical experiment performed four times with comparable results. (B) Co-immunoprecipitation of cell-surface GABA_\text{A}Rs and P2X_2Rs. The surface expression of GABA_\text{A}Rs but not that of P2X_2Rs decreased on co-expression with P2X_2Rs. Co-transfection with the trafficking-deficient \alpha_1(A160C) GABA_\text{A}R subunit caused a nearly 90% reduction of GABA_\text{A}R and a 21% reduction of P2X_2Rs at the cell surface. ***p<0.001, t-test, n=4 independent experiments).
Figure 5. $\alpha1$(A160C) mutation does not impair assembly of GABA$_A$R subunits. (A) Expression of the point-mutated $\alpha1$ subunit compared to wild-type subunit in HEK cells. (B, C) The assembly of $\alpha1$(A160C)-subunit with $\beta3$-subunit and the $\gamma2$-subunit is not impaired.

To investigate whether the two receptors co-trafficking to the cell surface, we generated a trafficking-deficient GABA$_A$R where the $\alpha1$-subunit had an alanine to cysteine mutation ($\alpha1$-A160C). This mutant assembles with other subunits of GABA$_A$Rs (Fig. 5) but does not reach the cell surface (surface level reduced by 91.4 ± 0.4 %, p<0.0001, n= 4 independent experiments; Fig. 4B, lane 1 and 3). We hypothesized that if the two receptors are co-trafficking, the intracellular-retention of GABA$_A$Rs should also retain the associated P2X$_2$Rs. In fact, trafficking-deficient GABA$_A$Rs reduced the cell surface expression of P2X$_2$Rs by 21 ± 2.7% (p<0.0001, n= 4 independent experiments; Fig. 4B, lane 4 and 6). Under these conditions, no associated GABA$_A$Rs and P2X$_2$Rs were detectable at the cell surface (Fig. 4B, lanes 3 and 6). Together, we conclude that GABA$_A$Rs and P2X$_2$Rs associate with each other in intracellular compartments and co-trafficking to the cell surface.

5.2. INTRACELLULAR AND SURFACE CO-LOCALIZATION AND FRET BETWEEN GABA$_A$RS AND P2X$_2$RS

To further characterize this interaction, we generated fluorescent constructs of GABA$_A$Rs having ECFP (or EYFP, experiments not shown) tags in the large intracellular loop of subunits (Fig. 6). P2X$_2$Rs having ECFP or EYFP tags in the intracellular C-terminal domain have been described previously (Schicker et al., 2009). HEK cells were then co-transfected with P2X$_2$-ECFP and P2X$_2$-EYFP subunits, or with GABA$_A$R $\alpha1$-ECFP, $\beta2$ and $\gamma2$ subunits as well as P2X$_2$-EYFP subunits.
Figure 6. Generation and characterization of the GABA<sub>A</sub>R α1-ECFP construct. (A) Location of ECFP tag in the intracellular loop (between amino acids 343 and 344) of the α1-subunit of GABA<sub>A</sub>Rs. (B) The presence of ECFP-tag did not impair the assembly with β2 and γ2-subunits of the receptor. (C) The α1-ECFP alone aggregated in the endoplasmic reticulum; once co-assembled with β2 and γ2-subunits, it is trafficked to the cell surface.

24 hours after transfection, receptor expression and distribution in living cells was imaged using a confocal microscope (Fig. 7). As expected, P2X<sub>2</sub>-ECFP and P2X<sub>2</sub>-EYFP subunits were strongly co-localized at the cell membrane, as well as in intracellular compartments. For cells expressing GABA<sub>A</sub>-ECFP and P2X<sub>2</sub>-EYFP receptors, we also observed co-localization in the intracellular compartment as well as at the cell surface.
Figure 7. Intracellular and membrane co-localization of receptors in living cells. HEK cells were co-transfected
with P2X$_2$-ECFP and P2X$_2$-EYFP or GABA$_A$R (α1-ECFP, β2, γ2) and P2X$_2$-EYFP subunits. P2X$_2$-ECFP and
P2X$_2$-EYFP assembled to form homo-trimeric receptors which are enriched at the cell membrane. Co-expression of GABA$_A$-ECFP and P2X$_2$-EYFP receptors resulted in co-localization in both intracellular and
membrane regions.

To investigate a possible direct interaction of GABA$_A$Rs and P2X$_3$Rs, we performed FRET
(flourescence resonance energy transfer) on appropriately transfected HEK. FRET images obtained
were processed using pixFRET plugin of ImageJ to visualize FRET signal in pseudo-color (Fig. 8A). Co-transfection of P2X$_2$-ECFP and P2X$_2$-EYFP subunits generated homo-trimeric P2X$_2$Rs where the
donor (ECFP) and the acceptor (EYFP) are sufficiently close to result in an intense FRET signal
(Khakh et al., 2005). Similarly, an intense FRET signal was observed for the GABA$_A$-ECFP/P2X$_2$-
EYFP pair whereas a negligible signal was observed when ECFP and EYFP were co-transfected
without being bound to receptor subunits (Fig. 8A). Similar to previous observation from confocal-imaging, FRET between P2X$_2$-ECFP/P2X$_2$-EYFP pair was not only observed at cell membranes.
(identified by their intense signal at the border of the cells) but also in intracellular regions (identified by a diffuse signal distributed within the cell).

