UNIVERZITA KARLOVA
2. lékařská fakulta

Autoreferát disertační práce

Úloha proteinu SGIP1 v řízení Kanabinoidního receptoru 1

The role of SGIP1 protein in the control of cannabinoid receptor 1

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Praha, 2023

Disertační práce byla vypracována v rámci prezenčního/ studia doktorského studijního programu Biochemie a patobiochemie. na Ústav Molekulárni Genetiky AV ČR

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Oponenti:

Obhajoba se bude konat před komisí pro obhajoby oborové rady Biochemie a patobiochemie dne ..... v ..... od ..... hod

Předsedou komise pro obhajobu disertační práce byl jmenován:

Předseda oborové rady a garant doktorského studijního programu:

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Tato práce vznikla za podpory grantu GAČR GA16-24210S, GAČR GA21-02371S. a IMG International Mobility 2 (CZ.02.2.69/0.0/0.0/16_027/0008512, Ministerstvo školství, mládeže a tělovýchovy České republiky)

S disertační prací je možno se seznámit na Oddělení Ph.D. studia děkanátu 2. lékařské fakulty Univerzity Karlovy, V Úvalu 84, 150 06,Praha 5 (tel. 224 435 836).

ABSTRAKT

Dvě oblasti na C-konci kanabinoidního receptoru 1 (CB1R), ^{425}SMGDS^{429} a ^{460}TMSVSTDTS^{468}, obsahují shluky serinových a treoninových zbytků, které mohou být fosforylovány a společně hrají zásadní roli při desenzitizaci a internalizaci aktivovaného CB1R. Pomocí metod bioluminiscenčního rezonančního přenosu energie spolu s farmakologickými inhibitory jsme v této práci zkoumali úlohu fosforylace těchto serinových/treoninových klastrů při zprostředkování protein-proteinových interakcí CB1R s molekulami důležitými pro desenzitizaci a internalizaci tohoto receptoru. Ukázali jsme, že interakce CB1R s G proteinem spřaženou receptorovou kinázou 3 (GRK3) a β-arrestinem2 závisí na odlišných vzorcích fosforylace C-konce CB1R. Kromě toho musí být GRK3 pro interakci s CB1R v aktivní formě. Dále byl zkoumán vliv proteinu SGIP1 (Src homology 3-domain growth factor receptor-bound 2-like (endophilin) interacting protein 1) na dynamiku signalizátoru CB1R a desenzitizaci CB1R. SGIP1 zásadně mění interakci CB1R s GRK3 a β-arrestinem2 a modifikuje vazbu GRK3 na βγ podjednotky G proteinů. V této práci charakterizujeme nově identifikované sestřihové varianty SGIP1 a ukazujeme, že změny způsobené alternativním sestřihováním v doménách SGIP1 nemají vliv na inhibiční účinek SGIP1 na internalizaci CB1R. Výsledky této studie podrobně popisují molekulární mechanismy zprostředkující signalizaci a desenzitizaci CB1R. Popis těchto jevů pomáhá pochopit kanabinoidní signalizaci a vývoj tolerance.

Klíčová slova: receptory spřažené s G proteinem, kanabinoidní receptor 1, desenzitizace receptoru, G proteinem spřažená receptorová kináza 3, β-arrestin2, fosforylace, SGIP1

ABSTRACT

Two regions within the cannabinoid receptor 1 (CB1R) C-tail, ^{425}SMGDS^{429} and ^{460}TMSVSTDTS^{468}, contain clusters of serine and threonine residues that can be phosphorylated and collectively play an essential role in the desensitization and internalization of activated CB1R. Firstly, we studied the role of phosphorylation of the aforementioned serine/threonine clusters in protein-protein interaction between CB1R and molecules relevant to the receptor desensitization and internalization using the bioluminescence Resonance Energy Transfer method in tandem with pharmacological inhibitors. We show that CB1R interaction with G protein-coupled receptor kinases 3 (GRK3) and β-arrestin2 depends on distinct C-tail phosphorylation patterns. Furthermore, the activation of GRK3 is required for its interaction with CB1R. Secondly, we studied the impact of Src homology 3-domain growth factor receptor-bound 2-like endophilin interacting protein 1 (SGIP1) on the dynamics of CB1R signalosome and CB1R desensitization. SGIP1 altered CB1R interactions with GRK3, β-arrestin2 and GRK3-Gβγ coupling. We characterize newly identified splice variants of SGIP1 and demonstrate that alternative splicing-based alterations in SGIP1 domains do not affect the inhibitory effect of SGIP1 on CB1R internalization. This study's outcomes describe the molecular mechanisms mediating CB1R signaling and desensitization. Such details help to understand cannabinoid signaling and tolerance development.

