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RECONSTITUTION OF GABAERGIC POSTSYNAPSES IN HOST CELLS

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Undergraduate Honors Thesis
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Abstract
Type A GABA receptors (GABAARs) can be found embedded in postsynaptic membranes or in a variety of extrasynaptic locations. Receptors with synaptic function are recruited to the postsynapse by submembranous scaffolds composed of gephyrin and collybistin (CB). This study was aimed at assessing whether the ability to interact with the scaffold differentiates synaptic from non-synaptic receptors. Using HEK293 cells as an expression system, and indirect immunofluorescence (IF), co-localization of extrasynaptic receptors α1β3δ and α4β3δ with the CB-gephyrin scaffold was assessed and compared with that of the synaptic receptor α1β3γ2. Results indicated that both extrasynaptic receptors were able to colocalize with the scaffold. This observation indicates that there must be some other mechanism responsible for keeping extrasynaptic receptors outside of the postsynapse.

Introduction
The type A GABA receptor (GABAAR) is a chloride channel found on the postsynaptic targets of GABAergic neurons in the central nervous system. The receptor is pentameric and composed of a combination of subunits, including α1-6, β1-3, and γ1-3. Additional subunits include δ, ε, θ, and π of which δ is extrasynaptic and responsible for forming channels that modulate neuronal excitability through tonic inhibition (Brickley and Mody, 2012). Most receptors in the CNS are composed of two α subunits, two β subunits, and a γ2 or δ subunit (Tretter et al., 2012). Different combinations can influence the function and behavior of GABAARs. According to Moss and Smart (2001), co-expression of α and β subunits is sufficient to produce a functioning GABAA chloride current in vitro. Likewise, the inclusion of a γ subunit is essential in producing a channel sensitive to benzodiazepines. However, the behaviors and interactions of the extrasynaptic δ, α4, and α5 subunits are yet to be determined.

GABAARs are localized at the synapse by interacting with a scaffolding protein, gephyrin, and collybistin (CB), a Rho-GTPase. These two proteins form submembranous clusters when co-expressed (Figure 1). Gephyrin homotrimers are transported
to the postsynapse where they are attach to the membrane with the help of CB (Schrader et al., 2004). All CB isoforms have a Rho guanine nucleotide exchange factor domain (RhoGEF), and a phosphoinositide-binding pleckstrin homology (PH) domain. The CB SH3+ isoform contains an additional src homology 3 (SH3) domain that autoinhibits CB phosphoinositide binding. In HEK293 cells, neuroligin 2 (NL2), or the α2 GABAAR receptor subunit are required to relieve this autoinhibition and induce the formation of gephyrin clusters. Conversely, the constitutively active SH3– variant, when co-transfected with gephyrin, has been shown to result in the formation of GABAAR superclusters in cultured hippocampal neurons (Chiou et al., 2011). These findings were mirrored in studies of chronic transgenic expression of CB2SH3+ and CB2SH3– in vivo (Fekete et al., 2017).

We hypothesized that synaptic receptors are localized at the synapse because of their ability to bind to the submembranous CB-gephyrin scaffold. Extrasynaptic receptors, on the other hand, do not have this property. The following study is interested in testing this hypothesis using HEK293 cells as a heterologous expression system.

**Materials and Methods**

**HEK293 Cell Culture.** Frozen 1 mL aliquots of HEK293 cells were thawed in ethanol at 36°C and added to 10% FBS/DMEM in a 15 mL Falcon tube. After centrifugation, the supernatant was aspirated with a sterile glass pipet and the cell pellet was resuspended in 12 mL of 10% FBS/DMEM prior to plating. Cells were split approximately once per week, or when 60-70% confluent. Growth media was replaced 24 hours after splitting. Wescodyne was added to tissue plates prior to discarding cultures if cells did not look healthy. Normally, cell cultures would be replaced with newer stocks once 16 passages were reached.

**Preparation of Plasmids and Experimental Layout.** DH5-Alpha *Escherichia coli* containing plasmids with kanamycin or ampicillin resistance markers were placed in 50 mL LB media inoculated with either kanamycin or ampicillin. Cells were rocked overnight at 250 rpm at 37°C. The following day, cells were pelleted and plasmids were isolated using a QIAGEN Midiprep Kit. The extent of colocalization of extrasynaptic receptors α1β3δ and α4β3δ with the CB-gephyrin scaffold was compared to that of the synaptic α1β3γ2 receptor. A list of all plasmids used in this study are provided in Table 1. Receptor subunits were transfected without eGFP-gephyrin and CB2SH3– first to assess whether the receptors could be reconstituted in cells and translocate to the membrane surface. Then, the receptors’ ability to interact with the synaptic scaffold was analyzed.

