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Interplay between Dopamine and γ2-AminoButyric Acid type A receptors' surface dynamics during maturation of neurons and development of hippocampal networks.

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D5R surface dynamics at hippocampal inhibitory synapses

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica do Professor Doutor François Maingret (Université de Bordeaux) e da Professora Doutora Ana Luísa Carvalho (Universidade de Coimbra)

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List of Abbreviations

AF	Alexa Fluor
BSA	Bovine Serum Albumin
cAMP	cyclic Adenosine MonoPhosphate
(c)DNA	(complementary) DeoxyriboNucleic Acid
D1R	Dopamine D1 Receptor
D5R	Dopamine D5 Receptor
DA	Dopamine
DARs	Dopamine Receptors
DIV	Days *in vitro*
DsRed	*Dicosoma sp.* Red (Fluorescent Protein)
E18	Embryonic day 18
eG/Y/C/mRFP	endogenous Green/Yellow/Cyan/monomeric Red Fluorescent Protein
EMCCD	Electron-Multiplying Charge-Coupled Device
ExtraSyn	ExtraSynapse
$GABA_AR$	Gamma-AminoButyric Acid Receptor Type A
γ2-$GABA_AR$	Gamma 2-containing $GABA_AR$
IgG	Immunoglobulin G
IQR	InterQuartile Range
LTP	Long-Term Potentiation
MSD	Mean Square Displacement
NMDAR	N-Methyl-D-Aspartate Receptor
PeriSyn	PeriSynapse
PFA	ParaFormAldehyde
PKA	Protein Kinase A

PKC	Protein Kinase C
PSD-95	PostSynaptic Density protein-95
QD	Quantum Dot
SEM	Standard Error of the Mean
SEP	super-ecliptic pHluorin
Syn	Synapse

Abstract

A dynamic synapse is crucial not only in the regulation of synaptic transmission but also for maturation and development of neurons and neuronal circuits. This is particularly important in the case of receptors, which must keep highly mobile and capable of exchanging between synapse and extrasynapse by lateral diffusion in order to modulate synaptic strength. This process must be well regulated and, one of the ways of doing so is through protein-protein interactions. Dopamine receptors are an interesting example by which this happens, providing at the same time a novel way of crosstalk between neurotransmitter systems and receptor dynamics regulation. First, a specific direct physical interaction was described between dopamine D5 receptor (D5R) and the γ2 subunit of γ-AminoButyric Acid type A Receptors ($GABA_A$Rs); this interaction could bi-directionally modulate the involved receptors' properties, inhibiting them upon agonist application. A couple of years later, another interaction, this time between dopamine D1 receptor (D1R) and N-Methyl-D-Aspartate Receptors (NMDARs), was also shown to modulate NMDARs function. Interestingly, our group showed that this interaction forms a perisynaptic reservoir of NMDARs that quickly diffuse to the synapse, enhancing synaptic strength, upon complex disruption by dopamine activation or interfering peptide action. This observation led us to wonder whether the D5R-$GABA_A$R interaction initially described was also capable of modulating these receptors surface dynamics. We here present preliminary data, obtained using super-resolution single nanoparticle tracking of D5/D1R and $GABA_A$R transfected in hippocampal neurons, which suggest that D5Rs show a confined and slow diffusion behavior in inhibitory but not glutamatergic synapses, in accordance with the clustering with $GABA_A$Rs but not NMDARs.

Keywords: lateral diffusion; single-molecule tracking; hippocampus; D5R; $GABA_A$R

Abstract

Uma sinapse dinâmica é fundamental não só na regulação da transmissão sináptica mas também para a maturação e desenvolvimento de neurónios e circuitos neuronais. Isto é particularmente importante no caso de recetores, que devem ser altamente móveis e capazes de transitar entre a sinapse e a extra-sinapse por difusão lateral de modo a modular a transmissão sináptica. Este processo tem de ser devidamente regulado e, uma das maneiras de o fazer é através de interações proteína-proteína. Um exemplo interessante é o caso dos recetores de Dopamina, proporcionando ao mesmo tempo uma nova forma de 'conversa-cruzada' entre sistemas de neurotransmissores e a regulação da dinâmica dos respetivos recetores. Inicialmente foi descrita uma interação física e direta entre o recetor de Dopamina D5 (RD5) e a subunidade γ2 dos recetores tipo A de ácido gama-aminobutírico ($RsGABA_A$); esta interação podia modular bidireccionalmente as propriedades dos recetores envolvidos, inibindo-os após administração dos agonistas. Um par de anos mais tarde foi descrita outra interação, desta vez entre o recetor de Dopamina D1 (RD1) e os recetores de N-Metilo-D-Aspartato (RsNMDA), capaz de modular a função destes últimos. Interessantemente, o nosso grupo mostrou que esta interação forma um reservatório peri-sináptico de RsNMDA que difundem rapidamente para a sinapse, aumentando a força sináptica, após rutura do complexo por ativação de Dopamina ou ação de um péptido de interferência. Esta observação levou-nos a pensar se a interação RD5-$RGABA_A$ descrita inicialmente é também capaz de modular a dinâmica de superfície destes recetores. Aqui apresentamos resultados preliminares, obtidos usando *super-resolution single nanoparticle tracking* de recetores D5, D1 e $GABA_A$ transfetados em neurónios de hipocampo, que sugerem que os recetores D5 exibem um comportamento de confinamento e difusão lenta em sinapses inibitórias, mas não excitatórias, acordante com a interação com $RsGABA_A$ mas não com RsNMDA.

Palavras-chave: difusão lateral; *single-molecule tracking*; hipocampo; RD5; $RGABA_A$

Introduction

Unlike what was though several decades ago, the synapse is a highly dynamic compartment, composed of constantly-rearranging receptors, anchoring and auxiliary proteins. This dynamic behavior is crucial to ensure and modulate synaptic transmission (Choquet and Triller, 2013). Conversely, impairment of these dynamic processes is associated with neurologic disorders such as Alzheimer's disease and Schizophrenia (Lau and Zukin, 2007).

Of course, such important processes must be highly regulated. Focusing on the dynamics of synaptic receptors, the aim of this project, one must consider the process of lateral diffusion - free movement on the membrane surface powered by thermal Brownian agitation; This process, however, is not exactly "free", as distinct membrane compositions and the presence of "obstacles" affect the diffusion speed and limit receptors' movement in the membrane (Renner et al., 2012). Furthermore, the localization and stability of receptors at the surface level can be regulated by anchoring proteins (e.g. PSD-95, gephyrin), activity-dependent signaling mechanisms (e.g. phosphorylation/dephosphorylation of subunits) and interacting partners, such as cell adhesions molecules, auxiliary subunits or even other receptors (Jacob et al., 2008; Luscher et al., 2011; Ladépêche et al., 2013). The later example provides an interesting way that allows specific cross-talk between directly and physically interacting neurotransmitter systems.

Dopamine, most likely the most versatile neuromodulator in the brain, has a very prominent role in synaptic transmission, in part due to its capacity to modulate receptor trafficking and properties. Dopamine receptors (DARs) are divided in D1-like (D1R and D5R) and D2-like receptors (D2-4 receptors) which mediate their effects mainly through Protein Kinase A (PKA)-dependent and PKA-independent intracellular signaling pathways (Tritsch and Sabatini, 2012). An unusual way of receptor's dynamics modulation by dopamine, independent of second-messenger systems, has been described few years ago (Liu et al., 2000): a direct physical interaction between the C-terminal domain of D5R and the second intracellular loop of γ2 subunit of $GABA_A$Rs. This interaction is specific as no interaction was detected with D1R. Moreover, this interaction exerts mutual inhibitory effect on both receptors: Dopamine causes a decrease in $GABA_A$R-mediated currents while GABA causes a decrease in D5R-mediated cAMP production. This effect was proved to be specifically due to the physical interaction between D5R and $GABA_A$R, and independent of second-messenger signaling (Liu et al., 2000).

Later, in 2002, another case of direct physical interaction between two distinct neurotransmitter systems, this time between the C-terminal tails of D1R (but not D5R) and GluN1-1a or GluN2A subunits of NMDARs was reported (Lee et al., 2002). Similarly to what Liu and colleagues

reported, the D1R-NMDAR interaction regulates NMDAR function: application of D1R agonist SKF 81297 resulted in a decrease of surface NMDARs and NMDAR-mediated current. This was independent of PKA or PKC but dependent on the interaction between D1R and GluN2A subunit. They also observed that activation of D1R caused dissociation between D1R and GluN1-1a subunit (Lee et al., 2002). More recently, using high resolution single-nanoparticle tracking, our team was able to further elucidate the dynamics of D1R-NMDAR complex assembly/disassembly and functional implications of said process. We first described the behavior of D1R at glutamatergic synapses as diffusive receptors yet confined due to what we later concluded to be clusters at perisynaptic sites, with which NMDARs were colocalized. Furthermore, we demonstrated that disruption of the complex either by SKF 81297 or an interfering peptide specifically and bidirectionally modulated D1R and NMDAR dynamics: D1Rs diffuse away from the synapse whereas NMDARs enrich the synapse. Therefore D1Rs provide a perisynaptic reservoir of NMDARs capable of quickly enriching the synapse and induce LTP (Ladepeche et al., 2013).