Key words: G protein-coupled receptors, cannabinoid receptor 1, receptor desensitization, G protein-coupled receptor kinase 3, β-arrestin2, phosphorylation, SGIP1

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1. INTRODUCTION

Cannabinoid receptor 1 (CB1R) is the principal constituent of the ECS system and one of the most abundant metabotropic receptors in the brain [1]. CB1R serves as a crucial retrograde messenger that suppresses neurotransmitter release and plays a key role in the regulation of anxiety, fear, stress, cognitive and motoric functions, appetite, energy balance and metabolism [2-4]. Given the vital role that CB1R plays in many physiological processes, its activity is tightly regulated mainly via receptor desensitization and internalization. Two regions within the CB1R C-tail, ^{425}SMGDS^{429} and ^{460}TMSVSTDTS^{468}, contain clusters of serine and threonine residues that can be phosphorylated and together with molecules G protein-coupled receptor kinase 3 (GRK3) and β-arrestin2 collectively play an essential role in the desensitization and internalization of activated CB1R [5-9]. Nevertheless, the relationship between the previously mentioned phosphorylation sites and the recruitment of GRK3 and β-arrestin2 is elusive.

Besides the aforementioned mechanisms, CB1R is also regulated by its interaction partner Src homology 3-domain growth factor receptor-bound 2-like (endophilin) interacting protein 1 (SGIP1). The presence of SGIP1 inhibits ligand-induced internalization in transfected human embryonic kidney cells and affects CB1R signaling in a biased manner: it augments β-arrestin2 interaction with CB1R, decreases ERK1/2 phosphorylation while leaving Gi/o-protein activation and Ca^{2+} release unmodified [10]. While the NCBI Gene database predicts as many as 20 possible mouse SGIP1 variants, only four have been reported, and solely two splice variant was studied with respect to CB1R [11]. As a result, the impact of other SGIP1 variants on the properties of CB1R is not known.

2. AIMS AND HYPOTHESES

This thesis aims to investigate the role of ^{425}SMGDS^{429} and ^{460}TMSVSTDTS^{468} phosphorylation sites of CB1R in the recruitment of GRK3 and β-arrestin2. The research also investigates the role of GRK3 in facilitating interactions of molecules following activation of the CB1R as it undergoes desensitization. Additionally, we hypothesize that the dynamics of these interactions are further modulated by SGIP1, an interaction partner of CB1R.

Mouse SGIP1 is coded by 27 exons, which allow the expression of SGIP1 variants of different lengths via alternative splicing. According to the NCBI Gene database, alternative splicing can hypothetically produce 20 possible mouse SGIP1 variants. However, only four have been described so far. In our laboratory, we have identified several SGIP1 mRNAs derived from the mouse brain, coding potential novel splice variants. This work analyzes the detected splice variants by testing their expression and CB1R-modifying features as previously described for 806 amino acids long SGIP isoform [10].

3. METHODS

3.1. Bioluminescence resonance energy transfer assays

To investigate the interactions between studied molecules, the bioluminescence resonance energy transfer (BRET) assay was used. BRET assay utilizes a phenomenon called Förster resonance energy transfer between two molecules – luciferase and yellow fluorescein protein (YFP). The bioluminescent enzyme luciferase (Rluc), delivered from *Renilla reniformis,* in the presence of substrate coelenterazine h, emits photons that are absorbed by YFP, resulting in excitation and subsequent emission of photons of different wave-length. This phenomenon occurs only when luciferase and YFP are close to each other. To study the interaction of two proteins, each is tagged with YFP or luciferase. Cells were seeded and transiently transfected using Lipofectamine™ 2000 according to the manufacturer's protocol. Twenty-four hours after transfection, cells were washed with PBS, and coelenterazine h was added to a final concentration of 5 μM. The stimulation of the cells by agonist was performed 5 min later. BRET signal detection was performed using Mithras LB 940 microplate reader (Berthold Technologies, Germany) equipped with donor (480 ± 20 nm) and acceptor (540 ± 40 nm) filters. The BRET signal ratio was calculated as the emission of the energy acceptor molecules (540 ± 40 nm) divided by the emission of the energy donor molecules (480 ± 20 nm). The data are presented as the agonist-promoted milliBRET (mBRET) change calculated by subtracting the BRET ratio obtained in the absence of an agonist from the one obtained following agonist application and multiplied by 1000 (Figure 10 E).

3.2. Microscopy

Cells were seeded onto culture dishes dedicated for microscopy and transfected by correspondent plasmids using polyethyleneimine. Live cells were imaged at 37°C using an inverted fluorescent microscope Leica DMI6000 with confocal extension Leica TCS SP5 AOBS TANDEM confocal superfast scanner, objective 63 × 1.4 oil (Leica Microsystems, Germany). Samples were excited with an argon laser 514 nm and detected with a HyD 4 detector in 535–545 nm range. Microscopic images were processed in ImageJ.