**Figure 8.** Intracellular and surface FRET between GABA<sub>A</sub>Rs and P2X<sub>2</sub>Rs. HEK cells were co-transfected with P2X<sub>2</sub>-ECFP and P2X<sub>2</sub>-EYFP, pECFP and pEYFP, or α1-ECFP, β2, γ2 and P2X<sub>2</sub>-EYFP subunits. (A) FRET images obtained are depicted in pseudo-color code. Results indicate a clear FRET signal between P2X<sub>2</sub>-ECFP and P2X<sub>2</sub>-EYFP, or GABA<sub>A</sub>-ECFP and P2X<sub>2</sub>-EYFP subunits. Examples of region of interest (white) on cell surface to compute FRET are shown (B) The average FRET intensity (± SE) for P2X<sub>2</sub>-ECFP/P2X<sub>2</sub>-EYFP receptors is not significantly different at the cell membrane and in the intracellular compartment (ns, p=0.922, n=48 cells, t-test). Similarly, a strong FRET signal was measured for GABA<sub>A</sub>-ECFP/P2X<sub>2</sub>-EYFP receptors at the cell membrane and in the intracellular compartment (ns, p=0.992, n=48 cells, t-test) (C, D) Cell-by-cell analysis indicated that the FRET intensity at the membrane of individual cells and in their intracellular compartment did not vary significantly for cells transfected with P2X<sub>2</sub>-ECFP/P2X<sub>2</sub>-EYFP receptors (ns, p=0.429, n=38 cells, paired t-test) and GABA<sub>A</sub>-ECFP/P2X<sub>2</sub>-EYFP receptors (ns, p=0.197, n=38 cells, paired t-test).
The average FRET intensity (± SE) measured for P2X$_2$-ECFP/P2X$_2$-EYFP pair in the cytosol (130.9 ± 5.1 arbitrary units, n=48 cells) and at cell membranes (131.6 ± 5.4 arbitrary units, n=48 cells) was similar (p=0.9, t-test). Similarly, FRET between donor protein, GABA$_A$-ECFP and acceptor P2X$_2$-EYFP was observed at cell membranes as well as in intracellular compartments. The FRET intensities for GABA$_A$-ECFP/P2X$_2$-EYFP receptors in the cytosol (104.4 ± 5.3 arbitrary units, n=46 cells) and at the cell surface (104.9 ± 4.2 arbitrary units, n=46 cells) were comparable (p=0.9, t-test) (Fig. 8B). The FRET intensity measured for cells expressing ECFP/EYFP was negligible (not shown, Fig. 8A).

To rule out that the similar FRET intensity values resulted from averaging data from different cells, we performed cell-by-cell FRET intensity analysis. This allowed us to calculate possible changes in FRET intensity between cytosol and membrane receptors due to a change in distance between fluorophores during co-trafficking. However, cell-by-cell intensity analysis revealed no significant difference between intracellular and membrane FRET for both P2X$_2$-ECFP/P2X$_2$-EYFP pair (p=0.9, n=38, paired t-test) and GABA$_A$-ECFP/ P2X$_2$-EYFP pair (p=1.0, n=38, paired t-test) (Fig. 8C and D). Altogether, these results suggest that GABA$_A$Rs associate with P2X$_2$Rs before reaching the cell surface, possibly in the endoplasmic reticulum. Moreover, the comparable FRET intensity in the cytosol and at the cell membrane suggests that there was no significant change in the distance between the donor and acceptor during trafficking from the cytosol to the cell membrane.

5.3. EXTRASYNAPTIC CO-LOCALIZATION OF GABA$_A$RS AND P2X$_2$RS IN SPINAL CORD NEURONS

P2X$_2$Rs are highly expressed in spinal cord either as homo-trimeric P2X$_2$Rs or as hetero-trimeric P2X$_{2/3}$Rs. To study whether these receptors interact with endogenous GABA$_A$Rs, we performed immunolabeling of receptors at the surface of spinal cord neurons. Such neurons were cultured from mRFP-gephyrin knock-in mice (Calamai et al. 2009) where the inhibitory synapses can be identified by visualizing mRFP-gephyrin clusters. 10-11 DIV old neurons were stained using anti-rabbit antibodies against the extracellular region of P2X$_2$Rs (Fig. 9) and mouse monoclonal anti-β2/β3 subunit specific antibodies to label the extracellular domain of β2/β3 subunits of GABA$_A$Rs. Immunostaining was performed in the absence of detergent to label only surface receptors. The images were acquired by a spinning-disk confocal microscope using a 63X magnification objective. Cells showing good fluorescence signal for both P2X$_2$Rs and GABA$_A$Rs were imaged and acquisition conditions were kept constant during the experiment.
**Figure 9.** Validation of P2X$_2$R antibody. (A) Western blot analysis to check the specificity of P2X$_2$R antibody. A single band at 55KDa was detected from protein extract prepared from rat brain. The predicted MW of P2X$_2$ is around 47KDa. (B) Western blot analysis of protein extracts from HEK cells transfected with different GFP-tagged P2X receptor subtypes shows that the antibody specifically detects P2X$_2$Rs at 85KDa (predicted 74KDa).