These findings seem to indicate that, similarly to the functional consequences of the D1R-NMDAR interaction, the D5R-$GABA_AR$ interaction might also affect these receptors' localization and dynamics. The fact that, in the hippocampus, D1Rs are enriched in spines while D5Rs are mainly present in dendritic shafts (Bergson et al., 1995), where glutamatergic synapses harboring NMDARs and inhibitory synapses harboring $GABA_ARs$ are found (Sheng and Kim, 2011), respectively, is a good evidence supporting our hypothesis. Besides canonical PKA-dependent mechanisms of receptor modulation by DA receptors, this could be a novel, intracellular signaling-independent mechanism by which two similar members of D1-like family differentially regulate two distinct neurotransmitter systems. To address this question, we here use high resolution single-nanoparticle tracking technique to characterize the dynamics of D5R and γ2-$GABA_AR$, together with immunocytochemistry to confirm localization and assess co-localization of these receptors at inhibitory synapses of cultured hippocampal neurons.

If hold true, we might be facing a novel way of regulating $GABA_ARs$ localization and exchanges between sub-synaptic compartments, which is of vital importance to ensure a healthy synaptic transmission and connectivity, either by defining extrasynaptic vs synaptic population of $GABA_ARs$ (tonic-activated vs phasic-activated) (Farrant and Nusser, 2005) or defining synaptic receptor numbers and regulating synaptic strength (Jacob et al., 2008; Luscher et al., 2011).

Materials and Methods

Cell Cultures

Cultures of hippocampal neurons and glial cells were prepared by certificated technicians from E18 Sprague–Dawley rats. All experiments were conducted in strict compliance with European Communities Council and French Directives for care of laboratory animals European directives and French laws on animal experimentation (approved by Bordeaux University Institutional Animal Care and ethics committee). Briefly, cells were plated at a density of 350×10^3 cells per ml on polylysine precoated coverslips. Cultures were maintained in serum free neurobasal medium (Invitrogen) and kept at 37 °C in 5% (vol/vol) CO_2 for 18 days in vitro (DIV) at maximum.

Transfection

Neurons were transfected at 7–10 DIV with cDNAs using the Effectene transfection kit (Qiagen). Initially 4 ml of culture medium (Neurobasal Medium + Neuromix + Glutamine), previously equilibrated in the incubator, were added to the dishes to be transfected. The dishes were put back in the incubator. The following was added in 1 eppendorf (per dish to be transfected):

1. 150µl of buffer;
2. 0,9µg of plasmid containing the receptor DNA and 0,3µg of plasmid containing the synaptic marker DNA;
3. 8µl of enhancer

The mix was vortexed and quickly centrifuged (short spin) followed by 2-5 minutes incubation at room temperature. 25µl of Effectene was then added to each eppendorf and a new incubation period of 5-10 minutes at room temperature followed. In the meanwhile, new dishes were prepared: 4 ml of cultured medium were transferred from the original to these new dishes, followed by the coverslips. 1 ml of cultured medium was added from these dishes to the eppendorf with the respective DNA and the solution was pipetted up and down. Afterwards, the final mixture was added drop by drop on top of each coverslip and allowed to incubate for 30 minutes-1 hour at 37°C. Finally, the coverslips were transferred back to the original dishes. The cultures were allowed 2-3 days of expression before imaging/staining.

Single-Particle (Quantum Dot) Tracking and Surface Diffusion Calculation

Quantum dot (QD) 655 coupled to goat anti-mouse IgG (Life Sciences) was incubated (1:10,000; dilution may have been adjusted depending on the amounts of QDs imaged) for 10 minutes on neurons previously exposed for 10 minutes to mouse monoclonal anti-GFP (1:1000; Roche) antibodies; Briefly, a drop of 100µl of incubation medium - cultured medium + 1% BSA + antibody - was added on top of parafilm and the coverslips were put on top of it (neurons facing the drop) and allowed to incubate 10 minutes at 37°C. Between primary and secondary antibody incubations, coverslips were washed 3 times in washing medium – cultured medium + 1% BSA. Nonspecific binding was blocked by adding BSA (1%; Sigma) to the incubation/washing medium. Finally, the coverslips were mounted in the microscope chamber (filled with 500µl of washing medium) and QDs were imaged using a mercury lamp and appropriate excitation/emission filters. Signals were detected using an electron-multiplying charge-coupled device (EMCCD) camera (Evolve™). 500 consecutive frames were acquired (20Hz acquisition rate). QDs were followed on successfully transfected, healthy dendritic regions for up to 20min. QD recording sessions were processed with the MetaMorph® software (Molecular Devices). The instantaneous diffusion coefficient, D, was calculated for each trajectory from linear fits of the first four points of the mean square displacement (MSD) versus time function using $MSD(t) = \langle r^2 \rangle(t) = 4Dt$. Synaptic dwell time was calculated for exchanging receptors and defined as the mean time spent within the synaptic area.

Live Immunocytochemistry

Briefly, surface D1R-CFP or D5R-YFP were specifically stained using a monoclonal mouse anti-GFP antibody (1:500 for saturating conditions; Roche) for 10 min on live neurons at 37 °C in culture medium; the mixture (100µl) was added on top of the coverslips on top of parafilm. The coverslips were then put into wells which were then filled with 1ml of PFA 4% + PBS during 15 minutes at room temperature for cell fixation. From then on, between each of the following steps, coverslips were washed 6 times 2 minutes each in PBS. After fixation and wash, NH_4Cl 50mM was added for 10 minutes at room temperature (enough to cover the coverslips) to reduce background fluorescence. Coverslips were blocked with 100µl of PBS + 10% BSA for 30 minutes at room temperature. Neurons were incubated with AlexaFluor (AF)-488 coupled to goat anti-mouse secondary antibodies (1:250 for saturating condition; Invitrogen) in PBS + 3% BSA for 1h at room temperature. To permeabilize cells, PBS + 0,25% Triton was added on top of each coverslip (enough to cover coverslips) for 3 minutes at room temperature. After permeabilization, to label postsynaptic areas, neurons were first incubated with rabbit anti-GFP antibody for 2h at room temperature (1:500; Invitrogen) in PBS + 3% BSA

followed by AF-568 coupled to goat anti-rabbit secondary antibodies for 1h at room temperature (1:500; Invitrogen) again in PBS + 3% BSA to stain gephyrin-mRFP. Coverslips were washed with sterile water, mounted with 8µl Mowiol (‘homemade’ mounting medium) and kept at 4°C until visualization.

Results

D5Rs move slower and have a bigger immobile fraction in inhibitory synapses

The first objective of this project is to characterize the dynamics of D5R at both inhibitory and glutamatergic synapses. In order to do so, we transfected hippocampal neurons with D5R-YFP (yellow fluorescent protein) and Homer-DsRed (a specific glutamatergic synapse component conjugated with a variant of red fluorescent protein) or Gephyrin mRFP (a specific glycinergic and GABAergic synapse component conjugated with a monomeric red fluorescent protein) - D5H and D5G, respectively - and tracked the receptors at 12-16 DIV.

The velocity of receptors at the surface – diffusion coefficient [mean square displacement (MSD) per time] - in the two synapses was significantly different: D5R was 2-fold slower at inhibitory extra- and peri-synapse and 5-fold slower at the synapse, when compared to glutamatergic synapses (Fig. 1A). Strikingly, more than half of the D5Rs were immobile (with diffusion coefficients < 0.005µm^2/s) at inhibitory synapses (52%), which was 2.5-fold more than in excitatory synapses (20%) (Fig. 1B).