**Transfection of HEK293 Cells.** Sterilized coverslips were added to wells in a 12-well plate and coated with poly-L-lysine. After washing twice, 1 mL HEK293 cells suspended in 1% FBS/DMEM were added to each well. Cells were allowed to incubate at 37° and 5% CO₂ for 48 hours. Transfection was carried out using the calcium phosphate method. Plasmids were mixed with CaCl₂ and sterilized water to give a total volume of 50 µL. An equivalent volume of HEPES buffered saline (HBS) was added dropwise while gently vortexing the reaction tube. Transfection
reagents were added to their respective wells and allowed to incubate for around four hours before the transfection media was aspirated and replaced with 0.25% FBS/DMEM. Coverslips were prepared for immunocytochemistry after allowing 48 hours for expression.

<table>
<thead>
<tr>
<th>Synaptic</th>
<th>Extrasynaptic</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1β3γ2</td>
<td>α1β3δ</td>
</tr>
<tr>
<td>pcDNA3.1α1</td>
<td>pcDNA3.1α1</td>
</tr>
<tr>
<td>pcDNA3.1β3</td>
<td>pcDNA3.1β3</td>
</tr>
<tr>
<td>pcDNA3.1γ2S</td>
<td>pRK5-myc-δ</td>
</tr>
</tbody>
</table>

**Table 1: Receptors and plasmids used for transfection of GABAAR subunits.** In some transfections, a β3-eGFP plasmid was used in lieu of pcDNA3.1β3. Scaffolds were produced by co-transfection of eGFP-gephyrin and pCAGGS-HA-CB2SH3– or pRK5-myc-CB2SH3–. We have chosen to include the α1β3γ2 and α4β3δ receptors in this study since they are the most abundant synaptic and extrasynaptic receptors, respectively, in the CNS.

**Immunocytochemistry and Fluorescence Microscopy.** Immunocytochemistry (ICC) was conducted using indirect immunofluorescence (IF) to label target antigens. For fluorescent labelling of extracellular N-terminal domains of certain subunits, a two-step capping method was used. Here, live cells were incubated with a mixture of primary antibodies, then with a mixture of secondary antibodies (with a washing step in between) prior to fixation and permeabilization (Christie, 2005). For tagging of intracellular antigens, cells were briefly fixed with 4% paraformaldehyde/4% sucrose/PBS, quenched with 50 mM NH₄Cl, and permeabilized with 0.25% Triton X-100/PBS. Donkey normal serum (DNS) was used in the blocking step. Primary antibodies were mixed together, diluted in 0.25% Triton X-100/PBS, added to each well, and allowed to incubate overnight at 4°C. Secondary antibodies were prepared in a similar fashion, added to their respective wells, and allowed to incubate for two hours at room temperature. After washing, coverslips were removed from each well and mounted on glass slides using Prolong Gold Antifade Mountant. Slides were cured for 1-2 days and viewed under a fluorescence microscope using a 60X oil immersion objective lens.

**Results**

HEK293 cells were first transfected with GABAAR subunits without the CB-gephyrin scaffold to assess reconstitution and translocation to the membrane. We have previously shown that the α1, β3, and γ2 subunits, when co-transfected, translocate to the membrane and assemble together. Figure 2, illustrates a cell co-transfected with plasmids encoding for the α1,
Figure 2: Reconstitution of the α1β3δ receptor. Colocalization of all subunits at the surface reflect formation of the α1β3δ receptor and insertion into the plasma membrane. Capping (clustering) of subunits as a result of surface-labelling was also observed.

Co-transfected Plasmids
pcDNA3.1α1
pcDNA3.1β3
pRK5-myc-δ

Figure 3: Reconstitution of the α4β3δ receptor. Similar to the result in Figure 2, colocalization of all transfected subunits at the surface was observed – indicating receptor formation at the membrane. In this assay, a β3-eGFP plasmid was used.

Co-transfected Plasmids
pcDNA3.1α4
β3-eGFP
pRK5-myc-δ
β3, and myc-δ subunits. The α1 subunit was labelled with an anti-α1-NH₂ primary antibody and a secondary antibody conjugated to an AMCA fluorophore. Likewise, the β3 subunit was labelled with an anti-β2/3 primary and a secondary antibody conjugated to a FITC fluorophore. Myc-δ was labelled using an anti-myc antibody in the red channel (AF594).