P2X$_2$Rs show a clear labeling over the surface of the cell body as well as over dendrites. In contrast, GABA$_A$Rs are highly enriched in dendrites, and much less over the cell body. Merged image demonstrates that indeed the two receptors co-localize with each other as indicated by the tightly associated red and green dots shown in (Fig. 10A, top).

Quantitative analysis (±SE) shows that 7.1 ± 0.5% of β2/β3 subunit containing GABA$_A$R clusters co-localize with P2X$_2$R clusters, whereas 20.6 ± 0.9% of P2X$_2$R clusters overlap with GABA$_A$R clusters (n=55 cells, 4 independent experiments from four different cultures). MIA images (see materials and methods) for a section of dendrite is shown for P2X$_2$Rs, GABA$_A$Rs and overlaid channels (Fig. 10A, bottom). mRFP-gephyrin (shown in blue) images were also acquired simultaneously along with the two receptors. Quantitative analysis of the merged images for gephyrin/P2X$_2$ (Fig. 10B), gephyrin/GABA$_A$ (Fig. 10C), and gephyrin/P2X$_2$/GABA$_A$ (Fig. 10D) demonstrate that only 3.4 ± 0.3% (±SE) of P2X$_2$Rs exist at inhibitory synapses (n=40 cells, 4 independent experiments from four
different cultures). Altogether, we demonstrate co-existence of GABA<sub>A</sub>R/P2X<sub>2</sub>R clusters in cultured spinal cord neurons. Further, as P2X<sub>2</sub>Rs are very rare at inhibitory synapses, we conclude that GABA<sub>A</sub>Rs and P2X<sub>2</sub>Rs co-localize mainly at extrasynaptic localizations.

**Figure 10.** Extrasynaptic co-localization of P2X<sub>2</sub>Rs and GABA<sub>A</sub>Rs in spinal cord neurons. Cultured spinal cord neurons (10-11 DIV) from mrfp-gephyrin knock-in mice were stained for P2X<sub>2</sub>Rs and GABA<sub>A</sub>Rs (β2/β3-subunit) without permeabilization. P2X<sub>2</sub>Rs (green-FITC), GABA<sub>A</sub>Rs (red-cy5) and gephyrin (blue-mRFP) were visualized using a spinning-disk confocal microscope. (A) P2X<sub>2</sub>Rs are expressed both over the surface of the cell body as well as over the dendrites, whereas GABA<sub>A</sub>Rs are mainly enriched over dendrites. Lower panel represents a section of a dendrite (boxed region) after multidimensional image analysis (MIA, See Materials and Methods). Two-representative co-localized clusters depict GABA<sub>A</sub>Rs and P2X<sub>2</sub>Rs co-localize at synaptic (line arrow) and extrasynaptic (block arrow) location. (B, C, D) Overlaid images for P2X<sub>2</sub>Rs and gephyrin, GABA<sub>A</sub>Rs and gephyrin and all three channels. Line arrow represents all three proteins co-localized while other arrows depict co-localization of only two proteins. Scale bar: 10 µm.
5.4. MODULATION OF GABA_A R AND P2X_2 R DISTRIBUTION BY PURINERGIC DRUGS IN SPINAL CORD NEURONS

Previous studies on GABA_A R and P2X_2 R interaction was performed in the presence of agonists and all our results so far suggest agonist independent interaction between the two receptors. We were interested to see if activation or deactivation of P2X_2 Rs has any effect on the strength of this association. 10-11 DIV spinal cord neurons were first incubated with the P2XR agonist 2MeS-ATP, or the antagonist TNP-ATP, for two hours followed by immunostaining. A section of dendrites stained for GABA_A Rs β2/β3 subunit (red) and P2X_2 Rs (green) is shown for all conditions (Fig. 11A).

