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Faculty of Medicine and Life Sciences
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Master of Biomedical Sciences

Master's thesis

Elucidating the Subcellular Localization of Glycine Receptor Alpha 2 in Animal Models of Schizophrenia: Insights from Striatal Immunohistochemistry

Joke Peeters

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Molecular Mechanisms in Health and Disease

SUPERVISOR :

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Elucidating the Subcellular Localization of Glycine Receptor Alpha 2 in an Animal Model of Schizophrenia: Insights from Striatal Immunohistochemistry*

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*Running title: S*ubcellular localization of glycine receptor α2*

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ABSTRACT

Glycine receptors (GlyRs) are ligand gated ion channels that mediate inhibitory currents upon activation in the adult brain. Recent studies have identified GlyRa2 as the primary GlyR in the adult dorsal striatum, a brain region involved in reward-motivated behavior. Here, sensory and motivational inputs are integrated, and significance is assigned to relevant stimuli. Overactivation of the striatum due to hyperdopaminergic inputs disrupts this system, resulting in psychosis. Considering the inhibitory nature of GlyRa2, and its presence in the striatum, GlyRa2 represents a promising potential antipsychotic treatment target. Before GlyRa2 can be used in therapeutic applications, more information on its properties is needed. This study aimed to investigate changes in subcellular localization of GlyRa2 in the striatum of animal models of schizophrenia (SCZ) compared to control mice. We hypothesized an extrasynaptic localization of GlyRa2. This was investigated through colocalization with the inhibitory vesicular GABA transporter (VGAT) in the striatum. Immunohistochemistry revealed no significant changes in colocalization between SCZ and control mice. However, a non-significant increase was observed of disrupted in schizophrenia 1 locus impaired (DISC1-Li) mice compared to wild-types. Although inconclusive, these findings suggest the possibility of altered GlyRa2 localization in the striatum of this genetic SCZ model. Further research is needed to validate and expand upon these findings. Understanding the subcellular localization of GlyRa2 in the striatum can provide insights into its functional properties, enabling the development of more targeted and effective treatments for psychosis.

INTRODUCTION

Glycine receptors (GlyRs) are pentameric ligand-gated ion channels (LGIC) that mediate chloride currents across the cell membrane. These currents are coordinated through rapid changes in chloride conductance in response to binding of an agonist (1, 2). When GlyR is activated, the receptor channel opens, allowing chloride to passively diffuse across the membrane (1). Since the chloride equilibrium potential in the adult brain usually lies more negative compared to the cell resting potential, GlyRs are generally considered as inhibitory (1, 2). To date, four functional agonist-binding α (α1-4) subunits and one structural β subunit are identified (3). GlyRs are either homopentameric, consisting of only alpha subunits, or heteropentameric, where alpha and beta subunits are combined. In heteropentameric GlyRs, the beta subunit anchors the receptor to the postsynaptic cytoskeleton via gephyrin, a cytoplasmatic scaffolding protein. Conversely, homopentameric GlyRs are located extrasynaptically due to their inability to bind to gephyrin at the synapse. (1, 4).

Prenatally, the alpha 2 subunit containing GlyR (GlyRα2) is abundantly expressed throughout

most of the central nervous system, but their numbers decline rapidly after birth (1, 3, 5). However, recent studies done in our research lab have uncovered that GlyRa2 is still expressed in the adult brain. Furthermore, their particular findings imply that GlyRa2 is the only functional GlyR in the adult dorsal striatum, a brain region that plays an important role in motor learning, voluntary movements and reward-motivated behavior (3, 6). The striatum processes pleasurable experiences through dopamine signaling and contributes to the initiation of a behavioral response (7). In psychosis, overactivation of the striatum through hyper-dopaminergic inputs causes dysregulation in the reward system, resulting in excessive attribution of significance to irrelevant stimuli. This may lead to delusions, hallucinations, and disorganized behavior (8, 9). Interestingly, GlyRs are able to decrease the firing probability of a cell through shunting inhibition, this way preventing excessive activity. This property, together with their presence in the striatum, makes GlyRa2 a unique potential future treatment target for psychosis (3).

However, before we can exploit GlyRa2 in therapeutic settings, further exploration is necessary to uncover significant information about the receptor. We aim to investigate the subcellular localization of GlyRa2 in medium spiny neurons (MSNs) through immunohistochemistry. MSNs represent 95% of all neurons in the striatum and can be identified by their distinct morphological features, including a medium-sized cell body, extensive dendritic branching, and a high spine density (3, 8, 10). The localization of GlyRα2 on these cells is important because it might influence the future development of new antipsychotics. The precise localization of a receptor within specific subcellular compartments influences its functional properties, as well as the activation of receptor mediated pathways (11, 12). One receptor can be involved in multiple distinct pathways, depending on their localization (13). An example of this is the N-methyl-D-aspartate (NMDA) receptor, which synaptically will promote cell survival in MSNs, whereas activation of extrasynaptic NMDA receptors will lead to cell death (14). Furthermore, drug response of a receptor can be influenced by the subcellular localization. For example, the heteromeric B-subunit containing GlyRs, which are only expressed synaptically, are not affected by low doses of picrotoxin (PTX), whereas homomeric extrasynaptic GlyRs are sensitive to even small doses (15, 16). Therefore, understanding the subcellular localization will help design drugs with enhanced efficacy, reduced side effects, and improved pharmacokinetics.

We hypothesize that the receptor is located extrasynaptically on the MSNs, based on previous research showing a tonic activation of the GlyRs in the striatum (3, 17). This tonic current arise through persistent activation of extrasynaptic receptors through spillover of neurotransmitters originating from neighboring synapses (18, 19). Additionally, our hypothesis is further supported by previous research indicating the lack of striatal GlyRB signal in immunohistochemical stainings (20), and the observed inhibition of tonic GlyR currents in the striatum when exposed to low concentrations of PTX. At these concentrations, PTX selectively blocks homomeric GlyRs, while leaving GlyRB heteropentamers unaffected (17).