3.3. SDS page and Western blot

Expression levels of CB1R-YFP mutant variants were characterized using the SDS page and subsequent western blot analysis of cell lysates. Briefly, HEK293 cells transfected with a particular CB1R variant or empty plasmid pRK6 (mock) were washed with ice-cold PBS and harvested in PBS complemented with cOmplete™ EDTA-free Protease Inhibitor Cocktail tablet followed by centrifugation 13,000 g for 10 min at 4 °C. Supernatants were decanted, and the pellets were resuspended in cold PBS with protease inhibitor. Afterward, the cells were disrupted by ultrasonication, and the total amount of protein in each lysate was determined using Bradford Reagent-based assay following the manufacturer's instructions. The samples were resuspended in SDS–PAGE treatment buffer (0.25 M Tris-Cl, 8% SDS, 20% glycerol, 0.02% bromophenol blue, 0.04 M DTT, pH 6.8) and boiled for 10 min at 85°C. Lysates were separated by 10% SDS–PAGE. Subsequently, the proteins were transferred to the nitrocellulose membrane using BioRad Trans-blot Turbo transfer system (semi-wet transfer) according to the manufacturer's instructions. The membrane was blocked in 5% blotting-grade powdered milk in PBST buffer. Afterward, the membrane was cut into two pieces and labeled either with primary antibody mouse anti-GFP (1:400) followed by secondary antibody labeling goat anti-mouse IgG-HRP antibody (1:10,000) for detection of CB1R-YFP variants or with primary antibody rabbit anti-actin (1:500) followed by secondary goat anti-rabbit IgG-HRP antibody (1:10,000) for the detection of actin to check the equal loading and protein transfer. The proteins of interest were visualized by chemiluminescence using the SuperSignal West PICO chemiluminescent substrate and detected on the LAS-300 system (Fujifilm, Japan).

3.4. Animals used

Mice were bred and group-housed in accordance with animal welfare rules. The animal care and experimental procedures used in this study complied with applicable laws, Guidelines of the National Institutes of Health on the Care and Use of Animals and to Directive 2010/63/EU. All animal models and experiments in this study were ethically reviewed and approved by the Institute of Molecular Genetics.

3.5. Inositol monophosphate accumulation

To measure the inositol monophosphate (IP1) release, IPOne HTRF kit (PerkinElmer - CisBio, France) was utilized accordingly to the manufacturer's recommendations. Briefly, cells were seeded and transiently co-transfected with CB1R variant and chimeric G protein $G\alpha_{qi9}$ (1:1 ratio), which permits $G_{i/o}$-coupled GPCRs to couple to $G\alpha_q$ and produce IP1 [12]. 24 hours after the transfection, cells were incubated in the presence of receptor agonist for 20 min at 37°C, and then cryptate-labeled anti-IP1 and D2-labeled IP1 antibodies were added for 1 h at the 21°C. Native IP1 produced by cells compete with d2-labeled IP1 (acceptor of energy) for binding of anti-IP1-Cryptate (donor of energy). The fluorescence was detected at 665 and 620 nm using a PHERAstar plate reader (BMG Labtechnologies, Germany). The HTRF signal was calculated as the 665/620 nm emission ratio multiplied by 10,000. The specific measured HTRF signal (energy transfer) is inversely proportional to the concentration of IP1 in the cells. The data were normalized against the minimal and maximal IP1 accumulation in cells driven by specific CB1R variant.

3.6. Internalization assay

The Homogenous Time-Resolved FRET (HTRF) technology was used to assess the cell surface receptor internalization rate. Briefly, HEK293 cells were seeded on 96-well plate and transiently transfected with SNAP-tagged CB1R plasmid and either with empty plasmid (pRK6) or SGIP1 splice variant (1:2 DNA mass ratio) using Lipofectamine™ 2000 according to the manufacturer's protocol. Twenty-four hours after the transfection, the cell culture medium was removed, and the cells were labeled with 100 nM SNAP-Lumi4-Tb, diluted in Tag-lite labeling medium, and incubated for 1 h at 37°C, 5% CO_2. Afterward, cells were washed four times with Tag-Lite buffer solution. The receptor internalization experiment was performed by adding Tag-lite buffer containing 24 μM fluorescein and agonist WIN 55,212-2 mesylate (WIN) or vehicle dimethyl sulfoxide (DMSO). HTRF signal was recorded over the course of 90 minutes at 37°C using Mithras LB 940 microplate reader equipped with HTRF module with relevant filters. After the donor (terbium cryptate) was excited at 340 ± 26 nm, the donor emission was measured at 520 ± 10 nm, and the acceptor (fluorescein) emission was measured at 620 ± 10 nm. The HTRF ratio was calculated as the donor emission divided by the acceptor emission multiplied by 10,000. Then, the ratios were normalized to maximal CB1R internalization values in the absence of SGIP1.

