Jonas Bienzeisler*, Marc Landry, Alberto Llorente and Gereon Hüttmann

Analysis of the *in-vivo* $GABA_B$ receptor relocalization and oligomerization in chronic pain conditions using spatial intensity distribution analysis

Abstract: The response to the synaptic release of γ-Aminobutyric acid (GABA) in the central nervous system is mediated by the $GABA_B$ receptor, inter alia dependent on the formation of receptor oligomers. Spatial intensity distribution analysis (SpIDA) is an image analysis method measuring the oligomerization and density of proteins. It was used to quantify the oligomerization of $GABA_B$ receptors in the rodent spinal cord under conditions of chronic pain and inflammation. SpIDA reports a lowered density of tetrameric $GABA_B$ receptor entities in GABAergic neurons of the dorsal horn under these conditions compared to sham samples. The soma of neurons in the ventral horn is shown to be populated by single entities of the $GABA_B$ receptor. The findings underline the role of receptor oligomerization, as well as the membrane environment in regulating receptor function.

Keywords: Spatial Intensity Distribution Analysis, $GABA_B$ receptor, oligomerization, chronic pain

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1 Introduction

The central nervous system (CNS) is formed by an information transmitting network of neurons, communicating mutually through the release and detection of neurotransmitters. Slight changes to how the system reacts to the release of these molecules have great physiological significance, but may also have patho-physiological implications. γ-Aminobutyric acid (GABA) is the most abundant inhibitory neurotransmitter in the mammalian CNS. The release is detected by the metabotropic $GABA_B$ and ionotropic $GABA_A$ receptor. Although the $GABA_B$ receptor (GBR) is suspected to be involved in neuronal malfunctions, the mechanisms regulating the function and distribution of the receptor are not yet well understood. The way of how many macromolecular complexes form the receptor, the oligomerization, is a promising candidate for playing a major role in this process [1]-[4]. Spatial Intensity Distribution Analysis (SpIDA) is a novel tool reporting the oligomerization and density of fluorophores [5]. Combined with GBR labelled with enhanced green fluorescent protein (eGFP), SpIDA becomes a powerful approach to target investigating the function of this receptor.

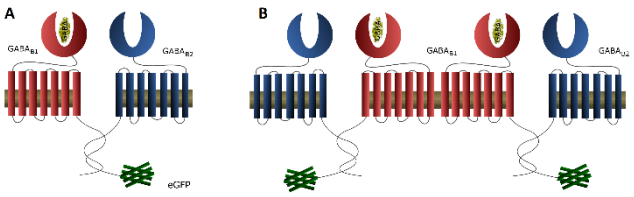

Figure 1: Detecting oligomerization of GBR by SpIDA. (A) A schematic heterodimeric GBR labelled with eGFP and (B), a tetrameric GBR labelled with two eGFP.

1.1 SpIDA

The number of observed photons per unit of time emitted from a single kind, excited, fluorophore fluctuates [6]. SpIDA is facilitated by the Poissonian behavior governing this process, thus empowering a super Poissonian fit to intensity histograms computed from confocal laser scanning microscopy images (CLSM) (Fig. 1) [5]. The intensity histogram of an imaged region of interest reports the number of pixels for each intensity value. It can be fitted either for the number of fluorophores per excitation volume N, or the

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quantal brightness ε_0 assuming a super-poissonian distribution. For known ε_0 values it is also possible to describe the histogram of mixed populations of fluorophores, since the probability distribution for the sum of two random variables is given by the convolution of the two individual distributions. This allows the use of SpIDA for reporting the oligomerization and densities of proteins labelled with fluorophores, as a labelled dimer has a monomeric brightness of $2\varepsilon_0$. In practice the quantal brightness for a monomeric protein sample is determined first and then protein densities can be assessed.

1.2 The $GABA_B$ receptor system

The GBR is a G-protein-coupled receptor mediating the response to the synaptic release of GABA. Affected by the sub-cellular localization, the GBR exerts distinct regulatory effects on the synaptic transmission making it a promising target for the treatment of psychiatric and neurological disorders [7]. Furthermore, GBRs are involved in the regulation and perception of pain [1]. The patho-physiological process of neuropathic pain is associated with neuronal sensitization in dorsal horn (DH) spinal networks, induced by a maladaptive $GABA_B$ inhibition of calcium-dependent intrinsic properties of DH neurons [3]. Albeit having diverse functionalities in the CNS, GBRs are invariably expressed as the same structure, being an obligate heterodimer of the two subunits $GABA_{B1}$ and $GABA_{B2}$ (see **Figure**). Coexpression of the subunits is mandatory for the functional expression and proper binding of GABA to the receptor [7]. It is suspected that diversified function is achieved inter alia by the cell surface expression and spatial organization of the receptor, as multiple receptors form higher-order oligomers in association with themselves [2], [8]. Transient interactions among the receptors result in a functional cooperativity. In this fashion, the formation of two GBR as a tetramer has been observed [2]. A key to understanding the physiological significance of the tetrameric organization and patho-physiological implications is studying the distribution of dimeric and tetrameric entities. If oligomerization is regulating receptor function, it can thus be postulated, that altered organization of GBR in the DH could play a role in the maladaptive $GABA_B$ inhibition under conditions of neuropathic pain in the CNS.