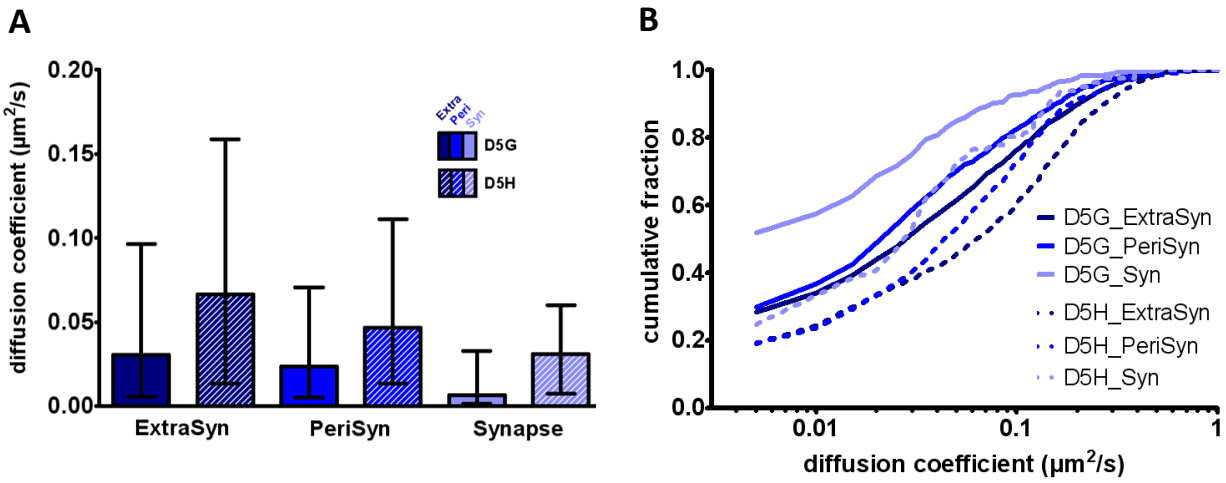

Figure 1. Diffusion coefficient and cumulative fraction of surface D5R-YFP show differences between Inhibitory (D5G, fully colored) and glutamatergic (D5H, dashed) synapses. (A) Diffusion coefficient, as median and Interquartile range (IQR), of D5R-YFP at Inhibitory (ExtraSyn: 3.04x10^{-2} µm^2/s; PeriSyn: 2.36x10^{-2} µm^2/s; Syn: 0.66x10^{-2} µm^2/s) and glutamatergic synapses (ExtraSyn: 6.64x10^{-2} µm^2/s; PeriSyn: 4.65x10^{-2} µm^2/s; Syn: 3.10x10^{-2} µm^2/s). (B) Cumulative fractions of the instantaneous diffusion coefficients of D5R-YFP at Inhibitory and glutamatergic synapses. The first points correspond to the percentage of immobile receptors (diffusion coefficient < 0.005µm^2/s). Inhibitory synapses (n=54 neurons) – ExtraSyn: 5300, PeriSyn: 1145 and Syn: 285 trajectories); Glutamatergic synapses (n=56 neurons) - ExtraSyn: 1549, PeriSyn: 589 and Syn: 81 trajectories.

D5Rs are more confined and spend more time in inhibitory synapses

By analyzing the mean square displacement (MSD - area explored by the receptor) we observed a tendency to a bigger confinement of D5R at inhibitory synapses, as seen by the plateau-forming curves deviating from the hypothetical free Brownian movement (gray dashed line) in D5G condition. We observed no differences between inhibitory and glutamatergic extra- or peri-synapse but a more confined behavior in inhibitory synapse: D5Rs explored $8.61x10^{-2}\mu m^2$ in glutamatergic synapses but just $5.86x10^{-2}\mu m^2$ in inhibitory synapses (values averaged from time points 0.40-0.55s) (Fig. 2A). In terms of time spent in each sub-synaptic compartment – dwell time – we can observe that, in both types of synapse, receptors spend increasingly less time as they go from extrasynapse to the synapse. Interestingly, D5Rs spend 1.5-fold more time across all inhibitory sub-synaptic compartments (Fig 2B).

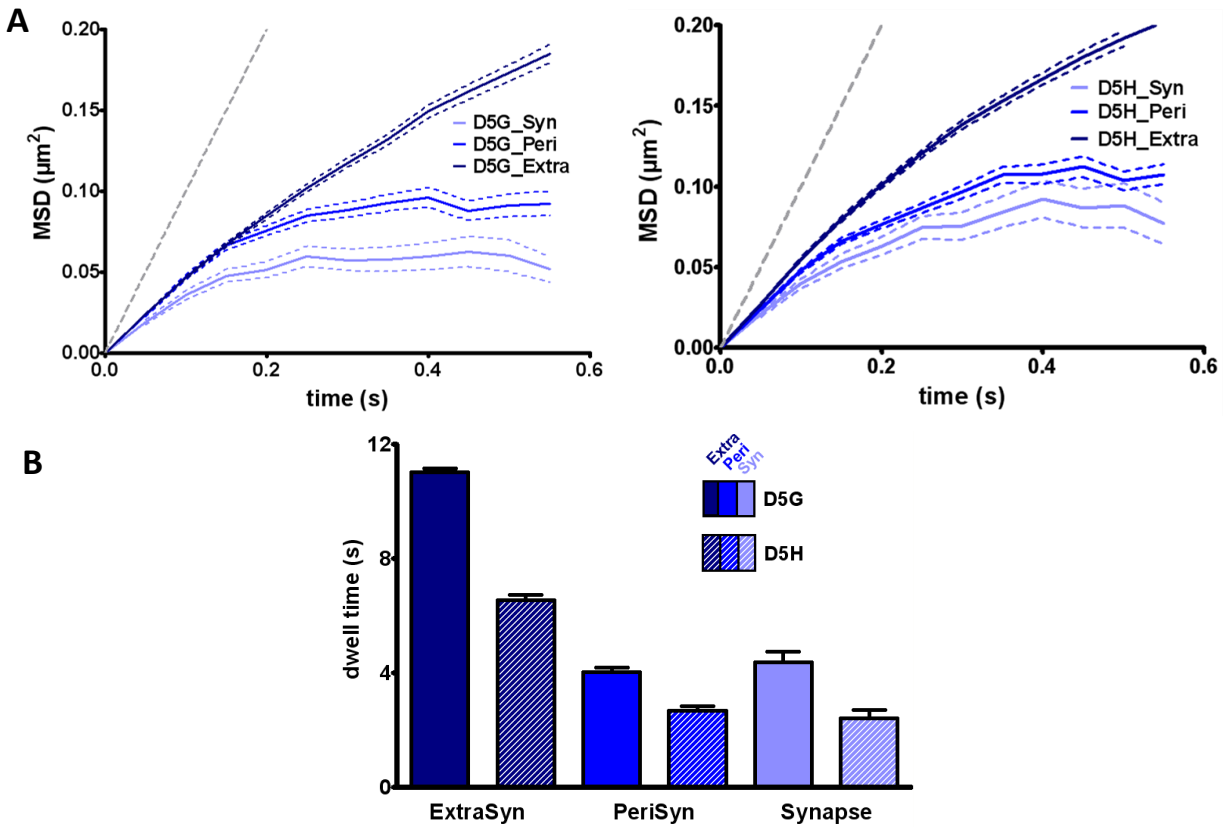

Figure 2. MSD reveals that D5Rs are more confined at inhibitory synapses but there are no major alterations in dwell time. (A) Plot of the MSD of D5R-YFP versus time segregated into Inhibitory (full line) and glutamatergic (dotted line) extra-, peri- and synapse. The standard error of the mean (SEM) is included alongside the mean value (n values from total trajectories). (B) Dwell time, as mean ± SEM, of D5R-YFP in different synaptic compartments of Inhibitory (ExtraSyn: 11.03±0.12s; PeriSyn: 4.03±0.16s; Syn: 4.38±0.36s) or glutamatergic synapses (ExtraSyn: 6.55±0.18s; PeriSyn: 2.69±0.15s; Syn: 2.42±0.29s). Inhibitory synapses (n=54 neurons) – ExtraSyn: 5300, PeriSyn: 1145 and Syn: 285 trajectories); Glutamatergic synapses (n=56 neurons) - ExtraSyn: 1549, PeriSyn: 589 and Syn: 81 trajectories.

D1R dynamics are similar at inhibitory and glutamatergic synapses

D1R and D5R, as members of the D1-like dopamine receptors, share similarities in terms of overall structure, pharmacological properties and signaling cascades activated (Vallone et al., 2000). The ability to bind to $GABA_A$Rs or NMDARs, however, is a property they do not share; most likely due to divergence within the C-terminal, domain shown to mediate the interaction with distinct neurotransmitter systems and effector proteins, D1R interacts specifically with NMDARs, but not $GABA_A$Rs (Lee et al., 2002b), whereas D5R interacts specifically with $GABA_A$Rs but not NMDARs (Liu et al., 2000). As such, we tracked D1R in both glutamatergic and inhibitory synapses as a negative control to our experiments with D5R. In order to do so, we transfected hippocampal neurons with D1R-CFP (cyan fluorescent protein) and Homer-DsRed or Gephyrin mRFP and tracked the receptor at 12-16 DIV.