4. RESULTS

To test whether GRK3 requires an active conformation to interact with Gβγ, I used cmpd101, a pharmacological inhibitor that binds to the GRK2/3 active site and renders the kinase catalytically inactive, together with a GRK3-$G\gamma_2$ BRET-based sensor. Application of the CB1R agonist WIN resulted in a rapid GRK3-Rluc8-$G\gamma_2$-YFP association, as observed by the increase of the BRET signal (Figure 1 A). The pretreatment of cells with of cmpd101 significantly reduced the interaction between GRK3-Rluc8 and $G\gamma_2$-YFP upon WIN stimulation. The application of cmpd101 did not alter the amount of CB1R (Figure 1 B). WIN potency was not modified in the presence of cmpd101 as shown by WIN-dose response assay in cells untreated and pretreated cells with cmpd101 (Figure 1 C).

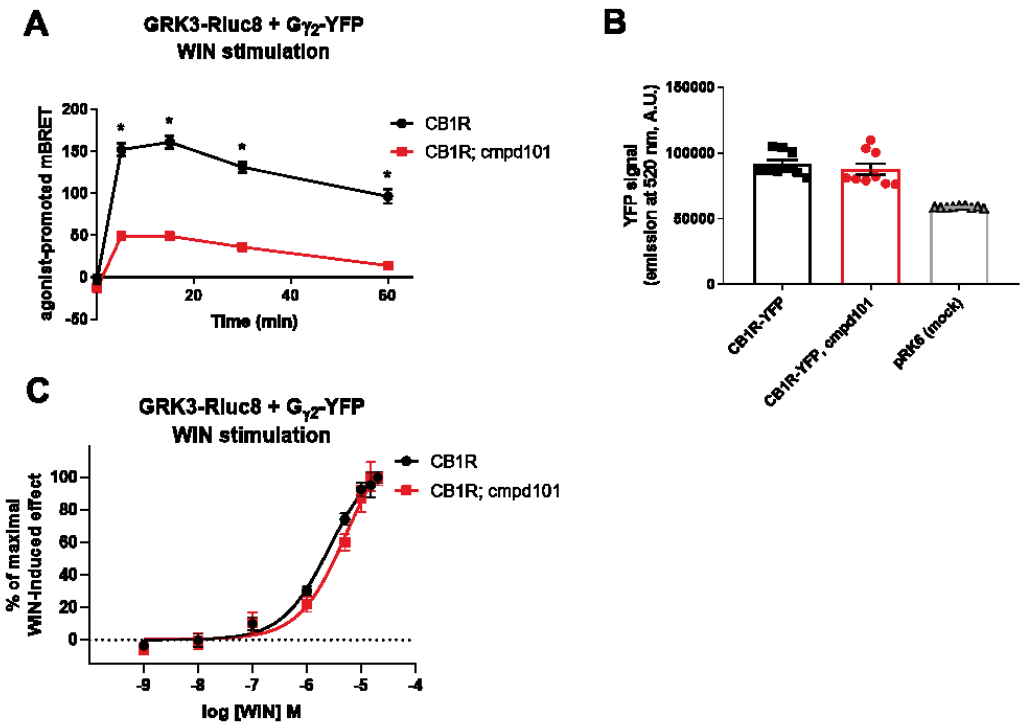

Figure 1. Inhibitor of GRK3 catalytic activity cmpd101 attenuates GRK3 interaction with $G\gamma_2$. A) Kinetic profiles of GRK3-RLuc8 and $G\gamma_2$-YFP association dynamics in cmpd101 treated and nontreated cells. **B)** cmpd101 does not affect the expression of CB1R-YFP. **C)** cmpd101 does not alter WIN potency. Dose-response curves of GRK3-RLuc8 and $G\gamma_2$-YFP association dynamics in cmpd101 treated and nontreated cells after CB1R stimulation with increasing concentrations of WIN. All data represent the mean ± SEM of three experiments of independent cell preparations performed in three technical replicates. *$p \leq 0.05$.

To study whether activation of GRK3 is required for the interaction with CB1R, HEK293 transiently expressing BRET pair CB1R-YFP and GRK3-RLuc8 were used to study their association. Stimulation of CB1R by WIN resulted in a rapid increase in BRET signal, implying a formation of CB1R-GRK3 complexes (Fig. 2 A). In contrast, pretreatment of cells with GRK2/3 activity blocker cmpd101 resulted in inhibited CB1R-GRK3 complex formation, as observed by decreased BRET signal (Fig. 2 A), suggesting that GRK3 has to be in active form to interact with CB1R. It was verified that WIN activation of CB1R specifically drives GRK3 recruitment to CB1R, as WIN application to mGluR1a-expressing cells did not produce change in BRET signal (Fig. 2 B). In addition, pretreatment of cells with the CB1R-selective inverse agonist rimonabant (SR141716) completely suppressed WIN-driven CB1R-GRK3 complex formation (Fig. 2 B).