**Figure 11.** Regulation of GABA_A and P2X_2 Rs immunoreactivity by purinergic drugs. Spinal cord neurons (10-11 DIV) were treated for 2hrs with 2MeS-ATP (100µM) or TNP-ATP (100µM) and stained for GABA_A Rs (β2/β3-subunit, red-cy5) and P2X_2 Rs (green-FITC). Inhibitory synapses were identified based on gephyrin
clusters. (A) A representative dendrite is shown for different conditions. (B) The normalized fluorescence intensity demonstrates that 2MeS-ATP has no significant effect on the immunoreactivity of synaptic and extrasynaptic GABA\(_A\)Rs whereas it significantly increased the fluorescence intensity of P2X\(_2\)Rs. In contrast, TNP-ATP treatment significantly elevated the fluorescent intensity of both receptors. The normalized fluorescence intensity value (± SE) for total GABA\(_A\)Rs: Control: 0.81 ± 0.02, n=43; 2MeS-ATP: 0.81 ± 0.02, n=44 (ns, p=0.205); TNP-ATP: 0.90 ± 0.02, n=44 (**, p<0.001), for synaptic GABA\(_A\)Rs: Control: 0.85 ± 0.02, n=15; 2MeS-ATP: 0.85 ± 0.03 (n=15; ns, p=0.851); TNP:ATP: 0.93 ± 0.03, n=15 (*, p<0.05), for extrasynaptic GABA\(_A\)Rs: Control: 0.90 ± 0.02, n=15; 2MeS-ATP: 0.91 ± 0.03, n=15 (ns, p=0.948); TNP:ATP: 1.04 ± 0.02, n=15 (***, p<0.0001), for total P2X\(_2\)Rs: Control: 0.76 ± 0.05, n=24, 2MeS-ATP: 1.25 ± 0.06, n=30 (***, p<0.0001); TNP:ATP: 1.24 ± 0.05, n=31 (***, p<0.0001). Scale bar: 10 µm

2MeS-ATP treatment had no visible effect on GABA\(_A\)R fluorescence intensity but at the same time P2X\(_2\)R clusters showed increased fluorescence intensity (Fig. 11A, second row). Quantitative analysis indicated no significant change in the intensity of both synaptic and extrasynaptic GABA\(_A\)Rs, but a significant up regulation of the fluorescence intensity of total P2X\(_2\)Rs (Fig. 11B). On the other hand, the competitive antagonist TNP-ATP strongly elevated the fluorescence signal for both GABA\(_A\)Rs and P2X\(_2\)Rs (Fig. 11A, third row). Quantitative analysis of fluorescence intensity of GABA\(_A\)R and P2X\(_2\)R clusters shows that TNP-ATP treatment significantly enhanced the fluorescence intensity of both the receptors (Fig. 11B). Even though pharmacological modulation resulted in re-distribution of GABA\(_A\)Rs and P2X\(_2\)Rs, we observed no significant change in the percentage of co-localized receptor clusters (not shown). Together, these results suggest that purinergic receptors can directly modulate the distribution of not only P2X\(_2\)Rs but also of GABA\(_A\)Rs.

**5.5. REGULATION OF GABA\(_A\)R DIFFUSION DYNAMICS BY DRUGS ACTING ON PURINERGIC RECEPTORS**

We performed single particle tracking using the quantum dot technique to evaluate the effects of P2XR agonists or antagonists on the lateral diffusion of GABA\(_A\)Rs. The experiments were performed on spinal cord neurons from mRFP-gephyrin knock-in mice allowing the direct visualization of inhibitory synapses. The drugs were added to the imaging medium after labeling the receptors with quantum dots (GABA\(_A\)R-QD). The effect of drugs on receptor diffusion could be evaluated from the area explored by GABA\(_A\)R-QD trajectories. The activation of P2XRs by 2MeS-ATP (100µM) increased, whereas the antagonist, TNP-ATP (100µM) decreased the surface explored by GABA\(_A\)Rs (Fig. 12A). The cumulative frequency distribution of diffusion
**Figure 12.** Modulation of GABA\textsubscript{A}R membrane dynamics by purinergic drugs. The $\gamma$2-subunit of GABA\textsubscript{A}Rs was labeled with QD in spinal cord neurons (DIV 11-12) from mrfp-gephyrin mice. (A) Examples of surface exploration by GABA\textsubscript{A}R-QDs (green) for 38.4s in presence of 2MeS-ATP (100µM) and TNP-ATP (100µM). mRFP-gephyrin (red clusters) represents inhibitory synapses. Notably, GABA\textsubscript{A}R-QDs explored a larger surface area in presence of agonist, while their surface exploration is greatly diminished in the presence of antagonist. (B) Cumulative frequency distribution of diffusion coefficient D for GABA\textsubscript{A}R-QD trajectories in the synapse (control, n= 293; 2MeS-ATP, n=279; TNP-ATP, n=150) and outside the synapse (control, n= 858; 2MeS-ATP, n=1016; TNP-ATP, n=1206). Agonist accelerated extrasynaptic receptors, while antagonist slowed down both synaptic and extrasynaptic receptors. Values are from four independent experiments from four different cultures, (*p < 0.05, ***p < 0.005; Kolmogorov-Smirnov test). (C) Average ($\pm$ SE) MSD over time for GABA\textsubscript{A}R-QDs at synaptic and extrasynaptic sites. (D) The average confinement size (µm) of synaptic and extrasynaptic GABA\textsubscript{A}R-QDs indicates that TNP-ATP treated extrasynaptic receptors were more confined in a micro domain. (E) Percentage of immobile (D < 10^{-4} µm$^2$/s) synaptic and extrasynaptic receptors. The number of immobile ($\pm$ SEM) synaptic receptors was 8.3 $\pm$ 3.4% for control (n=134), 9.4 $\pm$ 3.8% for 2MeS-ATP (n=139) and 5.2 $\pm$ 2.7% for TNP-ATP (n=97) treated cells and extrasynaptic receptors was 11.8 $\pm$ 0.9% for control (n=858), 11.7 $\pm$ 1.9% for 2MeS-ATP (n=1016) and 18.1 $\pm$ 1.9% for TNP-ATP (n=1377) treated cells. Note that the agonist had no distinct effect on the proportion of immobile GABA\textsubscript{A}R-QDs, whereas the antagonist significantly increased the number of immobile extrasynaptic GABA\textsubscript{A}R-QDs. (***, p<0.001, t-test). Scale: 5 µm.
coefficient (D), for extrasynaptic (p<0.05) and synaptic (not significant) receptors (Fig. 12B) indicated an overall but weak increase in the diffusion rate in presence of the P2XR agonist 2MeS-ATP. In contrast the antagonist, strongly reduced the diffusion of both synaptic (***, p<0.001) and extrasynaptic (***, p<0.001) GABA<sub>A</sub>R-QD (Fig. 12B, green).