Our results didn't show any major differences in receptor dynamics between the two types of synapses. The diffusion coefficient revealed a tendency to decrease the closer they get to the synapse although no changes were observed between synapses (Fig. 3A). The amount of immobile receptors at the synapse was also similar between synapses, with only a slight increase of immobile D1Rs at glutamatergic synapses - 40% vs 35% - and extrasynapses - 26% vs 21% (Fig. 3B).

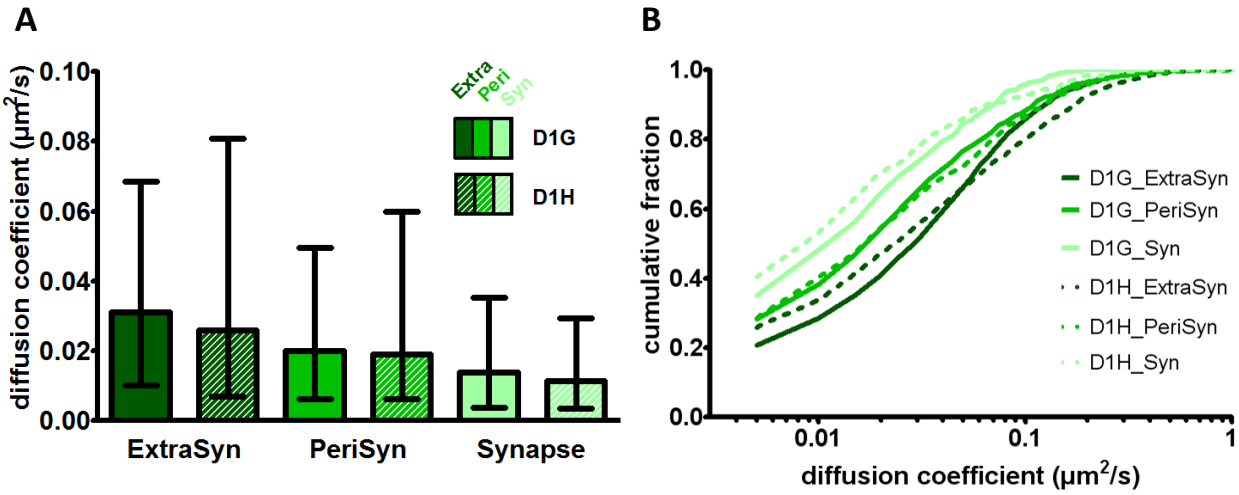

Figure 3. Diffusion coefficient and cumulative fraction of surface D1R-CFP do not show any major differences between Inhibitory (D1G, fully colored) and glutamatergic (D1H, dashed) synapses. (A) Diffusion coefficient, as median and Interquartile range (IQR), of D1R-CFP at Inhibitory (ExtraSyn: $3.11x10^{-2}$ $\mu m^2/s$; PeriSyn: $1.99x10^{-2}$ $\mu m^2/s$; Syn: $1.38x10^{-2}$ $\mu m^2/s$) and glutamatergic synapses (ExtraSyn: $2.59x10^{-2}$ $\mu m^2/s$; PeriSyn: $1.90x10^{-2}$ $\mu m^2/s$; Syn: $1.14x10^{-2}$ $\mu m^2/s$). (B) Cumulative fractions of the instantaneous diffusion coefficients of D1R-CFP at Inhibitory and glutamatergic synapses. The first points correspond to the percentage of immobile receptors (diffusion coefficient < $0.005\mu m^2/s$). Inhibitory synapses (n=65 neurons) – ExtraSyn: 7944, PeriSyn: 1273 and Syn: 299 trajectories); Glutamatergic synapses (n=29 neurons) - ExtraSyn: 2826, PeriSyn: 1041 and Syn: 290 trajectories.

D1R seem more confined in inhibitory synapses

By analysis of the MSD we could conclude that, D1R behaves distinctly in inhibitory or glutamatergic synapses. While we observe a more confined behavior in inhibitory extra- and peri-synapses (with a more prominent difference between extrasynapses), at glutamatergic synapses D1Rs are, however, more confined than at inhibitory synapses – $3.67x10^{-2}$ μm^2 vs $4.75x10^{-2}$ μm^2, respectively (values averaged from time points 0.40-0.55s) (Fig. 4A). Regarding the dwell time, we can conclude that receptors spend more time at the extrasynapse, but, again we observed no differences between inhibitory or glutamatergic synapses (Fig. 4B).

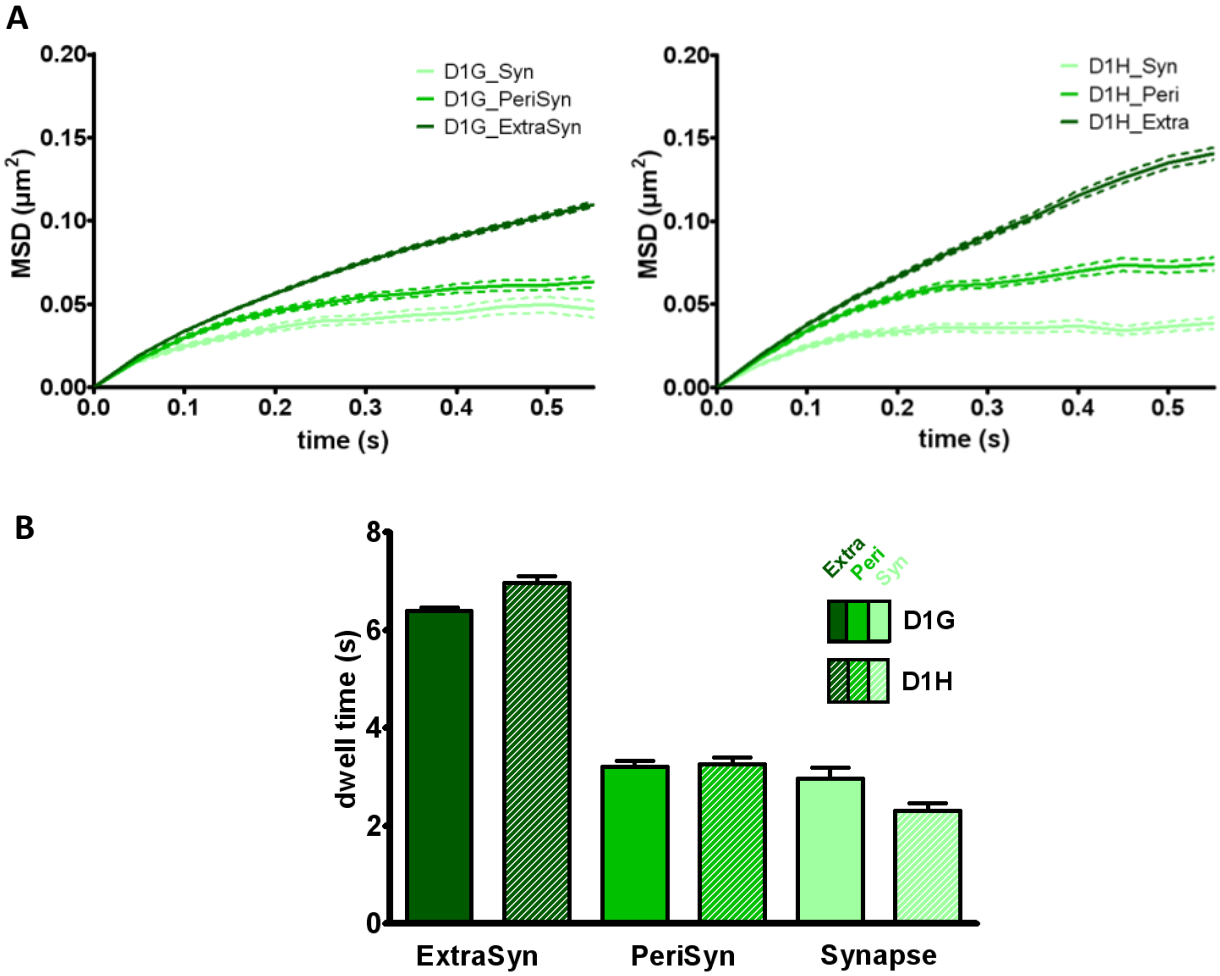

Figure 4. MSD shows a bigger confinement of D1R in inhibitory synapses yet no differences are observed in dwell time. (A) Plot of the MSD of D1R-CFP versus time segregated into Inhibitory (full line) and glutamatergic (dotted line) extra-, peri- and synapse. The standard error of the mean (SEM) is included alongside the mean value (n values from total trajectories). (B) Dwell time, as mean±SEM, of D1R-CFP in different synaptic compartments of Inhibitory (ExtraSyn: 6.39±0.08s; PeriSyn: 3.21±0.11s; Syn: 2.96±0.23s) or glutamatergic synapses (ExtraSyn: 6.97±0.14s; PeriSyn: 3.27±0.14s; Syn: 2.30±0.16s). Inhibitory synapses (n=65 neurons) – ExtraSyn: 7944, PeriSyn: 1273 and Syn: 299 trajectories); Glutamatergic synapses (n=29 neurons) - ExtraSyn: 2826, PeriSyn: 1041 and Syn: 290 trajectories.