In this research, we investigated the changes in colocalization of GlyRa2 and VGAT in the striatum of two different mouse models of schizophrenia, compared to control mice. Since VGAT is expressed at the synapse, we expect no colocalization between GlyRa2 and VGAT. However, we observed overlap in GlyRa2 and VGAT signal in all groups, indicating colocalization. Although we found no significant differences in colocalization, a non-significant increase was observed in both the dorsal and ventral striatum of disrupted in schizophrenia 1 (DISC1) locus impaired (Li) mice compared to wild type (WT) mice. These DISC1-Li mice served as a genetical schizophrenia animal model. Although not significant, our findings suggest changes in synaptic GlyRa2 levels in a genetic mouse model of schizophrenia.

EXPERIMENTAL PROCEDURES

Animals – All animal experiments were performed according to EU directive 2010/63/EU and Institutional Animal Care Committee guidelines, and approved by local ethics committees. C57BL/6 mice were group-housed on a 12h light-dark cycle with ad libitum access to food and water, unless stated otherwise.

Mouse models of schizophrenia – Two different mouse models of schizophrenia (SCZ) were implemented. A pharmacological dual hit

model (further referred to as PCP mice) was obtained through combination of single-housing and intraperitoneal (IP) injections with 5mg/kg phencyclidine hydrochloride (PCP; Tocris biosciences, 956-90-1) at postnatal day P7, P9 and P11. The control group consisted of group-housed littermates who received IP saline injections (further referred to as SAL). Additionally, DISC1-Li mice were used as a genetical model, with WT mice serving as a control. The DISC1-Li model was generated by a homogeneous deletions in the Disc1 gene, resulting in the absence of the full-length DISC1 isoform of 100 kDAa.

Brain slices – Brains were fixated with 4% paraformaldehyde (PFA) through cardiac perfusion, while animals were under deep anesthesia induced by IP injections of sodium pentobarbital (Dolethal, Vetoquinol). Next, brains were harvested and post-fixated overnight in 4% PFA at 4°C. Subsequently, they were consecutively saturated in 15% and 30% sucrose (VWR chemicals, 57-50-1) in phosphate buffered saline (PBS; Gibco, Thermo Fisher Scientific, 10010-015) for cryoprotection. Brains were embedded in TissueTek (Surgipath FSC 22 frozen section compound, Leica, 3801480) before snap freezing in liquid nitrogen.

Immunohistochemistry – 30µm thick brain sections were subjected to antigen retrieval in 10mM citrate buffer (pH 8.5; Citric acid, trisodium salt dihydrate, 99%, Janssen Chimica, 68-04-2) at 80°C for 30min. After cooling down, sections were permeated in 0.5% Triton X-100 (Sigma-Aldrich, 9002-93-1) in PBS for 20min. This permeation step, together with all further use of Triton X-100, was omitted in all sections which were only stained for GlyRB. Next, the brain slices were blocked in blocking buffer (0.5% Triton X-100, 5% normal goat serum (NGS; Merck Millipore, S26) and 5% normal donkey serum (NDS; Merck Millipore, S30) in PBS) for 1 hour at room temperature. Brains were incubated with primary antibodies directed against GlyRa2, VGAT, DARPP32, and GlyRB diluted in half blocking buffer (1/2 blocking buffer diluted in PBS) overnight at 4°C (table 1). Next, brain slices were washed with PBS before incubating with Alexa-conjugated secondary antibodies diluted in half blocking buffer for 1 hour at room temperature (table 2). Sections were counterstained for 15min with DAPI (1:1000, Thermo Fisher Scientific, 62248) and mounted using ImmuMount (Thermo Sccientific, 9990402).

Super resolution imaging – Super resolution images of the dorsal and ventral striatum were obtained using a confocal laser scanning microscope (LSM900, Carl Zeiss Microscopy GmbH) Airyscan 2 ((type 63x) 32 channel GaAsP array), equipped with a Plan-ApoChromat 63x/1.40 Oil DIC M27 objective. High-power 405nm, 488nm, 561nm, and 640nm diode lasers were used for excitation. Laser settings were kept the same throughout the same experiment. Z-stacks (0.60µm intervals) were acquired through the ZEN blue operating system (ZEN blue 3.1 software, Carl Zeiss Microscopy GmbH).

Statistics – Images were processed in Image J (21). The background was subtracted with a rolling bar radius of 50.0 pixels. Next, the Z-plane with the clearest and most distinct puncta in both the GlyRa2 and VGAT channels was chosen. The VGAT and GlyRa2 channels of this plane were made binary according to a self-set threshold (figure S1.B). Next, a new channel containing only the overlap in VGAT and GlyRa2 signal was obtained via the Image calculator tool (figureS1.C). A region of interest (ROI) was defined based on the DARPP32 signal and implemented on all relevant channels (VGAT, GlyRa2, and overlap) (figure S1.D). This ROI encompassed one clear DARPP32 stained cell body, indicating an MSN, and a part of its surrounding. The amount of signal in the ROI was determined through particle analysis in each relevant channel. Only particles with a size of $>4\mu m^2$ were counted. The area occupied by signal in the ROI was obtained by multiplying the number of particles in the ROI with the mean particle area. The percentage of GlyRa2 overlapping with VGAT was determined by dividing the area of overlap signal by the area of GlyRa2 signal. Statistical analysis was performed in GraphPad Prism 9.5.1. Normal distribution was confirmed via the Shapiro-Wilk test. The unpaired t-test was used to compare colocalization percentages between SCZ models and their representative controls.