Synaptic and extrasynaptic trajectories of GABA<sub>A</sub>Rs were further analyzed using the mean square displacement (MSD) plotted as a function of time (Fig. 12C). The negative bent of the MSD curve indicates the level of confinement of receptors in a given sub-domain (Bannai et al., 2009, Alcor et al., 2009). 2MeS-ATP treatment slightly increased the slope of the average MSD for both synaptic and extrasynaptic receptors (Fig. 12C, red). On the other hand, TNP-ATP decreased the average slope of the MSD for synaptic receptors and this effect was even more dramatic for extrasynaptic GABA<sub>A</sub>Rs (Fig. 12C, green). These changes in the GABA<sub>A</sub>R QDs MSD curves are consistent with the modulations observed for the diffusion coefficients (Fig. 12B).

MSD curves are consistent with the modulations observed for the diffusion coefficients (Fig. 12B). The size of the microdomains in which receptors are confined can be calculated from the MSD curves (Kusumi et al., 1993, Bannai et al., 2009, see materials and methods). At synapses the size of the GABA<sub>A</sub>R confinement domain was not modified by drug treatment (Control: 0.18 ± 0.02µm, n=93; 2MeS-ATP: 0.19 ± 0.02µm, n=111; TNP-ATP: 0.16 ± 0.02µm, n=69) (Fig. 12D, black). At extrasynaptic locations, agonist treatment had no effect on confinement domain, but it is significantly reduced by the antagonist indicating that the receptors were more confined (Control: 0.26 ± 0.01µm, n=545; 2MeS-ATP: 0.29 ± 0.01µm, n=615; TNP-ATP: 0.12 ± 0.01µm, n=718) (Fig. 12D, grey). These observations are in line with the modification of the shape of the MSD plots (Fig. 12C). The reduction in the size of the confinement domain suggested that some extrasynaptic receptors may be stabilized in the presence of TNP-ATP. This can be also estimated by the proportion of immobile (D < 10<sup>-4</sup> µm/s<sup>2</sup>) receptors. As expected, only TNP-ATP treatment increased the number of immobile extrasynaptic receptors, which almost doubled (Fig. 12E, gray).

5.6. MODULATION OF P2X<sub>2</sub>R DIFFUSION PROPERTIES BY PURINERGIC DRUGS

We then explored how the membrane dynamics of P2X<sub>2</sub>Rs itself could be modulated by purinergic drugs. SPT experiments were performed using P2X<sub>2</sub>-FLAG-EGFP receptor transfected in spinal cord neurons (see materials and methods). P2X<sub>2</sub>R-QDs were only rarely observed at gephyrin positive synapses (not shown); this is why we have analyzed global pool of P2X<sub>2</sub>Rs on the neuronal membrane
independent of inhibitory synaptic localization. The surface explored by QDs over the acquisition period emphasizes the effects of the drug on lateral diffusion (Fig. 13A) and showed that the overall explored surface area was reduced with both the agonist and the antagonist (Fig. 13A). The distribution of diffusion coefficient, D was not significantly different between the 2MeS-ATP and the control experiments (ns, p=0.226, n=495 for control and n=481 for 2MeS-ATP, KS test) (Fig. 13B, blue and red).

Figure 13. Regulation of P2X$_2$R diffusion dynamics by purinergic drugs. Spinal cord neurons (DIV 9) were transfected with P2X$_2$-FLAG-EGFP receptors having the FLAG-tag in the extracellular region and EGFP at the C-terminus. EGFP was used to identify transfected cells and single particle QD tracking of P2X$_2$Rs was performed by labeling the FLAG-tag of the receptor. (A) Examples of surface explored by P2X$_2$R-QDs (red) over transfected cell (green) for different conditions. (B) Cumulative frequency distribution of diffusion for P2X$_2$R-QDs trajectories shows no significant change in receptor diffusion on agonist treatment whereas antagonist treatment significantly slowed down the receptors (**p < 0.001, Kolmogorov-Smirnov test). (C) Average MSD plot for trajectories of P2X$_2$R-QDs shows that 2MeS-ATP as well TNP-ATP increased the confinement of the P2X$_2$Rs. These are typical results from three independent experiments.
However, as indicated by the MSD plot, the confinement of P2X₂-R-QDs increased in presence of 2MeS-ATP compared to the control (Fig. 13C, blue and red). The antagonist treatment lowered the diffusion of P2X₂-R-QDs (***, p < 0.005, n=495 for control and n=475 for TNP-ATP) (Fig. 13B, blue and green) and also increased the confinement (Fig. 13C, blue and green).