2 Material and methods

To study the distribution of GBR and role in pathophysiological processes, spinal tissue of transgenic mice expressing eGFP-tagged $GABA_{B1}$ receptor-subunits under varying conditions was imaged and probed with SpIDA.

2.1 Fluorescence microscopy

Samples were imaged with a true point-scanning, spectral confocal system (Leica, Mannheim) at a 63X magnification, NA 1.4. The eGFP was excited using a 488 nm diode laser at an intensity of 70 %. The fluorescence was detected by a PMT at a gain of 700 V. To calibrate the system for SpIDA and correct for error terms, the point spread function of the microscope was determined using 25 nm beads (Molecular Probes, Eugene). The slope of the linear correlation between the mean pixel value and the respective variance was asserted with a Argo-LM slide (Argolight, Pessac) [5],[9].

2.2 Measuring the monomeric brightness

Slides of viruses transfected with eGFP were prepared to measure ε_0 of eGFP under the given microscope setting. In these viruses the expression of eGFP can be assumed to be isolated and thus monomeric. SpIDA functions of the resulting images' intensity histograms were fit for ε_0.

2.3 Mouse model

A total number of ten transgenic mice expressing $GABA_{B1}$ receptor-subunits fused to eGFP were sacrificed [10]. Among these, persistent neuropathic and inflammatory pain was evoked in two mice with Spared Nerve Injury (SNI), two mice were injected with Complete Freund's Adjuvant (CFA), two mice were injected with NaCl for sham conditons, four mice were left untreated. SNI was induced by a lesion of the tibial and common peroneal nerve branches causing a partial denervation of the sciatic nerve and hence pain-like behaviour [11]. The intraplantar injection of CFA, triggering an immunoreaction, was applied to the mice's left paws.

2.4 Sample preparation

The mice were anesthetized with a 225 mg/kg lethal dose of sodium pentobarbital. After pinch response testing the

animals were perfused through the left ventricle of the heart; two minutes with phosphate-buffered saline (PBS) 1x, pH7.4 and two minutes with 4 % paraphormaldehyde, 0.1 % glutaraldehyde at pH 7.4 at room temperature. Brains and spines were extracted and post-fixated for two hours at 4°C in 4 % paraformaldehyde, 0.1 % glutaraldehyde at pH 7.4. The samples were put in a cryoprotective solution of 15 % sucrose in PBS overnight, subsequently embedded in Tissue-Tek R (Sakura Finetek Europe, Leiden) and frozen in isopentan (-90°C). The samples were stored at -20°C and cut into 10 µm thick cryostat sections for mounting onto gelatin covered slides with Fluorescence Mounting Medium (Dako, Hamburg).

2.5 Image analysis

The oligomerization of GBR was investigated in the ventral horn (VH) and the DH. The regions of interest for SpIDA were selected manually, considering a trade-off between specificity (small ROI) and optimal sampling (large ROI) [5]. Background noise was empirically determined by measuring the mean intensity of image regions without fluorescence signal and subtracted from the intensity histogram for final fitting with the SpIDA functions [5].

3 Results

Since the function of the GBR-system depends on the subcellular region, SpIDA was applied to ROIs where these could be observed. In the soma of motoneurons innervating skeletal muscle located in the VH, high densities of the GBR are almost exclusively found in the dimeric state (see **Figure**). Gaberic inhibitory interneurons, showing up as small regions with high fluorescence intensity in CLSM images, are found in the superficial DH (see **Figure**). These regions with high densities of GBR have a normal distributed population of dimeric or tetrameric receptors. A tendency towards more tetrameric entities can be observed, as well as a linear correlation of the data (r = -0.568, see **Figure**). GBR in the surrounding DH laminae with low fluorescence signal and thus low receptor densities are favourably organized dimeric.

Weakened $GABA_B$ inhibition in the DH of the rodent spine is observed under neuropathic pain conditions. Concurrently the oligomerization and the density of the GBR is altered under neuropathic pain, inflammatory and sham conditions (see **Figure**). Under SNI, CFA and sham conditions high densities of almost exclusively isolated, dimeric GBR are found in the soma of VH motoneurons, with no significant differences (see **Figure**). The same is the case for receptors in the surrounding laminae of the DH with low fluorescence signal and thus low receptor densities, which are also favourably organized dimeric (see **Figure**). Distinct changes in oligomerization were detected in the DH; the GABAergic inhibitory interneurons show significant alterations under neuropathic pain conditions. While under sham conditions a greater proportion of tetrameric organized GBR can be observed, under SNI conditions there are significantly more neurons in which the GBRs are organized predominantly dimeric (see **Figure**). The same is the case for CFA conditions, where a higher rate of neurons show rather dimeric GBR compared to sham conditions; however, there is no significant difference in data from the DH ipsilateral to the CFA injection (see **Figure**). In all data a linear correlation of dimeric and tetrameric oligomerization can be observed, while these distributions are also normal distributed.