Validation of GlyRa2 antibody – To validate the specificity of the GlyRa2 antibody (table 1), human embryonic kidney (HEK 293T) cells were

Table 1: Primary antibodies. Dilutions used in normal immunohistochemistry, tryout staining with two rabbit primary antibodies (Rb+Rb), GlyRa2-antibody validation in HEK cells (validation), and immunocytochemistry of primary cortico-striatal co-cultures (co-culture) are given. GlyRa2, glycine receptor alpha 2; VGAT, vesicular GABA transporter; DARPP32, Dopamine- and cyclic-AMP-regulated phosphoprotein of molecular weight 32 kDa; GlyRB, glycine receptor beta, pAb; primary antibody; IgG1, Immunoglobulin G1; Rb, rabbit

Target	Host species	Name pAb	Company	Reference number	Dilution
GlyRa2	Rabbit	Rabbit anti glycine receptor alpha 2 pAb	Novus bio	NBP3-03685	1/1000 (Rb+Rb: 1/200) (Validation: 1/3000)
VGAT	Guinea pig	Anti-vesicular GABA transporter	Alomone labs	AGP-129	1/1000 (co-culture: 1/500)
DARPP32	Mouse	DARPP-32 (H-3) mouse monoclonal IgG1	Santa Cruz biotechnology	Sc-271111	1/300 (co-culture: 1/500)
GlyRB	Rabbit	GLRB antibody	biorbyt	Orb 157165	1/600
PSD95	Rabbit	Rabbit anti-PSD95 antibody	Alomone labs	APZ-009	(Tryout Rb+Rb and co-culture:1/500)
VGLUT1	Mouse	VGLUT1 Antibody (S28-9)	Invitrogen	MA5-27614	(co-culture: 1/500)
Gephyrin	Rabbit	Anti-Gephyrin	Alomone labs	AIP-005	(co-culture: 1/500)

Table 2: Secondary antibodies. Dilutions used in normal immunohistochemistry, tryout staining with two rabbit primary antibodies (Rb+Rb), GlyRa2-antibody validation in HEK cells (validation), and immunocytochemistry of primary cortico-striatal co-cultures (co-culture) are given. IgG, immunoglobulin; F(ab')2, Fragment Affinity-Purified Secondary Antibodies

color	Target species	Host species	Name sAB	Company	Reference number	Dilution
555	Rabbit	Donkey	Donkey anti-rabbit IgG-555	invitrogen	A31572	1/400 (Rb+Rb: 1/500) (validation: 1/600)
647	Guinea pig	Goat	Goat pAB to Gpig IgG (Alexa fluor 647)	abcam	Ab 150187	1/400
488	Mouse	Goat	Alexa fluor 488 F(ab')2 fragment of goat anti-mouse	Invitrogen	A11017	1/400
647	Rabbit	Goat	Alexa fluor 647 goat anti-rabbit IgG (H+L)	Invitrogen	A21245	1/500 (Rb+Rb: 1/500)
488	Rabbit	Goat	Alexa fluor TM 488 goat anti-rabbit IgG (H+L)	Invitrogen	A11008	(co-culture: 1/600)
488	Guinea pig	Goat	Alexa fluor 488 goat anti-guinea-pig IgG (H+L)	Life technologies	A11073	(co-culture: 1/600)
555	Mouse	Goat	Alexa fluor TM 555 F(ab')2 fragment of goat anti-mouse IgG	Invitrogen	A21425	(co-culture: 1/600)

cultured in HEK medium ((DMEM (Dulbeco's Modified Eagle Medium; Gibco, Thermo Fisher Scientific, 41966-029) supplemented with 10% foetal bovine serum (FBS (Biowest, S181B-500) and 1x Penicillin-Streptomycin). Cells were seeded onto of 12mm glass coverslips in a 24-well plate at a final concentration of 30 000 cells per well. The next day, the cells were either co-transfected with

GFP and a human GlyR construct on a PRK5 backbone (either hGlyRa1, hGlyRa2, hGlyRa3L, or hGlyRB), or transfected with only GFP or hGlyRa2 with a intracellularly fused monomeric chloride-sensing YFP label. (IC hGlyRa2-mCl-YFP). All GlyR constructs were provided by Prof. Robert J Harvey (Queensland, Australia) 20µl transfection mix (18µl HBS, 250ng of the desired constructs, and 1µl CaCl2) was added to each well and incubated overnight at 37°C and 5% CO_2. The day after, cells were fixed with 4% PFA for 4min at room temperature. After washing the coverslips with PBS, the cells were blocked in blocking buffer (10% BSA (bovine serum albumin; Sigma Aldrich, A7906-1006) in PBS) for 30 min at room temperature. Cells were incubated with 1:3000 anti-GlyRa2 primary antibody diluted in blocking buffer overnight at 4°C (table 1). After washing with PBS, cells were incubated with 1:600 555 Alexa-conjugated secondary antibody diluted in blocking buffer for 2 hours at room temperature (table 2). Nuclei were stained with DAPI for 10 min and coverslips were mounted on glass slides with ImmuMount. Coverslips were imaged using a Leica fluorescence microscope (Leica microsystems).

RESULTS

Colocalization VGAT and GlyRa2 – To analyze the colocalization of GlyRa2 and VGAT in the dorsal and ventral striatum, immunohisto-chemical analysis was performed on murine brain slices. Images were processed in image j and the area of GlyRa2, VGAT, and overlap signal was acquired and the percentage of GlyRa2 that overlapped with VGAT was calculated.

Pharmacological SCZ model – An unpaired t-test was used to investigate differences in overlap of GlyRa2 and VGAT signal between PCP and SAL mice. No significant differences in dorsal (SAL 19.92 +/- 3.806%, PCP 20.91 +/- 5.514%, $t(6) = 0.1487$, $p = 0.8866$) nor ventral (SAL 14.23 +/- 2.384%, PCP 14.59 +/- 4.416%, $t(6) = 0.07171$, $p = 0.9452$) overlap of GlyRa2 and VGAT was found (figure 1A).

Genetical SCZ model – DISC1 Li mice appeared to have an increased overlap in signal in both dorsal (WT 19.56 +/- 1.535%, DISC1-Li 22.69 +/- 1.7%, $t(5) = 1.314$, $p = 0.2458$) and ventral (WT 10.84 +/- 1.202%, DISC1-Li 17.10 +/- 4.056%, $t(6) = 1.479$, $p = 0.1895$) striatum compared to WT mice, although this shift did not reach significance when subjected to an unpaired t-test (figure 1B).