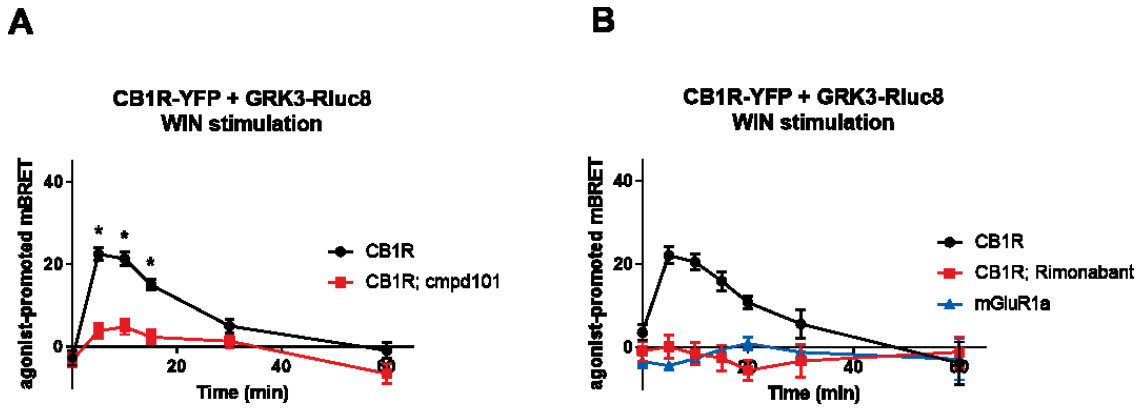

Figure 2. GRK3 catalytic activity is required for its association with the activated CB1R. A) Kinetic profiles of GRK3-RLuc8 recruitment by WIN-activated CB1R-YFP in HEK293 cells pretreated or not treated with cmpd101. **B)** GRK3 recruitment to CB1R is driven by WIN stimulation of CB1R. Kinetic profiles of GRK3-RLuc8 recruitment by WIN-activated CB1R-YFP/mGluR1a-YFP in HEK293 cells pretreated or not with rimonabant. Data represent the mean ± SEM of three experiments of independent cell preparations performed in three technical replicates. *$p \leq 0.05$.

To investigate if β-arrestin2 binding to CB1R is dependent on the kinase activity of GRK2/3, HEK293 expressing BRET pair β-arrestin2-Rluc and CB1R-YFP were treated or not with cmpd101. Upon activation of CB1R by

WIN, an apparent increase in BRET signal was recorded due to the formation of CB1R-β-arrestin2 complexes, whereas application of cmpd101 resulted in inhibited β-arrestin2 recruitment (Fig. 3), implying that recruitment of β-arrestin2 to WIN-stimulated CB1R depends on the catalytic activity of GRK2/3.

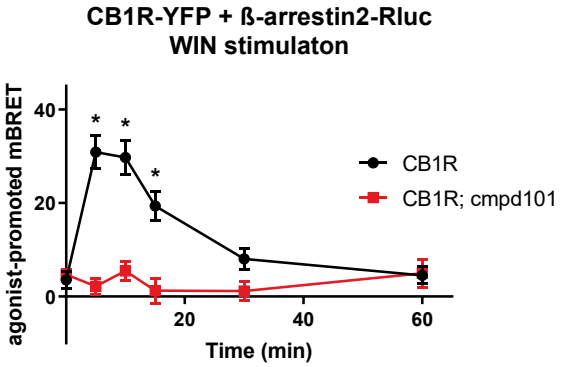

Figure 3. β-arrestin2 recruitment to CB1R is dependent on the activity of GRK2/3. β-arrestin2-Rluc recruitment by activated CB1R-YFP in cmpd101 pretreated and non-pretreated cells. Data represent the mean ± SEM of three experiments of independent cell preparations performed in three technical replicates. *p ≤ 0.05.

For the characterization of the role of CB1R C-tail phosphorylation in mediating interactions with molecules involved in receptor signaling and desensitization, a set of CB1Rs mutated within the ^{425}SMGDS^{429} and ^{460}TMSVSTDTS^{468} was created. Serine and threonine residues were mutated either into alanine residues, which cannot be phosphorylated, or into negatively charged aspartic acid, which partially mimics a phosphorylated state (Fig. 4). CB1R variants with mutations within ^{425}SMGDS^{429} region are termed as CB1R_2X, mutants in ^{460T}MSVSTDTS^{468} region as CB1R_6X. Receptors simultaneously mutated in both regions are labeled as CB1R_8X. Based on the amino acid substitution, X is either A (mutation into alanine) or D (aspartic acid mutations). The western blot analysis demonstrated that all mutant receptors have similar expression levels to wild type CB1R (Fig 5 A). Imaging by confocal fluorescent microscopy showed the proper receptor localization on the

cellular membrane (Fig 5 B). All the CB1R mutant variants remain functional and maintain the ability to activate the $G\alpha_{i1}$ protein signaling pathway, as receptors stimulation led to the activation of G proteins and subsequent dissociation of $G\alpha_{i1}$ and $G\beta\gamma$ subunits (Fig. 6).