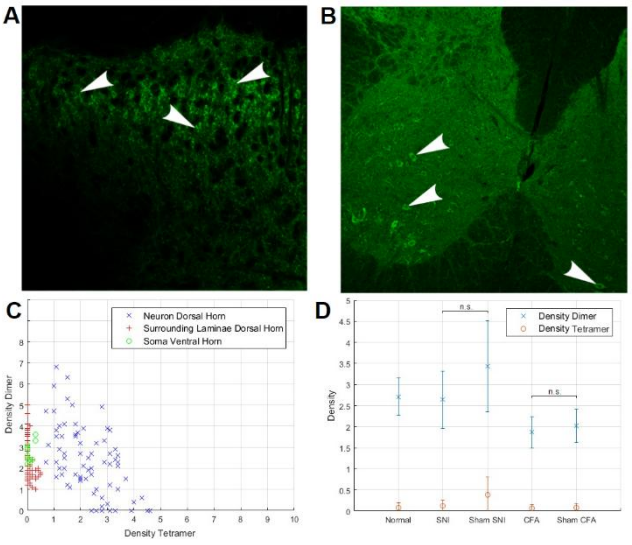

Figure 2: The Oligomerization of GBR in the DH and VH. (A) GABAergic inhibitory interneurons in the superficial DH show high intensities of fluorescence (arrows). (B) The soma of motoneurons innervating skeletal muscle located in the VH (arrows). (C) GBR densities in fluorescent molecules per laser beam-effective focal volume. Each data point represents one ROI, respectively one neuron (x), the soma of one nerve cell (circle) or surrounding laminae in the DH (plus). (D) The averaged oligomerization (with standard deviation) of the soma of nerve cells in the VH shows no significant differences. Scale bar: 100 µm. * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001; ns P > 0.05.

4 Conclusion

Under all conditions there are two consistent observations: almost exclusively dimeric GBRs are found in the soma of

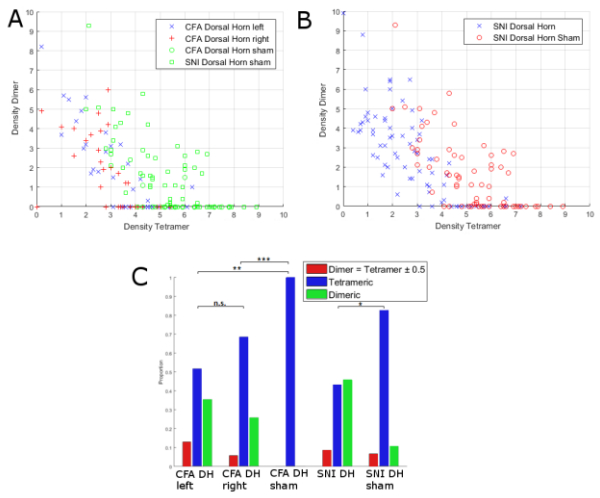

Figure 3: Oligomerization of GBR in pain and inflammation. (A) Correlation in the scatterplot of GBR densities in fluorescent molecules per laser beam-effective focal volume in the DH of mice under CFA conditions (r_{DHleft} = -0.83, $r_{DHright}$ = -0.79) and (B) under SNI conditions (r_{SNI} = -0.763, $r_{SNIsham}$ = -0.651) . (C) Summary of the data from (A) and (B) in the form of a box plot. The plots show significant differences in the proportion of neurons that had a higher amount of dimeric, tetrameric GBR, and evenly distributed tetrameric or dimeric GBR. * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001; ns P > 0.05.

VH motoneurons without significant changes due to SNI or CFA conditions, and all DH scatter plots show linearly correlated data of dimeric and tetrameric oligomerization. The former observation can be explained with the endocytotic mechanism that is involved in the control of cell surface expression of GBR. Surface trafficking of GBR means assembly of both the $GABA_B$ subunits [12]. This demonstrates that inactive GBR subunits are internalized in the soma of neurons isolated from each other, thus the SpIDA results report single receptors. Dissociation apparently occurs preferentially at the plasma membrane, which is in accordance with previous findings [4]. The linear correlations observed in the DH data indicate that the amount of receptors on the plasma membrane surface is limited and that the spatial configuration of receptors is altered under conditions of chronic pain or inflammation. Both observations suggest that the dimerization of the receptors is a dynamic process taking place on the membrane surface; changes in the membrane environment through lateral diffusion might also regulate function. It also hints that the assembly of $GABA_B$ subunits and endocytotic pathways might not be involved in altering the oligomerization of GBR on the membrane surface. Unequivocal is the physiological significance of the tetrameric organization and patho-physiological implications of changed distribution and hence the oligomerization of GBR. Tetrameric organization as a dimer of two GBR apparently plays a role in regulating the function of the receptor. A lower proportion of tetrameric organization in the DH is seemingly intertwined with impeded $GABA_B$ inhibition and the pathological conditions of chronic pain or inflammation. These conclusions give further inside into the function of the GBR system and may help new ways of targeting neuropathic pain pharmacologically. However, the results need to be investigated on a greater scale in vivo, as the conclusions drawn need to be further evidenced.

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Author's Statement

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