Comparison of SCZ models - An unpaired t-test was performed to test if there are any changes in colocalization of GlyRa2 and VGAT in the dorsal (PCP 20.91 +/- 5.514%, DISC1-Li 22.69 +/- 1.7%, $t(6) = 0.3083$, $p = 0.7682$) and ventral (PCP 14.59 +/- 4.416%, DISC1-Li 17.10 +/- 4.056%, $t(6) = 0.4192$, $p = 0.6897$) striatum of both SCZ models. No significant differences were observed in overlap percentages (figure 1C).

Comparison of control groups – Percentages of colocalization of GlyRa2 and VGAT were compared between both control groups via unpaired t-test. No significant differences were observed in either dorsal (SAL 19.92 +/- 3.806%, WT 19.56 +/- 1.535%, $t(5) = 0.07717$, $p = 0.9415$) nor ventral (SAL 14.23 +/- 2.384%, WT 10.84 +/- 1.202%, $t(6) = 1.267$, $p = 0.2520$) striatal GlyRa2 and VGAT overlap (figure 1D).

GlyRa2 antibody specificity - To assess whether the GlyRa2 antibody we used was specific, HEK 293 T cells were transfected with different GlyR constructs before staining with anti-GlyRa2. GFP was used as a visual control to verify whether the transfection worked. We saw GFP signal in all GFP-transfected wells, indicating that the transfection did work. We expected to only see signal originating from GlyRa2 staining in the wells that were transfected with GlyRa2 constructs. However, we found that transfection with GlyRa1 and GlyRB constructs also stained positive for GlyRa2 (figure 2).

DISCUSSION

Psychosis is a mental syndrome characterized by a distorted perception of reality, leading to difficulties in distinguishing what is real and what is not (22). It is primarily characterized by positive (e.g. hallucinations and delusions) and cognitive (e.g. thought disorganization, difficulties with concentration, disorganized speech patterns,

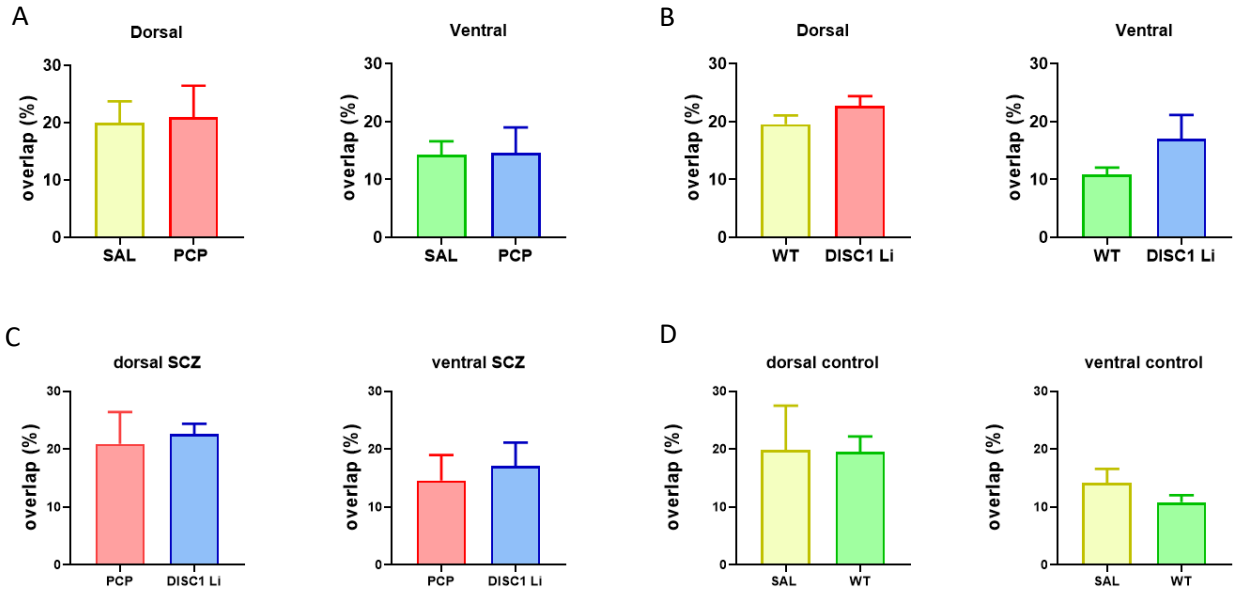

Figure 1: There is no significant difference in GlyRa2/VGAT signal overlap between schizophrenia mouse models and their relative control. Unpaired t-test indicated no significant differences in dorsal nor ventral overlap of GlyRa2 and VGAT in any of the compared groups. A) pharmacological dual hit schizophrenia mouse model and saline injected littermates: dorsal: SAL 19.92 +/- 3.806%, PCP 20.91 +/- 5.514%, t(6) = 0.1487, p = 0.8866; ventral: SAL 14.23 +/- 2.384%, PCP 14.59 +/- 4.416%, t(6) = 0.07171, p = 0.9452; B) genetic mouse model for schizophrenia compared to WT mice: dorsal: WT 19.56 +/- 1.535%, DISC1-Li 22.69 +/- 1.7%, t(5) = 1.314, p = 0.2458; ventral: WT 10.84 +/- 1.202%, DISC1-Li 17.10 +/- 4.056%, t(6) = 1.479, p = 0.1895; C) PCP vs. DISC1 Li mice: dorsal: PCP 20.91 +/- 5.514%, DISC1-Li 22.69 +/- 1.7%, t(6) = 0.3083, p = 0.7682; ventral: PCP 14.59 +/- 4.416%, DISC1-Li 17.10 +/- 4.056%, t(6) = 0.4192, p = 0.6897; SAL vs. WT mice: dorsal: SAL 19.92 +/- 3.806%, WT 19.56 +/- 1.535%, t(5) = 0.07717, p = 0.9415; ventral: SAL 14.23 +/- 2.384%, WT 10.84 +/- 1.202%, t(6) = 1.267, p = 0.2520. Percentage of GlyRa2 which overlapped with VGAT. All groups contained two mice. Overlap of GlyRa2 and VGAT signal was calculated on two brain slices of each mouse, resulting in 4 measurements per group with the exception of the WT mice, in which only 3 measurements could be obtained. GlyRa2, glycine receptor alpha 2; VGAT, vesicular GABA transporter; SAL, saline; PCP, phencyclidine hydrochloride; WT, wild type; DISC1 Li, disrupted in schizophrenia 1 locus impaired

and inappropriate behavior) symptoms (23, 24). These symptoms arise through dysregulation in the striatum, the major reward-related signal integration hub in the brain (9). Contradicting to prior believes, recent studies established that the primary dopamine-related abnormalities in psychosis are present within the dorsal, rather than in the ventral striatum (25).