Thus, the mechanisms leading to reduced diffusion of P2X₂-Rs in presence of 2MeS-ATP or TNP-ATP are different (Holcman and Triller, 2006). In both cases there is an increase in confinement, but decrease in diffusion coefficient was observed only for latter. In case of 2MeS-ATP, this indicates that the diffusion rate was not affected by the binding of agonist, but that the surface area in which diffusion take place was reduced. This is in favor of multiple binding events of short dwell time with unbiased diffusion between them. In case of TNP-ATP, the antagonist rather led to long binding events which reduced the overall lateral diffusion and increase the confinement.

5.7. MODULATION OF GABAₐR AND P2X₂R INTERACTION BY PURINERGIC DRUGS

SPT experiments suggested that an agonist of P2X₂-Rs increased, while an antagonist decreased the mobility of extrasynaptic GABAₐRs at the cell surface. To further study this effect, we investigated whether pharmacological regulation of P2X₂-Rs by purinergic drug alters the amount of associated GABAₐRs at the surface of HEK cells. Cell surface co-immunoprecipitation was performed as described in Fig. 4. Cells expressing GABAₐR α₁, β₂ and γ₂ subunits as well as P2X₂-EYFP subunits were incubated with either 2MeS-ATP (30µM) or TNP-ATP (10µM) for 1 hr. Surface GABAₐRs were immunolabeled by α₁-subunit specific antibodies followed by extraction and precipitation of antibody-labeled receptors by adding immunoprecipitin. The remaining intracellular GABAₐRs were subsequently precipitated by incubating with α₁-subunit specific antibodies. Changes in surface levels of GABAₐ receptors following drug treatment were then measured by Western blotting. 2MeS-ATP treatment reduced the level of surface GABAₐRs and at the same time, we observed an increased degradation of intracellular receptors (Fig. 14, lanes 1 and 2). The co-associated P2X₂-Rs were detected using digoxigenin-labeled anti-EGFP-antibodies. Similar to previous observation (Fig. 4B, lane 2), we observed a very weak association of P2X₂-Rs with GABAₐRs for control and 2MeS-ATP treated cells. (Fig. 14, lane 1 and 2). In contrast, TNP-ATP treatment had no effect on the surface level of GABAₐRs but highly up regulated the associated P2X₂-Rs (Fig. 14, lane 1 and 3). Taken together we
demonstrate here that agonist binding on P2X\textsubscript{2}Rs reduce the surface expression of GABA\textsubscript{A}Rs and targets it for degradation whereas antagonist binding highly stabilizes the interaction.

**Figure 14.** Modulation of direct association of GABA\textsubscript{A}Rs and P2X\textsubscript{2}Rs by purinergic drugs. HEK cells were co-transfected with GABA\textsubscript{A}R α1, β2, and γ2 subunits and EYFP-tagged P2X\textsubscript{2}R subunits. 48 hours after transfection, cells were treated with either 2MeS-ATP (30\textmu M) or TNP-ATP (10\textmu M) for 1 hour followed by surface precipitation of GABA\textsubscript{A}R using α1-subunit specific antibodies. Precipitated GABA\textsubscript{A}Rs were detected using digoxigenin-labelled α1 antibodies and co-precipitated P2X\textsubscript{2}Rs were detected using digoxigenin-labelled EGFP antibodies. 2MeS-ATP, but TNP-ATP treatment, decreased the surface the level of GABA\textsubscript{A}Rs. While we detected very weak association of P2X\textsubscript{2}Rs with GABA\textsubscript{A}Rs for control and agonist treated cells, we observed a very strong association when the cells were treated with TNP-ATP.
6. DISCUSSION

6.1. INTRACELLULAR ASSOCIATION AND CO-TRAFFICKING OF GABA\textsubscript{A}RS AND P2X\textsubscript{2}RS ENSURES SPECIFIC TARGETING OF P2X\textsubscript{2}RS