γ2-GABA$_A$Rs move slower and have a higher immobile fraction in Inhibitory synapses

The next step was to study the dynamics of γ2-GABA$_A$Rs in both inhibitory, where they are known to interact with scaffolding machinery, and glutamatergic synapses, where they are not expected to form interactions. If indeed γ2-GABA$_A$Rs interact with D5Rs, we should expect similar dynamics for the two receptors or, at least, similar behavior in the same type of synapse. In order to do so, we transfected hippocampal neurons with γ2-SEP (super-ecliptic pHluorin, a pH sensitive derivate of eGFP) and Homer-DsRed or Gephyrin mRFP - G2H and G2G, respectively - and tracked the receptors at 12-16 DIV.

γ2-GABA$_A$Rs diffusion coefficient was markedly reduced across all sub-synaptic compartments of inhibitory synapses relative to glutamatergic ones, ranging from 3-fold decrease in extra- and peri-synapse to 3.5-fold decrease at the synapse (Fig. 5A). Likewise, we found a substantially bigger fraction of immobile receptors in inhibitory extra-, peri- and synapses (39%, 43% and 58% respectively) compared to glutamatergic ones (24%, 28% and 36%, respectively) (Fig. 5B).

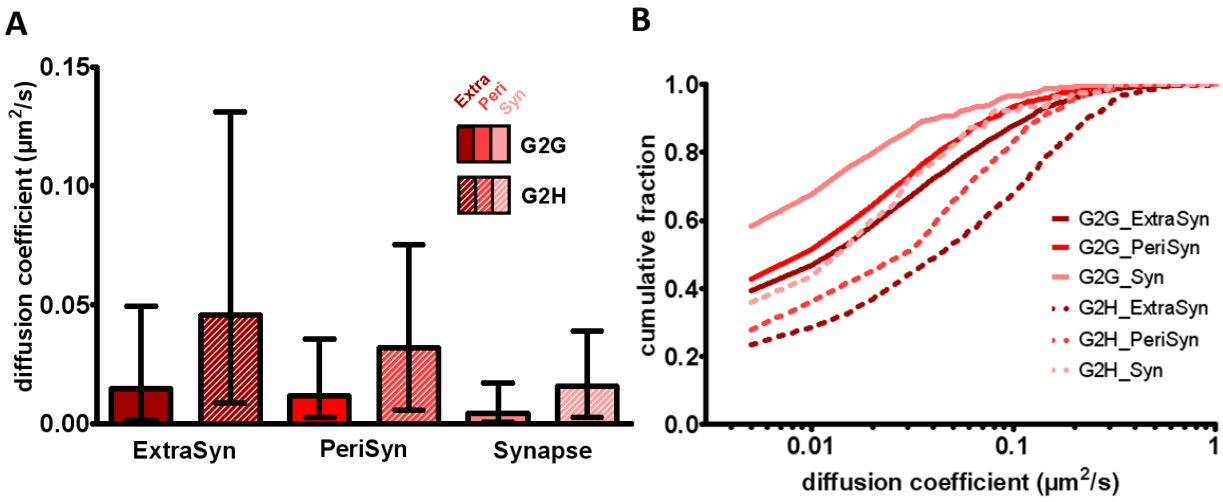

Figure 5. Diffusion coefficient and cumulative fraction of surface γ2-SEP indicate that γ2-containing GABA$_A$Rs move slower and have a bigger immobile fraction at inhibitory (G2G, fully colored) rather than glutamatergic (G2H, dashed) synapses. (A) Diffusion coefficient, as median and Interquartile range (IQR), of γ2-SEP at inhibitory (ExtraSyn: $1.48x10^{-2}$ µm^2/s; PeriSyn: $1.15x10^{-2}$ µm^2/s; Syn: $0.43x10^{-2}$ µm^2/s) and glutamatergic synapses (ExtraSyn: $4.57x10^{-2}$ µm^2/s; PeriSyn: $3.18x10^{-2}$ µm^2/s; Syn: $1.58x10^{-2}$ µm^2/s). (B) Cumulative fractions of the instantaneous diffusion coefficients of γ2-SEP at Inhibitory and glutamatergic synapses. The first points correspond to the percentage of immobile receptors (diffusion coefficient < 0.005µm^2/s). Inhibitory synapses (n=73 neurons) - ExtraSyn: 6689, PeriSyn, 1538 and Syn: 381 trajectories. Glutamatergic synapses (n=4 neurons) - ExtraSyn: 1139, PeriSyn: 297 and Syn: 78 trajectories.

γ2-GABA$_A$Rs are more confined in inhibitory synapses

Once again, after analyzing the MSD, we could observe that γ2-GABA$_A$Rs exhibited a confined behavior in both synapses, but even more so in inhibitory synapses. This difference was particularly accentuated between extrasynaptic sites while at the synapse the difference was smaller but γ2-GABA$_A$Rs were still more confined in inhibitory than glutamatergic ones: $2.31x10^{-2}\mu m^2$ vs $3.56x10^{-2}\mu m^2$, respectively (values averaged from time points 0.35-0.50s) (Fig. 6A).

In terms of time spent in the synapse, again we observe a bigger dwell time in both inhibitory and glutamatergic extrasynapses relative to other compartments and more time spent by γ2-GABA$_A$Rs at inhibitory synapses (Fig. 6B).