Current antipsychotics lack striatum-specific targeting, resulting in a significant risk of off-target side effects (8, 26). Furthermore, they primarily address symptoms rather than the underlying disease, and often yield incomplete response, with a notable proportion of patients showing resistance (22). These challenges highlight the necessity for new, more effective psychosis treatments. We propose GlyRa2 as a promising potential treatment target for psychosis.

In this article, we aimed to determine the subcellular localization of GlyRa2 in MSNs in two different animal models of schizophrenia. Although we were not able to come up with a clear conclusion about this location, we were able to take some steps in the right direction. Through colocalization with VGAT, a protein expressed in the presynaptic nerve endings of inhibitory synapses (27), we were able to further explore the potential synaptic localization of GlyRa2. All groups showed partial colocalization with VGAT in both dorsal and ventral striatum, indicating the presence of GlyRa2 in inhibitory synapses. However, as most GlyRa2 did not colocalize with

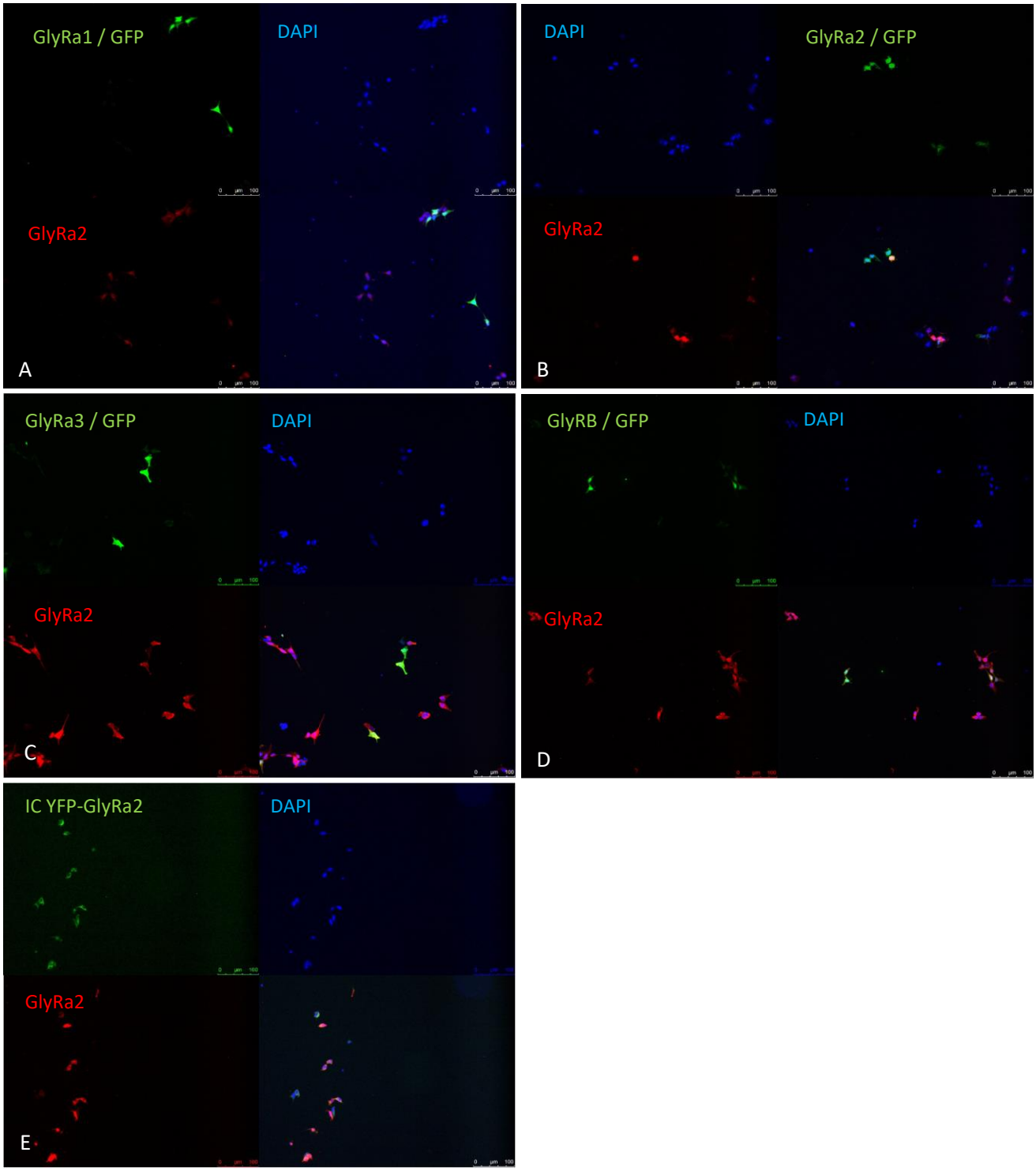

Figure 2: Validation of GlyRa2 antibody in transfected HEK 293T cells. HEK 293T cells were either transfected with A) GlyRa1 and GFP, B)GlyRa2 and GFP, C) GlyRa3 and GFP, D) GlyRB and GFP, or E) IC YFP-GlyRa2 through calcium phosphate precipitation, and stained with anti-GlyRa2 (red). Anti-GlyRa2 signal could be observed in cultures transfected with GlyRa2, GlyRa3, GlyRB, and IC YFP-GlyRa2. GlyR, glycine receptor; HEK, human embryonic kidney; GFP, green fluorescent protein; IC, intracellular; YFP, yellow fluorescent protein.