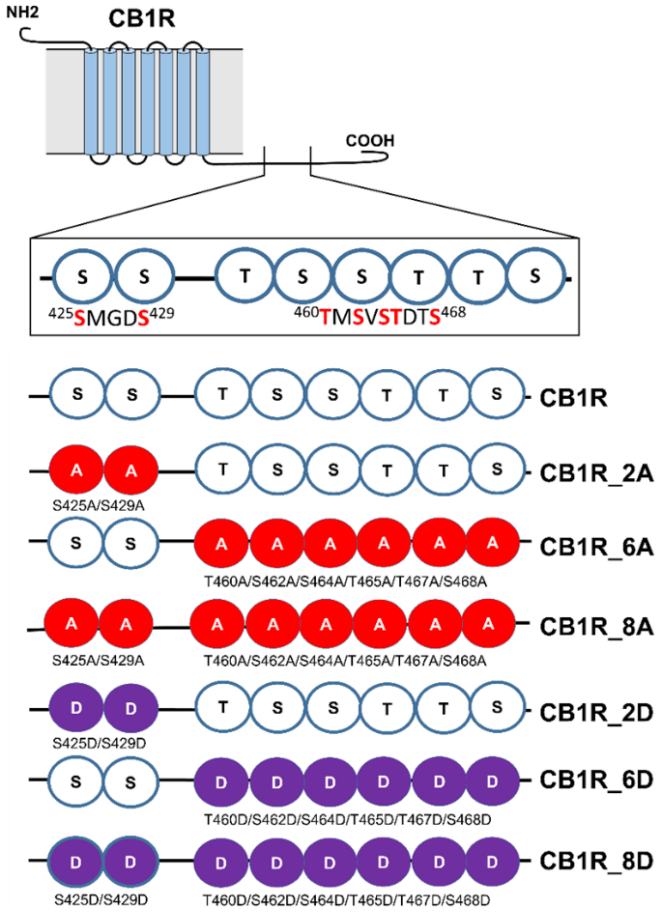

Figure 4. List of constructed CB1R variants mutated within the C-tail. Schematic depiction of CB1R mutants with corresponding sequences. Two regions of CB1R contain serine/threonine residues that are possibly phosphorylated during the desensitization of CB1R. CB1R C-tail phosphorylation mutants were constructed according to the following scheme: A - alanine mutation, D - aspartic acid mutation.

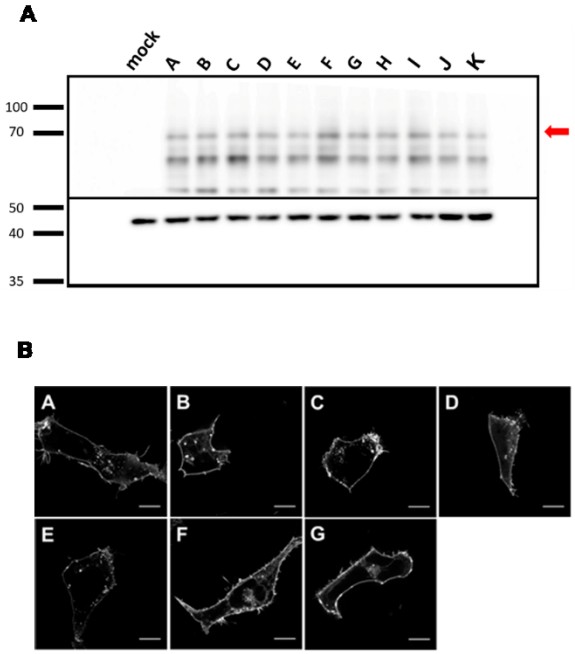

Figure 5. Mutant CB1Rs variants have similar levels of expression and cellular localization as wild type CB1R. **A)** Mutant CB1Rs variants have similar levels of expression to wild type CB1R. Membranes were stained with either anti-GFP antibody for detection of CB1R-YFP variants (top blot) or anti-Actin antibody (actin) to normalize for loading and transfer of proteins (bottom blot). Legend: mock (pRK6 empty vector transfection), A) CB1R, B) CB1R_2A, C) CB1R_6A, D) CB1R_8A, E) CB1R_2D, F) CB1R_6D, G) CB1R_8D, H) CB1R_^{425}SMGDS^{429}_^{460}TMAVATDTA^{468}, I) CB1R_^{425}AMGDA^{429}_^{460}TMAVATDTA^{468}, J) CB1R_^{425}SMGDS^{429}_^{460}AMSVSADAS^{468}, K) CB1R_^{425}AMGDA^{429}_^{460}AMSVSADAS^{468}. **B)** CB1R and mutant CB1Rs are predominantly localized on the cellular membrane. A single confocal section through the equatorial plane of the cells is shown. Legend: (A) CB1R, (B) CB1R_2A, (C) CB1R_6A, (D) CB1R_8A, (E) CB1R_2D, (F) CB1R_6D, (G) CB1R_8D. The scale bar represents 10 μm.