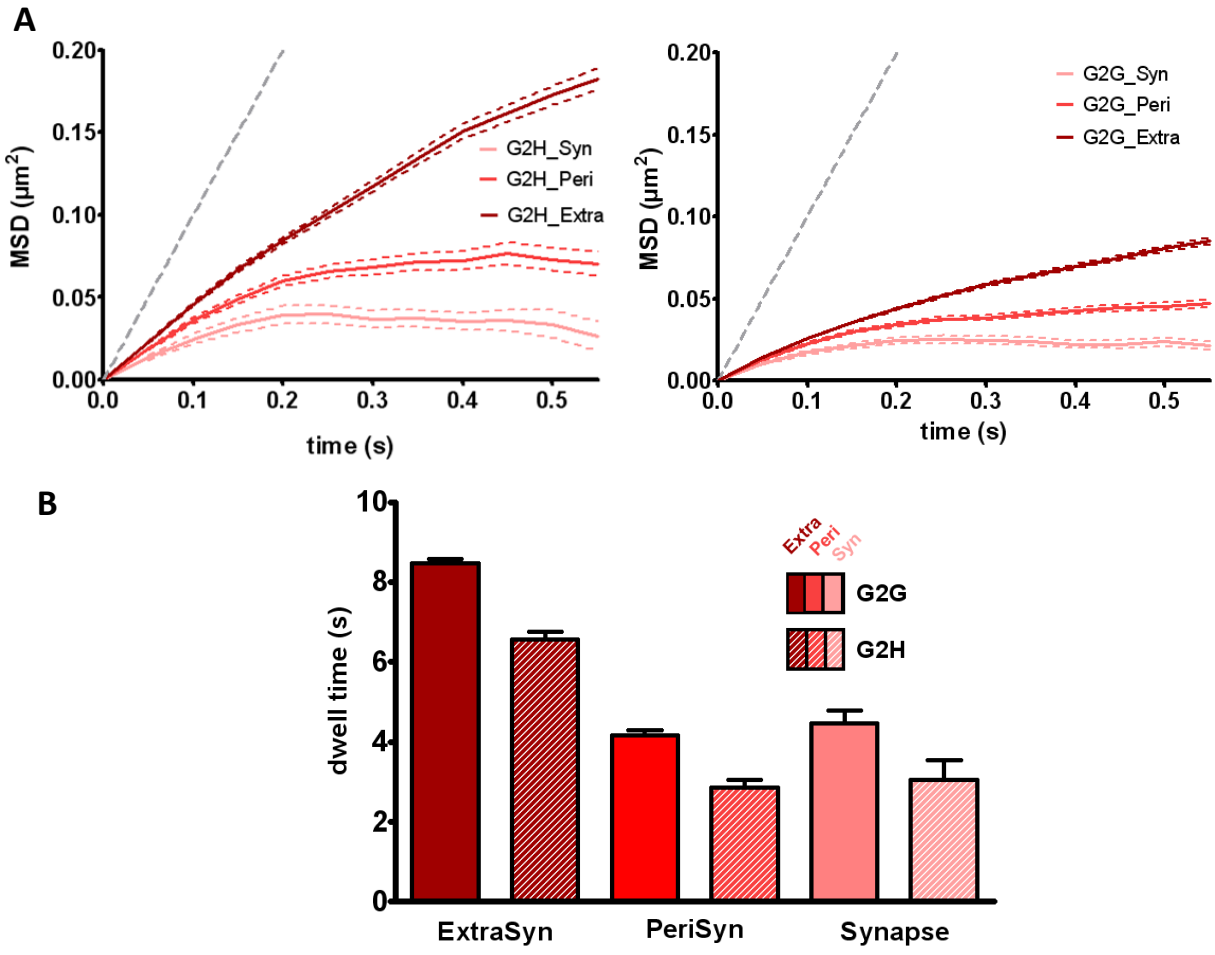

Figure 6. MSD shows a bigger confinement of γ2-GABA$_A$Rs in inhibitory synapses yet no differences are observed in dwell time. (A) Plot of the MSD of γ2-SEP versus time segregated into Inhibitory (full line) and glutamatergic (dotted line) extra-, peri- and synapse. The standard error of the mean (SEM) is included alongside the mean value (n values from total trajectories). (B) Dwell time, as mean±SEM, of γ2-SEP in different synaptic compartments of Inhibitory (ExtraSyn: 8.47±0.10s; PeriSyn: 4.17±0.14s; Syn: 4.47±0.31s) or glutamatergic synapses (ExtraSyn: 6.56±0.20s; PeriSyn: 2.87±0.19s; Syn: 3.05±0.50s). Inhibitory synapses (n=73 neurons) - ExtraSyn: 6689, PeriSyn, 1538 and Syn: 381 trajectories. Glutamatergic synapses (n=4 neurons) - ExtraSyn: 1139, PeriSyn: 297 and Syn: 78 trajectories.

D5Rs seem to be more co-localized with gephyrin than D1Rs

Until this point we just followed the movement of the different receptors at inhibitory and glutamatergic synapses. Now we wanted to confirm that indeed, D1Rs are enriched in spines, where glutamatergic synapses are established, whereas D5Rs mostly remain in the shafts, where inhibitory synapses are established (Bergson et al., 1995). In order to do this we performed immunocytochemistry of hippocampal neurons, co-staining the transfected receptor and the synaptic marker 3-7 days after transfection. So far, we only have preliminary data from D5Rs or D1Rs at inhibitory synapses, but from that data we can observe that D5Rs co-localize more frequently with gephyrin spots than D1Rs (e.g. circles in Fig. 7A). In fact, in the D1G condition, almost all synaptic areas are completely devoid of receptors (e.g. arrows and inset in Fig. 7B).

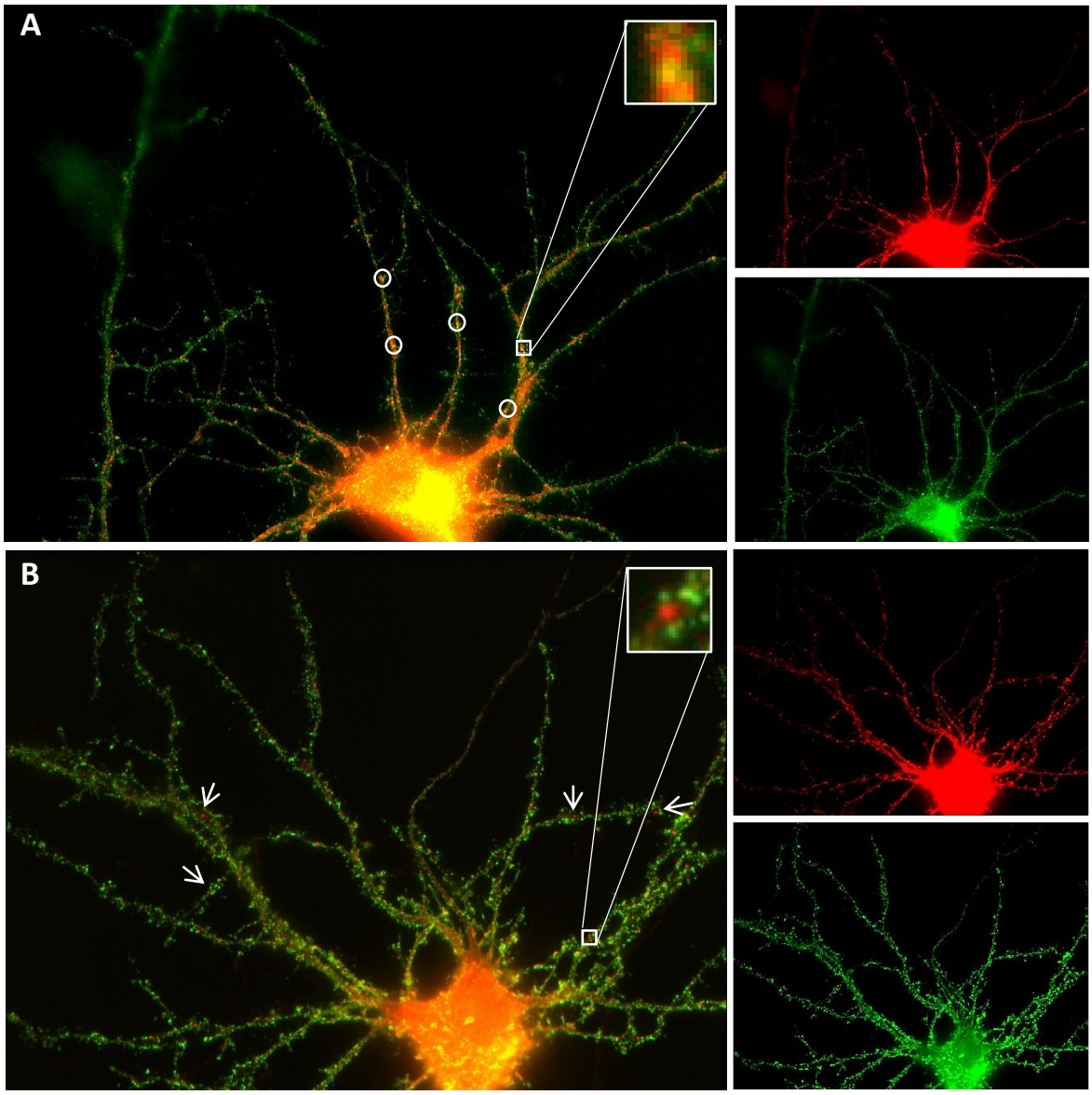

Figure 7. Immunocytochemistry of hippocampal neurons show that D5Rs colocalize more with gephyrin than D1Rs. (A) Neuron stainined for D5R-YFP (AF-488, in green; bottom right image) and gephyrin-mRFP (AF-568, in red; top right image). Circles highlight areas of colocalization. (B) Neuron stainied for D1R-CFP (green; bottom right image) and gephyrin-mRFP (red; top right image). Arrows indicate gephyrin clusters. Insets: Higher magnification of areas presenting or not colocalization.

Discussion and Conclusion

Using high-resolution single nanoparticle tracking of D1-like and γ2-containing $GABA_A$ Receptors in Homer- and Gephyrin-stained synapses, we here provide good evidences that indeed DARs are present at inhibitory synapses, where D5Rs, but not D1Rs, form stable clusters, most likely due to the direct physical interaction with $GABA_A$Rs previously described (Liu et al., 2000).

Our preliminary results on the behavior of D1R led us to the surprising conclusion that there are no major differences in the dynamics of this receptor between the two synapses. Since D1R binds specifically to NMDARs, and not $GABA_A$Rs, we were expecting a more confined D1R at glutamatergic synapses than at inhibitory ones, together with a bigger number of immobilized receptors at peri- or synaptic areas of glutamatergic synapses, where the localization of D1R-NMDAR was described (Ladepeche et al., 2013). Particularly, the fraction of immobilized D1R at inhibitory synapses was bigger than we expected, when compared to glutamatergic synapse, since, to our knowledge, there are no reports of interaction between this receptor and inhibitory-specific synaptic proteins (Bergson et al., 2003; Wang et al., 2008). The existence of uncharacterized D1R partners at inhibitory synapses or even the presence of NMDARs themselves, which has been already described (Cserép et al., 2012), are possibilities that cannot be ruled out and might explain the unexpected confinement observed. There is also the possibility of trajectory miss-readings due to high amounts of QDs and low sample diversity that could ultimately result in misleading or unreliable results. Indeed, regarding the D1R condition, we have a rather small amount of peri- and synaptic trajectories compared to extra-synaptic ones, which indicate that there might have been some kind of mistake during the analysis. Either way, these results certainly need to be further (re)analyzed, complemented with more experiments using a bigger number of cultures and smaller amounts of QDs to access whether this behavior is in fact due to interaction of D1Rs with other proteins or just a consequence of “poor” experimental procedure.