1.202% to 22.69 +/- 1.7%), the greater proportion of GlyRa2 is assumed to be expressed extrasynaptically. Comparison of SCZ models to controls provided no significant differences in striatal colocalization of GlyRa2 and VGAT. However, we saw a non-significant increase in colocalization in the ventral, and to lesser extend in the dorsal, striatum of DISC1 Li mice compared to WTs. However, this increase might be influenced by differences in sex and age between DISC1 Li mice and their WT controls, as DISC1 Li mice were females of 6.5 and 8 weeks old, whereas two 13 week old WT males were used as a control. Furthermore, some additional considerations need to be made when interpreting our results. First of all, due to the small sample size, results might not be reliable. The experiment should be repeated with a larger set of mice to improve statistic reliability. Furthermore, the observed GlyRa2 signal might not have been specific. This was tested in HEK 293T cells transfected with multiple different GlyR constructs. Positive GlyRa2 staining in GlyRa3 and GlyRB transfected cultures indicated aspecific binding of the GlyRa2 antibody, probably due to the large similarities between GlyRs subunits (1). However, this problem was omitted by the assumption that GlyRa2 is the only functional GlyR in the striatum (3), and therefore all signal induced by GlyRa2-antibody binding in the striatum should be GlyRa2. However, other insights might contradict this hypothesis (17). McCracken et al. shows evidence of tonic currents presumably generated by alpha3-subunit containing GlyRs in the striatum, and even states that the function of GlyRa3 outweighs the function of GlyRa2 (17). Considering the aspecificity of the GlyRa2 antibody we used, together with the insights provided by McCracken et al., we cannot be sure that the signal we saw originates from only GlyRa2, or includes GlyRa3 as well. This information needs to be taken into consideration when forming a conclusion based on our own data.

Further research is needed to determine the subcellular localization of GlyRa2 in MSNs. Additional colocalization studies using immuno-histochemistry and immunocytochemistry could be performed. By investigating colocalization of GlyRa2 with markers that are present in validated subcellular compartments, more information on GlyRa2 localization could be obtained. For example, colocalization analysis of GlyRa2 and gephyrin could provide information on the level of GlyRa2 present at inhibitory post-synapses (28). PSD95 is present in the postsynaptic density of excitatory synapses, whereas VGAT and VGLUT1 are expressed in inhibitory and excitatory presynaptic nerve endings, respectively (27, 29, 30). Unfortunately, we were not able to make relevant combinations of these antibodies with anti-GlyRa2, as the available antibodies (except for anti-VGAT and anti-VGLUT1) had the same host species as anti-GlyRa2. Nevertheless, we did test two modified protocols in an attempt to omit this problem, although not successful. Sequential immunohistochemical staining of rabbit anti-GlyRa2 and rabbit anti-PSD95 via both adapted protocols resulted in an almost complete overlap of PSD95 signal with GlyRa2 signal. Since PSD95 is expressed in excitatory synapses, the overlap in signal is most probable due to aspecific binding of the second set of antibodies (anti-PSD95 together with Alexa Fluor 647 goat anti-rabbit). Presumably, Alexa Fluor 647 goat anti-rabbit also detected rabbit anti-GlyRa2 primary antibodies. As both protocols for combining primary antibodies of the same host failed, we decided to focus on primary antibody combinations with different host species. This left us with a limited choice of available antibodies. Furter research including more relevant antibodies should be conducted.

Potential next steps to uncover the subcellular localization of GlyRa2 include colocalization studies in primary cortico-striatal co-cultures. These co-cultures develop more complex dendritic branches and have a higher density of dendritic spines compared to striatal monocultures, making them more physiologically relevant. Furthermore, they are confirmed to be suitable for studying the morphological development of MSNs (10, 31). As there is no GlyRa2-specific antibody available on the market, transfection with fluorescently-labeled GlyRa2 could be used to visualize the receptor (32). We were able to develop and partially characterize these cultures, with the goal of future use in continued research on the subcellular localization.

CONCLUSION

In present research, we found significant differences in colocalization GlyRa2 with VGAT in the dorsal and ventral striatum of SCZ models compared to controls. This implements no changes in proportions of synaptic compared to non-

synaptic GlyRa2 expression. However, further research in needed to validate these results and to further explore the subcellular localization of GlyRa2.

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Author contributions – **Who did what**? JH and JdR conceived and designed the research. JH and BvH performed experiments and data analysis. EvS and DD provided assistance with FLIM-FRET. JH and JdR wrote the paper. All authors careful