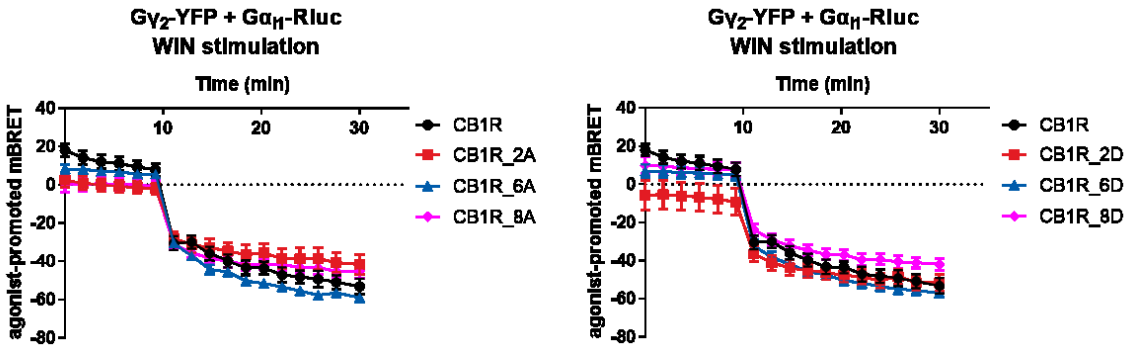

Figure 6. CB1Rs mutated in C-tail preserve the ability to activate G proteins. Data represent the mean ± SEM of three experiments of independent cell preparations performed in three technical replicates.

Afterward, I investigated the impact of CB1R C-terminal tail mutations on signaling by employing two different G protein-activation assays. The first BRET-based assay utilized $G\alpha_{i1}$-Rluc8 and $G\gamma_2$-YFP sensors to monitor G protein activation. The second assay measured the levels of inositol monophosphate (IP1) release via CB1R-driven activation of chimeric $G\alpha_{qi9}$. Both assays assessed the CB1R response on gradually increasing WIN

concentrations (WIN dose-response). The extent and potency of $G\alpha_{i1}$ activation and the production of IP1 driven by $G\alpha_{qi9}$ was similar in all tested CB1R variants (Fig. 7 A& B). Inability to phosphorylate serines and threonines within CB1R C-tail regions ^{425}SMGDS^{429} and ^{460}TMSVSTDT^{S468} does not affect G protein signaling.

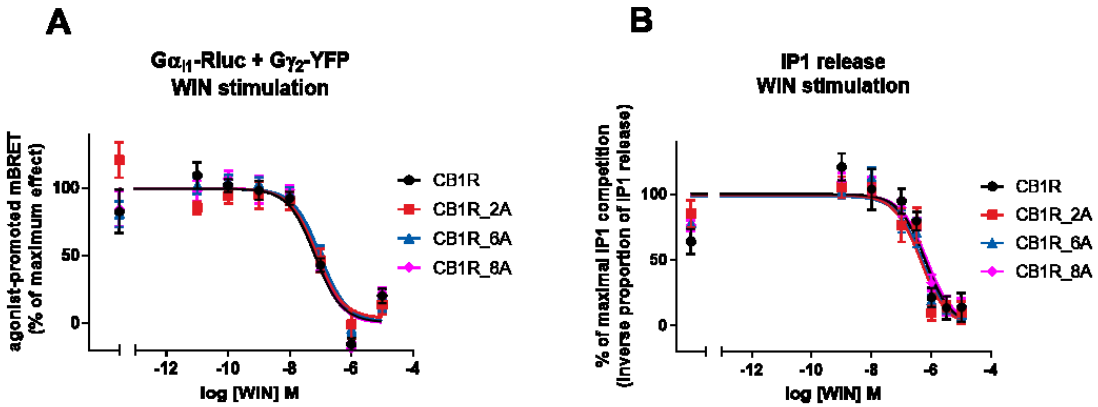

Figure 7. CB1R mutants do not have altered G protein signaling. A) C-tail mutations do not influence CB1R mediated $G\alpha_{i1}$ protein activation. Cells were stimulated with increasing concentrations of WIN and the decrease in BRET signal was measured 15 min after WIN application. **B)** CB1R mutants release comparable levels of IP1 as WT CB1R. Data represent the mean ± SEM of three experiments of independent cell preparations performed in three technical replicates. Data were normalized against the maximal WIN-induced response.

Next, the impact of CB1R C-tail phosphorylation on the interaction of the GRK3 with Gβγ subunits was studied using GRK3-Rluc8 and $G\gamma_2$-YFP BRET pair. All tested CB1R mutants were able to initiate recruitment of GRK3-Rluc8 to Gγ-YFP, albeit with different efficiency (Fig. 8 A - D). Consequently, the phosphorylation of the CB1R C-terminal motif is not required for GRK3-Gβγ interaction but is partially affected by it.