Regarding D5R dynamics at glutamatergic and inhibitory synapses, we obtained some interesting preliminary data. As we expected, D5Rs show a confined behavior, substantially slower diffusion and a much higher fraction of immobilization in inhibitory synapses relative to glutamatergic. These results are in accordance with the existence of clusters and/or regulatory mechanisms of D5R in inhibitory synapses, most likely mediated, at least in part, by their direct physical interaction with $GABA_A$Rs. D5Rs still present some degree of confinement at glutamatergic synapses which may be explained by the crowded environment and the shape of this.

Comparing D1 and D5 receptors in the same synapse also provides some interesting conclusions. At glutamatergic synapses D1Rs are more confined and slower compared to D5Rs. The decrease in

diffusion speed is most likely due to a 2-fold increase in immobile D1Rs relative to D5Rs (Supplementary Fig. 1). These results agree with the D1R-specific clustering with NMDARs previously reported (Lee et al., 2002a; Ladepeche et al., 2013). In inhibitory synapses, D5Rs are 2-fold slower exclusively at the synapse, have a bigger synaptic immobile fraction relative to D1Rs (52% versus 35%; respectively) and spend almost twice more time at the extrasynapse (Supplementary Fig. 2). These results seem to suggest the existence of a clustered population of D5Rs at the inhibitory synapse and the D5R-GABAAR interaction seem a probable cause for such behavior. Intriguingly, D1Rs are more confined in the inhibitory synapse than D5Rs. Once again this is likely due to errors during analysis.

We next tracked γ2-$GABA_A$Rs in both glutamatergic and inhibitory synapses. $GABA_A$Rs containing this subunit were shown to directly interact with D5Rs as well as to localize mainly at the synapse (Liu et al., 2000; Alldred et al., 2005). As expected, γ2-$GABA_A$Rs are highly confined, immobile and slowly diffusive at inhibitory synapses, to a much bigger degree than at glutamatergic ones; since they are in their "natural habitat", these receptors have several interacting partners (Jacob et al., 2008) contributing to an overall highly stable behavior at inhibitory synapses. This is most likely also the reason why γ2-$GABA_A$Rs are slightly more confined and immobile in inhibitory synapses than D5Rs. Moreover, the D5R-$GABA_A$R complex might only comprise a small fraction of the population, where the majority of D5Rs do no interact with $GABA_A$Rs and may even not be present at this synapses.

Interestingly, albeit different experimental setups, the MSD and diffusion coefficient values we obtained for γ2-$GABA_A$Rs, and even D5Rs, are in the same range of values as previously reported in the literature for the same type of $GABA_A$Rs (Renner et al., 2012). Moreover, the same study also characterized the dynamics of these receptors in "non-canonical" environment - excitatory synapses – and reported a slowed-down behavior, even though, as far as we know, said receptors do not form interactions at these synapses. These results strengthen the notion that receptors' dynamics are no straightforward matter and that they can be present outside their "natural habitat" with limited diffusion coefficient, which may explain some of our results with D1R in inhibitory synapses. Again, there is still the possibility of a faulty analysis that may have introduced errors in our values, something that we intend to verify in the future. Regarding the immunocytochemistry results, with so few experiments and preliminary results we cannot make any definitive statements about the localization of the receptors. These are ongoing experiments that need to be further complemented and analyzed. Our team had previously studied the co-localization of D1R and Homer or GluN1 and concluded that indeed D1Rs are clustered at the peri-synapse where they co-localize with NMDARs (Ladepeche et al., 2013); now we intend to complement those findings with the characterization of D1 and D5 receptors' co-localization with gephyrin or $GABA_A$Rs.

Again, these results are only preliminary raw data that need a bigger number of experiments and cultures to further complement them and allow a more significant insight on the dynamics of D1-like receptors. If verified, however, this study may prove valuable in uncovering a novel, signaling cascade-independent mechanism of receptor dynamics and localization modulation by the dopaminergic system. Besides the canonical role of DARs regulation of ionotropic receptors through PKA cascades, members from the same family may be able to differentially modulate distinct neurotransmitter systems through direct physical interactions with these receptors. Whereas D1Rs cluster excitatory NMDARs, controlling synaptic plasticity by directly affecting the number of receptors at the synapse and their turnover, D5Rs may have a similar function, this time with inhibitory $GABA_A$Rs. This may be functionally relevant in terms of synaptic plasticity, which, as we know, requires dynamic regulation of receptors at the synaptic surface (Luscher et al., 2011). In addition to the late effects of dopamine activation, that require a signaling cascade and phosphorylation of proteins, direct interaction between dopamine and GABAergic systems may serve as a fast first effector of synaptic plasticity and, consequently, impact the development and maturation of neurons, as was also proposed for the D1R-NMDAR case (Ladepeche et al., 2013).

While the preliminary data here shown cannot prove all these conjectures, it surely gives us some interesting possibilities to work with and to keep with the project. After confirming these results, we intend to study the functional consequence of the assembly/disassembly of D5R-$GABA_A$R complex. To do so, we will track D5Rs or γ2-$GABA_A$Rs in both synapses before and after the addition of D5R agonist and/or an interfering peptide capable of disrupting their interaction. We expect to observe alterations on the dynamics and localization of one or both receptors that may impact synaptic transmission and explain the decrease in $GABA_A$R-mediated current observed after D5R activation (Liu et al., 2000). We also intend to use dual-tracking of the receptors, which we will accomplish by transfecting hippocampal neurons with both D5R and γ2-$GABA_A$R, stain them with distinct QDs and track them simultaneously in the presence or absence of agonists or interfering peptides. Furthermore, to keep with our objective of uncovering the significance of D5R-$GABA_A$R assembly during synaptic development, we will also expand our experiments to other time windows, from 7 DIV immature neurons to 21 DIV fully matured neurons. This way we expect to understand at what time this interaction starts to occur and if and at what time this crosstalk ceases to exist.

