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Ensemble and single particle fluorimetric techniques in concerted action to study the diffusion and aggregation of the glycine receptor α3 isoforms in the cell plasma membrane

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ABSTRACT

The spatio-temporal membrane behavior of glycine receptors (GlyRs) is known to be of influence on receptor homeostasis and functionality. In this work, an elaborate fluorimetric strategy was applied to study the GlyR α3K and L isoforms. Previously established differential clustering, desensitization and synaptic localization of these isoforms imply that membrane behavior is crucial in determining GlyR α3 physiology. Therefore diffusion and aggregation of homomeric α3 isoform-containing GlyRs were studied in HEK 293 cells. A unique combination of multiple diffraction-limited ensemble average methods and subdiffraction single particle techniques was used in order to achieve an integrated view of receptor properties. Static measurements of aggregation were performed with image correlation spectroscopy (ICS) and, single particle based, direct stochastic optical reconstruction microscopy (dSTORM). Receptor diffusion was measured by means of raster image correlation spectroscopy (RICS), temporal image correlation spectroscopy (TICS), fluorescence recovery after photobleaching (FRAP) and single particle tracking (SPT). The results show a significant difference in diffusion coefficient and cluster size between the isoforms. This reveals a positive correlation between desensitization and diffusion and disproves the notion that receptor aggregation is a universal mechanism for accelerated desensitization. The difference in diffusion coefficient between the clustering GlyR α3L and the non-clustering GlyR α3K cannot be explained by normal diffusion. SPT measurements indicate that the α3L receptors undergo transient trapping and directed motion, while the GlyR α3K displays mild hindered diffusion. These findings are suggestive of differential molecular interaction of the isoforms after incorporation in the membrane.

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1. Introduction

The diffusion and aggregation of receptors in the cell membrane have received considerable attention in the last decade [1–4]. In addition to endo- and exocytotic cycling of these membrane proteins, their movement and distribution in the plasma membrane can considerably contribute to the homeostasis of these receptors in the membrane [5–7]. In this way, the cell possesses extra tools to fine-tune receptor-mediated signaling events [8–10]. An example of this, is surface trapping of neurotransmitter receptors at post-synaptic sites for the facilitation of neurotransmission [11–14]. Hence, a study of the spatiotemporal membrane behavior of neurotransmitter receptors is important in fully comprehending the physiological receptor function.

The transmembranous glycine receptor (GlyR) α3 is a neurotransmitter receptor subtype for which membrane properties are implicated in regulating signaling events. Post-transcriptional processing of the *GLRA3* gene transcript [15] gives rise to two isoforms identified as GlyRs α3K and α3L [16]. The former is the short isoform exhibiting a diffuse membrane staining and fast desensitization kinetics, while the latter contains a 15 amino acid insert (Fig. 1a), exhibits a clustered membrane appearance and slow desensitization kinetics [16,17]. An altered expression ratio of these isoforms has

Abbreviations: $\langle r^2 \rangle$, mean square displacement; ω_0, laser beam radius at $1/e^2$ of its maximum intensity; CLSM, confocal laser scanning microscope; D′, time-dependent diffusion coefficient; D_{1-3}, diffusion coefficient derived from the time lags 1 to 3; DC, dichroic mirror; dSTORM, direct stochastic optical reconstruction microscopy; FRAP, fluorescence recovery after photobleaching; GlyR, glycine receptor; HA, hemagglutinin; HEK 293, human embryonic kidney 293; ICS, image correlation spectroscopy; MWPR, medium without phenol red; PFA, paraformaldehyde; RICS, raster image correlation spectroscopy; ROI, region of interest; SPT, single particle tracking; TICS, temporal image correlation spectroscopy; TIRFM, total internal reflection fluorescence microscope; t_{lag}, time lag

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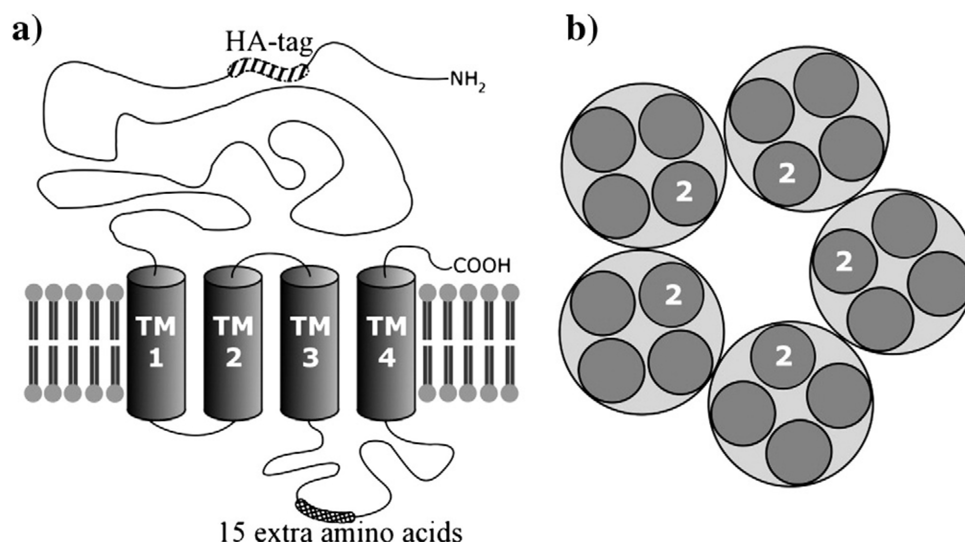