SUPPLEMENTARY INFORMATION

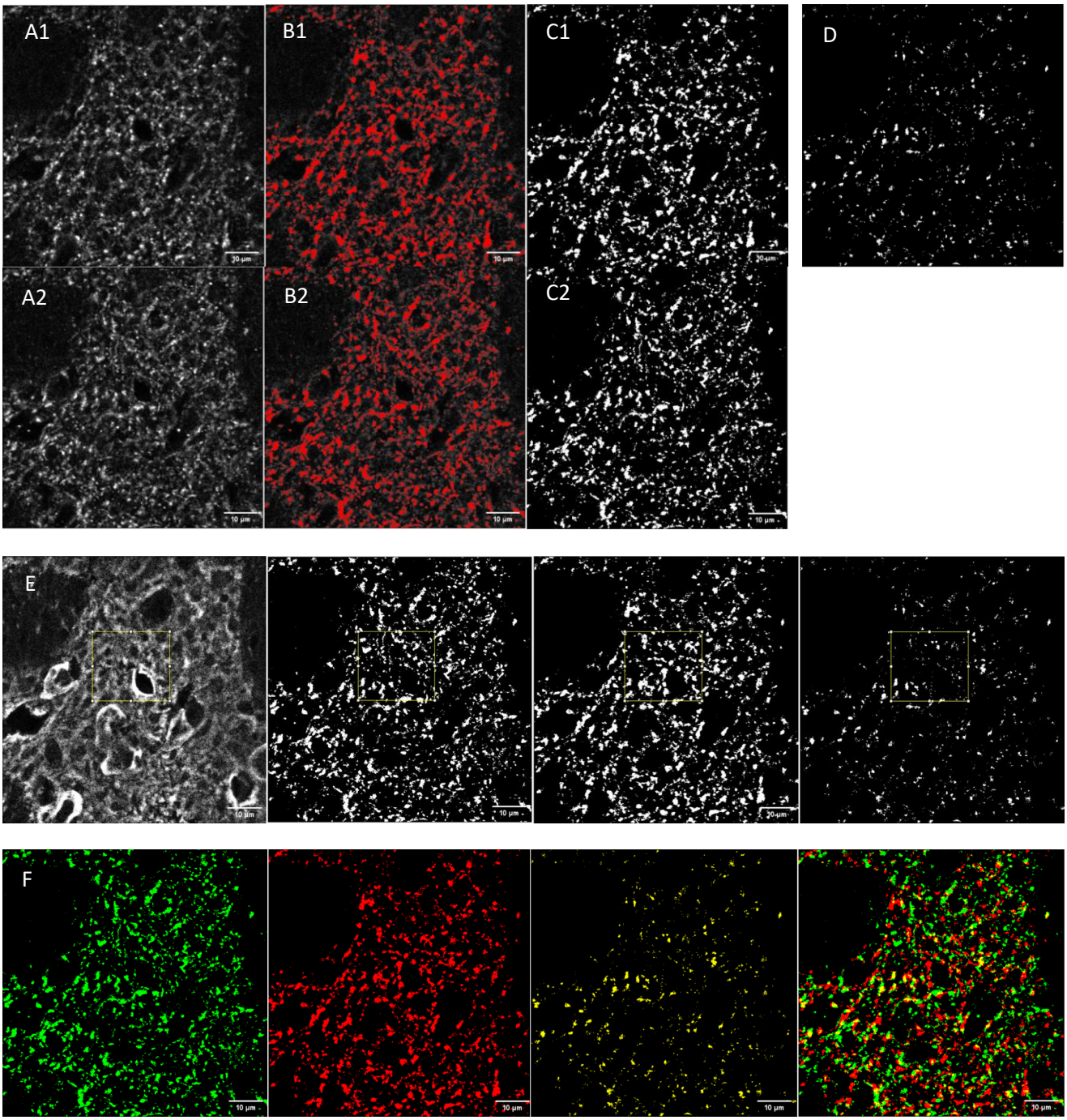

Figure S1: Representative images of image j processing. A1) Original VGAT channel image. A2) Original GlyRa2 channel. B) Threshold was set. Only red indicated areas will be read as signal. C) Binary image of VGAT channel. D) overlay of VGAT and GlyRa2 channel. E) ROI (yellow box) was set based on DARPP32 signal and implemented in all relevant channels. F) color-coded composite of VGAT (red), GlyRa2 (green), and their overlap (yellow). Images were obtained via super resolution imaging at 63x magnification. ROI, region of interest; VGAT, vesicular GABA transporter; GlyRa2, glycine receptor alpha 2

SUPPLEMENTARY METHODS

Sequential staining – Two different protocols were followed in an attempt to perform a staining using a combination of primary antibodies from the same host (rabbit). Both protocols were modified based on the previously described protocol ('Immunohistochemistry'). Following incubation with the first primary antibody (anti-GlyRa2, 1:200) and its corresponding secondary antibody (Alexa-Fluor 555 donkey anti-rabbit, 1:500), brain slices were blocked again. Next, either all the steps were consecutively repeated for the second set of antibodies (anti-PSD95, 1:500; Alexa fluor 647 goat anti-rabbit, 1:500), or the second set of antibodies was incubated together overnight at 4°C before adding on top of the brain slices at once. Lastly, nuclei were visualized with DAPI and mounted with ImmuMount as described above.

Primary cell cultures – Primary cortico-striatal cultures were prepared as followed. Pregnant female mice were sacrificed at embryonic day E14.5 – 15.5 by cervical dislocation. After decapitation of the embryos, the ganglionic eminence and cortex were isolated. Tissues were separately digested in a final concentration of 25% trypsin-EDTA (Gibco, Thermo Fisher Scientific, 25300-062) and 10% DNAse (Roche Diagnostics GmbH, 10104159001) in calcium magnesium free Hanks balanced salt solution (CMF-HBSS; HBSS (Gibco, Thermo Fisher Scientific, 14025-050) supplemented with 1% HEPES (VWR Chemicals, 7365-45-9) for 30min at 37°C. After rinsing with CMF-HBSS, the tissues were further dissociated by trituration in plating medium (0.6% glucose (D-glucose anhydrous, VWR chemicals, 50-99-7), 2mM glutamine (Sigma-Aldrich, G3126-100G), 10mM HEPES, 10% FBS and 10mM sodium pyruvate (Sigma-Aldrich, 113-24-6) in minimal essential media (MEM; Gibco, Thermo Fisher Scientific, 11090-081)) using fire-polished pipettes. Cells were plated on top of 12mm coverslips coated with 100µg/ml poly-D-lysine (PDL; Gibco, Thermo Fisher Scientific, A38904-01) and 4µg/ml laminin (Natural, mouse, Invitrogen, 23017-015) in a 24-well plate. A 3:2 cortex to striatum ratio in plating medium was used. Well plates were incubated at 37°C and 5% CO_2. After 2-4h, plating medium was replaced with pre-conditioned growth medium (neurobasal medium (Gibco, Thermo Fisher Scientific, 21103-049), 0.5mM glutamine and 1x B27 (Gibco, Thermo Fisher Scientific, 17504-001)). Pre-conditioned growth medium was prepared by incubating growth medium on confluent astrocytes for 48h. Every 7 days, half of the medium was replaced with fresh, unconditioned growth medium.