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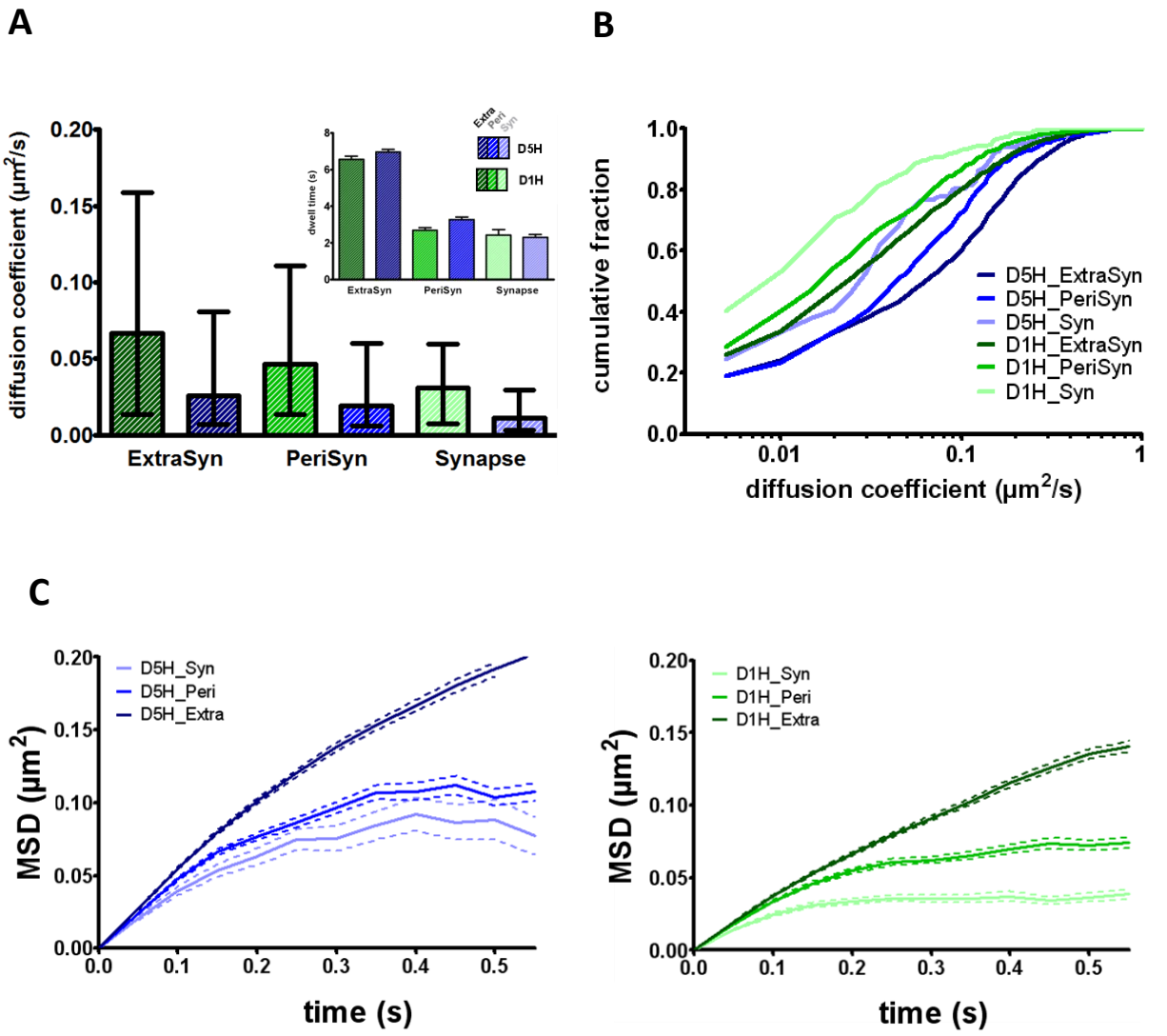
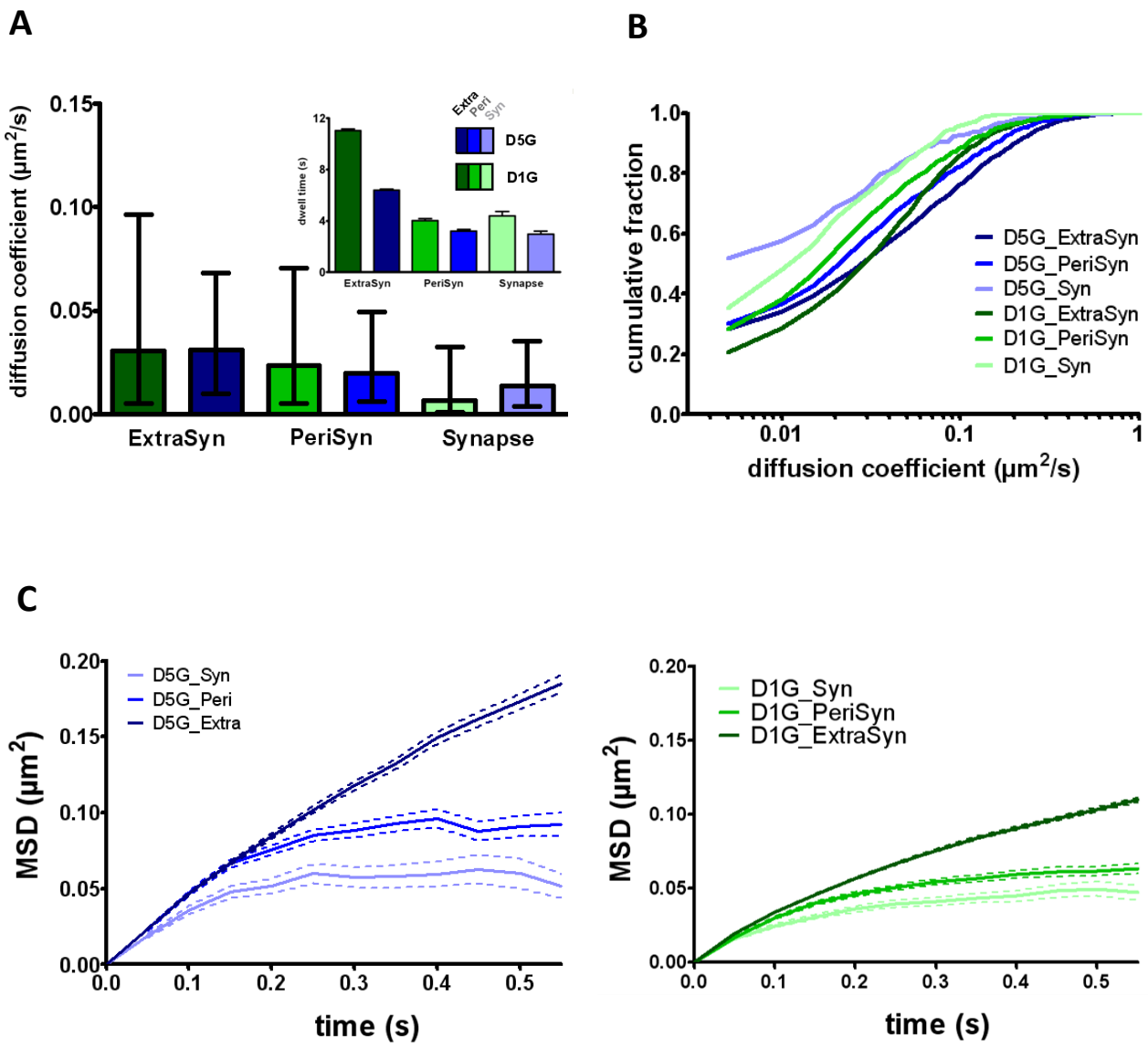
Supplementary Materials



Supplementary Figure 1. Comparison of D5 (shades of green) and D1 (shades of blue) receptor dynamics at glutamatergic synapses. (A) Diffusion coefficient, as median and Interquartile range (IQR), of D5R (ExtraSyn: $6.64x10^{-2}$ µm²/s; PeriSyn: $4.65x10^{-2}$ µm²/s; Syn: $3.10x10^{-2}$ µm²/s) and D1R (ExtraSyn: $2.59x10^{-2}$ µm²/s; PeriSyn: $1.90x10^{-2}$ µm²/s; Syn: $1.14x10^{-2}$ µm²/s) at glutamatergic synapses. Inset: Dwell time, as mean±SEM, of D5R (ExtraSyn: 6.55±0.18s; PeriSyn: 2.69±0.15s; Syn: 2.42±0.29s) and D1R (ExtraSyn: 6.97±0.14s; PeriSyn: 3.27±0.14s; Syn: 2.30±0.16s) at glutamatergic synapses. (B) Cumulative fractions of the instantaneous diffusion coefficients of D5R-YFP and D1R-CFP at glutamatergic synapses. The first points correspond to the percentage of immobile receptors (diffusion coefficient < 0.005µm²/s). (C) Plot of the MSD of D5R and D1R versus time segregated into glutamatergic extra-, peri- and synapse. The standard error of the mean (SEM) is included alongside the mean value (n values from total trajectories). D5H (n=56 neurons) - ExtraSyn: 1549, PeriSyn: 589 and Syn: 81 trajectories; D1H (n=29 neurons) - ExtraSyn: 2826, PeriSyn: 1041 and Syn: 290 trajectories.



Supplementary Figure 2. D5 and D1 receptors dynamics' comparison at inhibitory synapses. (A) Diffusion coefficient, as median and Interquartile range (IQR), of D5R (ExtraSyn: $3.04x10^{-2}$ µm²/s; PeriSyn: $2.36x10^{-2}$ µm²/s; Syn: $0.66x10^{-2}$ µm²/s) and D1R (ExtraSyn: $3.11x10^{-2}$ µm²/s; PeriSyn: $1.99x10^{-2}$ µm²/s; Syn: $1.38x10^{-2}$ µm²/s) at inhibitory synapses. Inset: Dwell time, as mean±SEM, of D5R (ExtraSyn: 11.03±0.12s; PeriSyn: 4.03±0.16s; Syn: 4.38±0.36s) and D1R (ExtraSyn: 6.39±0.08s; PeriSyn: 3.21±0.11s; Syn: 2.96±0.23s) at inhibitory synapses. (B) Cumulative fractions of the instantaneous diffusion coefficients of D5R-YFP and D1R-CFP at inhibitory synapses. The first points correspond to the percentage of immobile receptors (diffusion coefficient < 0.005µm²/s). (C) Plot of the MSD of D5R and D1R versus time segregated into inhibitory extra-, peri- and synapse. The standard error of the mean (SEM) is included alongside the mean value (n values from total trajectories). D5G (n=54 neurons) – ExtraSyn: 5300, PeriSyn: 1145 and Syn: 285 trajectories) D1G (n=65 neurons) – ExtraSyn: 7944, PeriSyn: 1273 and Syn: 299 trajectories)