Fig. 1. Schematic representation of the glycine receptor structure. a) General structure of a GlyR subunit showing the four transmembrane α helices (TM1–TM4) and the large, extracellular N-terminal domain. The location of the HA-tag used in this study is indicated, as well as the region of the 15 extra amino acids of the L isoform. b) A functional, homomeric GlyR comprises five subunits, with TM2 of each subunit lining the pore.

been measured in patients with a severe form of temporal lobe epilepsy (TLE) [17–19]. A differential synaptic localization of the isoform subunits, has been associated with this observation [17]. In order to improve the understanding of these isoform-related characteristics, this work investigates the spatiotemporal membrane behavior of homomeric [20] (Fig. 1b) α3K or α3L GlyRs on different time and spatial scales.

The GlyR α3 diffusion and aggregation were studied by means of both ensemble average and single particle fluorimetric techniques, either static or dynamic. Earlier work combining fluorescence recovery after photobleaching (FRAP) [21,22] and single particle tracking (SPT) [23–27] measurements for characterization of protein diffusion was inspirational for our fluorimetric approach [28–30]. In this way an integrated view of receptor properties is obtained and possible technical bias in the interpretation is reduced. However both the confocal laser scanning microscope (CLSM) [31,32] and total internal reflection fluorescence microscope (TIRFM) [33–35], used respectively for FRAP and SPT, provide several more techniques for receptor characterization, without requiring drastic changes to the set-up. In this work the CLSM was used not only for FRAP, but also for image correlation spectroscopy (ICS) [36,37] and raster image correlation spectroscopy (RICS) [38,39]. The range of techniques applied on the TIRFM was extended beyond SPT, with temporal image correlation spectroscopy (TICS) [40,41] and single particle based localization microscopy [42–46], in this case direct stochastic optical reconstruction microscopy (dSTORM) [47–49].

The aggregation state of both α3 isoforms was investigated by ICS and dSTORM. ICS has the advantage that it can be applied on a commercial CLSM and that it can determine the aggregation state of membrane proteins with very low detection limits. However, ICS is diffraction-limited and does not allow for the direct estimation of the cluster size. This hurdle was overcome by dSTORM, which generates a subdiffraction image. Since the biological samples of both techniques are identical, except for the addition of a reducing agent to the measuring solution, the extra workload to apply both techniques is minimal once they are operational. The diffusion of the receptors was studied at various length and time scales using, in order of decreasing scale, FRAP (> 1 μm, seconds to minutes), TICS (diffraction limited, ms to s), RICS (diffraction limited, μs to s) and SPT (subdiffraction technique, ms to s). In addition to the different scales they cover, two fundamentally different types of results are obtained: FRAP, TICS and RICS return all an ensemble averaged result, while SPT generates individual information for every tracked particle. For all fluorescence based techniques employed, an organic fluorophore coupled to a primary antibody was used for receptor labeling, allowing for the use of similarly sized labels for all techniques. Furthermore all dynamic measurements were carried out at 37 °C, in order to mimic the effect of body temperature on receptor movement and membrane viscosity.

2. Materials and methods

2.1. Cell culture

Human embryonic kidney 293 cells (HEK 293, kindly provided by Dr. R. Koninckx, Jessa Hospital, Hasselt, Belgium) were maintained at 37 °C in a humidified incubator at 5% CO_2 in Dulbecco's modified eagle's medium (Ref. 41966, Gibco BRL, Paisley, UK) supplemented with 10% fetal calf serum (Biochrom AG, Berlin, Germany) and a penicillin (100 IU/ml)-streptomycin (100 μg/ml) mixture (Invitrogen, Merelbeke, Belgium). The cells used for microscopic observation were plated 2 days before the experiment in 8-well Lab-Tek™ II chambered coverglass (Nalge Nunc International, Rochester, NY, USA) seeded at a density of 20,000 cells per well in transfection medium. This is Dulbecco's modified eagle's medium (Ref. 41965, Gibco BRL, Paisley, UK) supplemented with 10% fetal calf serum but without the penicillin/streptomycin mixture.

2.2. Transfection

After overnight incubation, the cells were transfected using calcium phosphate co-precipitation with plasmids encoding for the hemagglutinin (HA)-tagged (Fig. 1a) splice variants α3K and α3L of the mouse GlyR α3 [17]. The HA-tag was located in the extracellular N-terminal domain between amino acids 35 and 36 [17]. The plasmids were diluted in 250 mM $CaCl_2$ buffer at a concentration of 20 ng/μl and an equal amount of HEPES buffered saline solution (HBS, pH 7.05) was added dropwise. After incubation of 15 min, this mixture was added to the culture medium of the cells. All cell recipients used in the various experiments received a final amount of DNA of 0.21 μg/cm^2. After 6 h, the transfection medium was replaced with transfection medium supplemented with 200 nM strychnine. All measurements occurred within 24 to 36 h after transfection.

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