Several lines of evidence indicate that GABA\textsubscript{A}Rs and P2X\textsubscript{2}Rs directly associate with each other in the endoplasmic reticulum in an agonist independent way: First, both receptors could be co-immunoprecipitated from the cell surface or from a total extract of appropriately transfected HEK cells, using antibodies directed against either one of these receptors (Fig. 4); Second, both receptors are co-localized in membranes and the cytoplasm of HEK cells co-transfected with ECFP-tagged GABA\textsubscript{A}Rs and EYFP-tagged P2X\textsubscript{2}Rs as demonstrated by confocal microscopy (Fig. 7). Third, FRET experiments performed in these cells resulted in a similar FRET signal in the cytosol and at the membrane, suggesting that the same type of association of the GABA\textsubscript{A}/P2X\textsubscript{2}R complex is observed in these compartments (Fig. 8). Fourth, transfecting HEK cells with P2X\textsubscript{2}Rs and a trafficking deficient GABA\textsubscript{A}R mutant resulted in 90% reduction of GABA\textsubscript{A}Rs and a 21% reduction of P2X\textsubscript{2}Rs at the cell surface, providing direct evidence for co-trafficking of these receptors (Fig. 4B). Fifth, co-localization of endogenous GABA\textsubscript{A} and P2X\textsubscript{2}Rs in the endoplasmic reticulum (Protein disulfide isomerase, PDI) or Golgi apparatus (Golgi-matrix protein, GM130), the two crucial check-points of forward trafficking, was demonstrated in neurons (experiments not shown).

Interestingly, co-expression of P2X\textsubscript{2}Rs resulted in a 3-fold up-regulation in the expression of GABA\textsubscript{A}Rs in HEK cells (Fig. 1A), whereas the number of P2X\textsubscript{2}Rs expressed was not significantly influenced by the co-expression of GABA\textsubscript{A}Rs. This seems to indicate that P2X\textsubscript{2}Rs might have a stabilizing or chaperone function on a GABA\textsubscript{A}R subpopulation, preventing them from degradation. Such an intracellular function of P2X\textsubscript{2}Rs is supported by the fact that these receptors are highly enriched in the cytoplasm of the cell body of spinal cord neurons (not shown). Once associated, the complex is then co-trafficked to the cell surface and inserted extrasynaptically, where we observed them to be co-localized (Fig. 7). Interestingly, previously it was demonstrated that co-expression of P2X\textsubscript{2}Rs resulted in distal targeting of GABA\textsubscript{C} and GABA\textsubscript{A}Rs (Boué-Grabo et al., 2004a, 2004b). Together with our results, this indicates that GABA\textsubscript{A}Rs stabilized by P2X\textsubscript{2} receptors help to traffic these receptors to specific localizations at the cell surface.
6.2. PURINERGIC TRANSMISSION DECREASES GABAERGIC INHIBITION IN SPINAL CORD NEURONS

It was possible that GABA$_A$Rs and P2X$_2$Rs co-inserted into the cell membrane became enriched at inhibitory synapses. Actually, however, we observed only a very small fraction of co-localized receptors at inhibitory synapses (Fig. 14). This is consistent with previous studies indicating that P2X$_2$Rs are mainly enriched at glutamatergic synapses in the brain (Rubio and Soto, 2001; Masin et al., 2006). These observations indicate that the two associated receptors either function at extrasynaptic regions or they dissociate and subsequently exhibit functions independent from each other. In fact, several lines of evidence indicate that upon activation of P2X$_2$Rs by an agonist, the co-associated GABA$_A$Rs dissociate and are internalized and degraded whereas P2X$_2$Rs are stabilized. First, application of the P2X$_2$R agonist 2MeS-ATP increased the mobility of extrasynaptically located GABA$_A$Rs (Fig. 12), but did not change the mobility of P2X$_2$Rs and on the contrary, increased their confinement (Fig. 13) and cluster size (Fig. 11). This is consistent with a previous study where it was reported that ATP treatment resulted in hot-spots of P2X$_2$-GFP receptors (Khakh et al., 2001). Second, immunoprecipitation of receptors at the surface and intracellular compartments of HEK cells co-transfected with GABA$_A$ and P2X$_2$Rs indicated that incubation with 2MeS-ATP reduced the number of GABA$_A$Rs at the cell surface and increased the formation of GABA$_A$R degradation products in the cytosol (Fig. 14). The amount of P2X$_2$Rs was not changed under these conditions (Fig. 14), indicating that the increased clustering of these receptors (Fig. 11) on 2MeS-ATP treatment was not caused by newly incorporated receptors but by an increased confinement (Fig. 13) of freely diffusing P2X$_2$Rs at pre-existing P2X$_2$R clusters. And third, co-transfection of HEK cells with GABA$_A$Rs and P2X$_2$Rs increased the expression of GABA$_A$Rs in intracellular compartments, but caused a reduction of GABA$_A$Rs at the cell surface (Fig. 4). Since there was no change in the number of P2X$_2$Rs in intracellular compartments and at the cell surface under these conditions, the increased amount of GABA$_A$Rs in the intracellular compartments did not result in an increase of P2X$_2$R incorporation into the cell membrane. The reduced number of GABA$_A$Rs at the cell membrane then probably was caused by a dissociation of GABA$_A$Rs from the associated P2X$_2$Rs mediated by endogenous ATP present in the cell culture medium, followed by their internalization and degradation.