Immunocytochemistry primary cell culture – At 14 days in vitro (DIV14) and DIV19, coverslips containing primary cocultures were washed with PBS and fixed at 4°C with fixation buffer (4% PFA, 5% sucrose and 0.3% triton X100 in PBS) for 15min. Following fixation, cells were rinsed in PBS and washed with washing buffer (0.2% Triton X-100 *Staining primary cell culture* – At 14 days in vitro (DIV14) and DIV19, coverslips containing primary cocultures were washed with PBS and fixed at 4°C with fixation buffer (4% PFA, 5% sucrose and 0.3% triton X100 in PBS) for 15min. Following fixation, cells were rinsed in PBS and washed with washing buffer (0.2% Triton X-100 in PBS). After 1 hour blocking in blocking buffer (5% BSA, 0.3% Tween 20 (Sigma-Aldrich, 9005-64-5) and 0.2% Triton X-100) at 4°C, cultures were incubated overnight with primary antibodies diluted in blocking buffer at 4°C (table 1). Next coverslips were washed in washing buffer before incubation with secondary antibodies diluted in blocking buffer for 1 hour at room temperature (table 2). Cultures were counterstained with DAPI for 15min. Finally, coverslips were washed in washing buffer and rinsed with milliQ, before mounting with ImmuMount. Images were through a Leica fluorescence microscope

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SUPPLEMENTARY RESULTS

Sequential staining – Two different staining protocols were implemented to check whether it was possible to perform staining with multiple antibodies of the same host. In the first protocol, anti-PPSD95 and Alexa Fluor 647 were incubated together overnight before being added to the brain slices at once (figure S2.A). During the second staining, all antibodies were added consecutively (figure S2.B) Images of both protocols showed an almost complete overlap in signal of both GlyRa2 and PSD95. Alexa Fluor 647 signal was artificially made green in Image J, in order to make the overlap in signal more clear (red + green = yellow (figure S2).

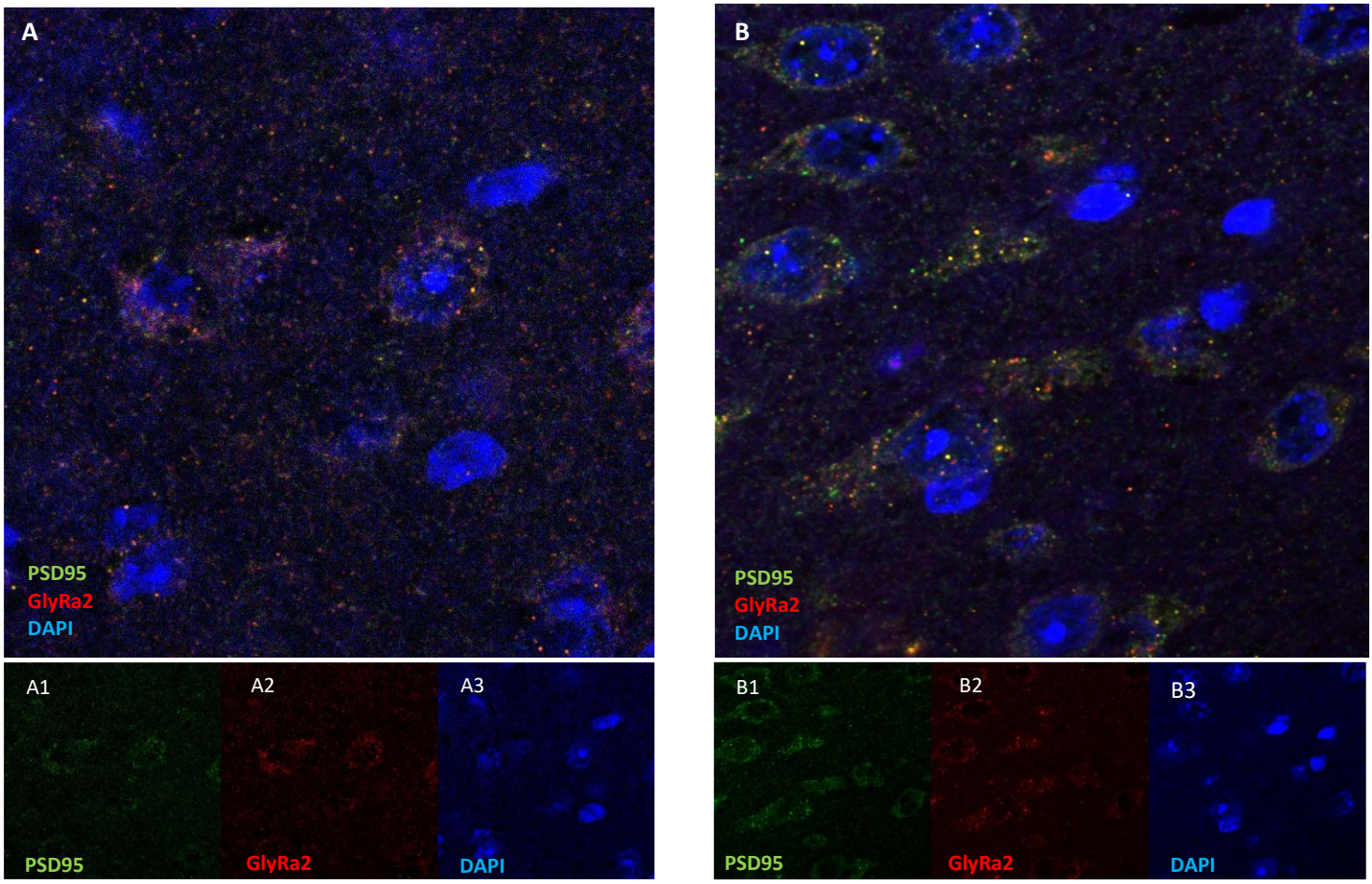

Figure S2: Tryout combination staining with rabbit anti-GlyRa2 (red) and rabbit anti-PSD95 (green). Alexa Fluor 647 signal was converted to green in Image J to facilitate the visual analysis of colocalization. A) PSD95 staining performed through overnight incubation of primary anti-PSD95 and secondary Alexa 647-conjugated antibodies before adding on top of brain slices. B) PSD95 staining was performed by consecutive incubation of primary and secondary antibodies. An almost complete overlap (yellow) is acknowledgable. Confocal images are taken at 63x magnification. GlyRa2, glycine receptor alpha 2; PSD95, post-synaptic density 95.