Cells co-transfected with the α4, β3-eGFP, and myc-δ subunits showed similar results (Figure 3). During ICC, the α4 subunit was labelled using an anti-α4-NH₂ primary and an AF594-conjugated secondary antibody. Results indicate that subunits were trafficked to the plasma membrane and, as evidenced by colocalization, had assembled into receptors.

Co-transfection of the subunits listed in Table 2 with the CB-gephyrin scaffold showed colocalization of the subunits at the surface in all cases. Figure 4 shows reconstituted α1β3γ2 receptors colocalized with the eGFP-gephyrin lattice. Here, the γ2 subunit has been labelled using an anti-γ2-NH₂ primary with a secondary antibody conjugated to an AF594 fluorophore. The result of this experiment was similar to that of the extrasynaptic α1β3δ and α4β3δ receptors co-transfected with CB and gephyrin (Figure 5 and Figure 6, respectively).

![Figure 4: HEK293 cell with the reconstituted α1β3γ2 synaptic receptor and CB-gephyrin scaffold. Colocalization of the γ2 and α1 subunits at the surface along with submembranous eGFP-gephyrin clusters indicate interaction between the α1β3γ2 receptor and the synaptic scaffold. This result is expected since both the receptor and scaffold are localized at the synapse in vivo.](image-url)

**Co-transfected Plasmids**
- pcDNA3.1α1
- pcDNA3.1β3
- pcDNA3.1γ2S
- eGFP-gephyrin
- pRK5-myc-CB2SH3–
Figure 5: HEK293 cell with the reconstituted extrasynaptic α4β3δ receptor and CB-gephyrin scaffold. IF images showed colocalization of the α4 and δ subunits at the surface with eGFP-gephyrin clusters. This indicates that there is interaction between the α4β3δ receptor to the CB-gephyrin scaffold.

**Co-transfected Plasmids**
- pcDNA3.1α1
- pcDNA3.1β3
- pRK5-myc-δ
- eGFP-gephyrin
- pCAGGS-HA-CB2SH3

Figure 6: HEK293 cell with the reconstituted extrasynaptic α1β3δ receptor and CB-gephyrin scaffold. IF images illustrate colocalization of the α1 and δ subunits with the eGFP-gephyrin scaffold. This, therefore, shows that there is interaction between the extrasynaptic α1β3δ receptor with the synaptic gephyrin lattice.

**Co-transfected Plasmids**
- pcDNA3.1α4
- pcDNA3.1β3
- pRK5-myc-δ
- eGFP-gephyrin
- pCAGGS-HA-CB2SH3
Discussion

The preceding results illustrate that (1) GABAAR subunits are inserted into the plasma membrane surface and (2) that subunits form receptors. Since all cells were non-permeabilized or fixed for the duration of the ICC protocol, labelling of antigens occurred only at the cell surface. Subunit colocalization indicates that co-transfected subunits assembled as receptors. This is consistent with previous studies on GABAAR reconstitution (Dong et al., 2007). For all receptors tested – the synaptic $\alpha_1\beta_3\gamma_2$ receptor and the extrasynaptic $\alpha_1\beta_3\delta$ and $\alpha_4\beta_3\delta$ receptors – colocalization of surface-capped subunits with the submembranous eGFP-gephyrin scaffold was observed. This indicates that there is interaction between surface receptors and internal gephyrin.

If both synaptic and extrasynaptic receptors are able to interact with gephyrin scaffolds, then there must be an alternate system in place that excludes extrasynaptic receptors from the postsynapse. A proposed mechanism involves the relative affinities of each type of receptor for binding to gephyrin. However, more testing is needed to determine whether this is true.

Conclusion

The preceding study has shown that reconstituted GABAARs interact with the submembranous CB-gephyrin scaffold in vivo. We had initially hypothesized that synaptic receptors are localized at the synapse because of their ability to interact with the gephyrin scaffold and that extrasynaptic receptors do not have this property. However, our experimentation has shown that the synaptic receptor $\alpha_1\beta_3\gamma_2$ and the extrasynaptic receptors $\alpha_1\beta_3\delta$ and $\alpha_4\beta_3\delta$ are all able to interact with gephyrin. Therefore, the ability to interact with the scaffold does not determine whether a GABAAR is synaptic or extrasynaptic. There must be another mechanism by which extrasynaptic receptors are kept outside of the postsynapse.

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