Dissociation of GABA$_A$Rs from associated P2X$_2$Rs can either be elicited by an ATP-induced conformational change in P2X$_2$Rs, or by the subsequent P2X$_2$R-mediated Ca$^{2+}$ influx into the cell.
Although it was demonstrated that GABA\textsubscript{A}R dynamics was unaltered by NMDA-elicited changes of the intracellular Ca\textsuperscript{2+} concentration in spinal cord neurons (Lévi et al., 2008), we cannot exclude conformational changes elicited by local Ca\textsuperscript{2+} influx via associated P2X\textsubscript{2}Rs. Rapid dissociation and internalization of GABA\textsubscript{A}Rs of course could be one of the mechanisms playing a role in the cross-talk of GABA\textsubscript{A} and P2X\textsubscript{2}Rs. But this for sure is not the only mechanism involved and it has to be emphasized that the present results describe the effects of 2MeS-ATP on GABA\textsubscript{A} and P2X\textsubscript{2}R distribution in neurons or HEK cells after 2 h treatment with high drug concentrations. In addition, the single particle tracking experiments were also performed over a time period of 5-20 min during drug application. These data, thus, describe long term effects of drugs and cannot necessarily provide explanations for a possible mechanism of the cross-talk between GABA\textsubscript{A} and P2X\textsubscript{2}Rs.

As members from P2XR family have been demonstrated to show cross-talk with not only GABA\textsubscript{A}Rs (Sokolova et al., 2001; Boué-Grabort et al., 2004b; Karanjia et al., 2006; Toulmé et al., 2007) but also with several other members of cys-loop receptors which includes 5-HT\textsubscript{3} receptors (Zhou and Galligan, 1998; Boué-Grabort et al., 2003) GABA\textsubscript{C} receptors (Boué-Grabort et al., 2004a) and nACh receptors (Khakh et al., 2000; Khakh et al., 2005), association of P2X\textsubscript{2}Rs with other receptors and co-trafficking to specific locations at the cell surface in fact may be a general phenomenon. Indeed, such cys-loop receptor mediated recruitment to the cell surface is possibly not restricted to P2X\textsubscript{2}Rs as several other members of P2XR family are now known to interact with GABA\textsubscript{A}Rs (P2X\textsubscript{1}, P2X\textsubscript{4} and P2X\textsubscript{5}: experiments not shown; P2X\textsubscript{3}: Sokolova et al., 2001; Toulmé et al., 2007) and nicotinic receptors (P2X\textsubscript{2}, P2X\textsubscript{3} and P2X\textsubscript{4}: Decker and Galligan, 2009). Such a receptor-mediated targeting of P2XRs becomes further important due to a lack of specifically defined synapses for these receptors. In any case, in the absence of P2X\textsubscript{2}R-selective drugs we cannot exclude that part of the agonist or antagonist-induced changes observed were elicited via other P2XR subtypes present in spinal cord.

6.3. Blocking P2XRs as a dual therapeutic strategy for spinal pain processing

Inflammatory diseases and neuropathic injury are frequently accompanied by severe and debilitating pain. Loss of synaptic inhibition by GABAergic and glycinergic spinal dorsal horn neurons has been proposed as one of the key pathways in propagation of nociceptive and neuropathic information (Knabl et al., 2008; Zeilhofer and Zeilhofer, 2008; Zeilhofer et al., 2009). A key molecule involved in spinal pain processing is ATP. It was established long back that exogenously applied ATP can induce pain sensation (Bleehen and Keele, 1977; Nagagawa et al., 2007) by activating P2XRs in lamina II of
spinal cord (Bardoni et al., 1997; Jo and Schlicter, 1999; Souslova et al., 2000). Our study demonstrates that the two pathways mediated by P2X and GABA\(_A\)Rs overlap and a fine balance between the two systems is essential to maintain homeostasis. Excess ATP released in diseased states will not only activate P2XR, but also result in a dissociation, internalization and degradation of P2XR-associated surface GABA\(_A\)Rs, thus further strengthening of P2XR activity by spinal dis-inhibition. Further experiments with spinal cord slices will have to strengthen this hypothesis.

The present finding that treatment with the competitive P2XR antagonist, TNP-ATP (Trujillo et al., 2006) resulted in increased clustering and slowing down of both GABA\(_A\)Rs and P2X\(_2\)Rs (Fig. 11, 12, 13), in cultures suggests a dual mechanism of action: blockade of the excitatory actions of P2XRs and strengthening of GABAergic inhibition by preventing degradation (Fig. 14). In fact, TNP-ATP has been shown to suppress the ATP induced effect in acute inflammatory or visceral pain following injury (Jarvis et al., 2001; Honore et al., 2002). The short half-life of TNP-ATP, however, does not make it suitable for therapeutic studies (Ding et al., 2000). P2X\(_2\)R antagonists exhibiting a longer half-life, however, might be useful candidates for preventing spinal-excitation as well as GABAergic disinhibition.
7. REFERENCES


Khakh BS, Fisher JA, Nashmi R, Bowser DN, Lester HA (2005) An angstrom scale interaction between plasma membrane ATP-gated P2X2 and alpha4beta2 nicotinic channels measured with


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