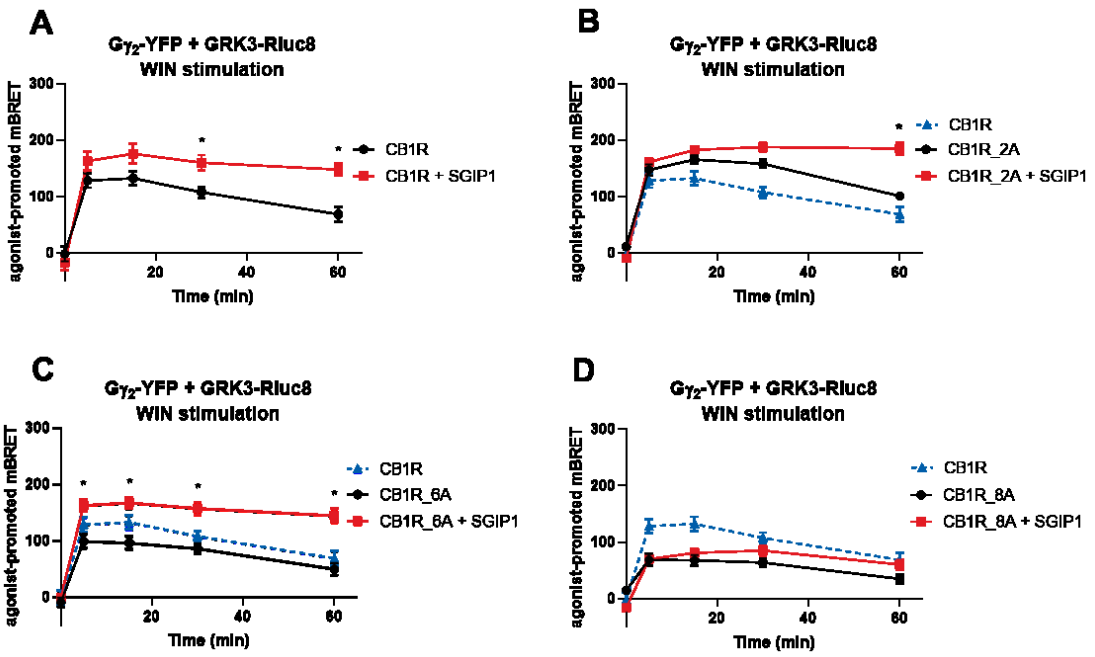

Figure 8. GRK3-Gβγ association is partially affected by CB1R C-tail phosphorylation. The interaction of GRK3 and Gβγ induced by the mutant receptors is modified by SGIP1. A) Kinetic profile of GRK3 recruitment to $G\gamma_2$ in CB1R in cell expressing WT CB1R and WT CB1R + SGIP1. **B)** Kinetic profile of GRK3 recruitment to $G\gamma_2$ driven by CB1R, CB1R_2A, and CB1R_2A + SGIP1. **C)** Kinetic profile of GRK3 recruitment to v driven in CB1R and CB1R_6A in the presence/absence of SGIP1. **D)** Kinetic profile of GRK3 recruitment to $G\gamma_2$ driven by CB1R and CB1R_8A in the presence/absence of SGIP1. Data represent the mean ± SEM of three experiments of independent cell preparations performed in three technical replicates. *$p \leq 0.05$.

As SGIP1 enhances CB1R and β-arrestin2 interaction [10], it was hypothesized that GRK3-Gβγ association could also be affected by SGIP1. Indeed, CB1R-induced interaction of GRK3-Rluc8 and $G\gamma_2$-YFP was significantly augmented and prolonged in the presence of SGIP1 (Fig. 8 A). Next, the effect of SGIP1 on GRK3-Gβγ complex formation driven by CB1R phosphorylation mutants was examined. Co-expression of SGIP1 with CB1R_2A or CB1R_6A mutants resulted in stronger receptor-driven GRK3-Gβγ association (Fig. 8 B & C). On the other hand, activation of CB1R_8A in the presence of SGIP1 resulted in similar GRK3-Gβγ interaction as in the cells without SGIP1 (Fig. 8 D).

Phosphorylation of serine/threonine residues within the receptor's third intracellular loop and C-tail by GRKs represents a pivotal step in the desensitization of GPCR. Therefore, the relationship between the phosphorylation pattern of CB1R and the recruitment of GRK3 was examined. Stimulation of WT CB1R by WIN increased BRET signal, which peaked at around 10 minutes and gradually diminished (Fig. 9 A). Activation of CB1R_2A-YFP induced increased and prolonged interaction (Fig. 9 B), while stimulation of phosphomimetic mutant CB1R_2D led to similar CB1R-GRK3 interaction as in a case of WT CB1R (Fig. 9 E). These observations suggest that the phosphorylation of serines in ^{425}SMGDS^{429} decreases GRK3 interaction with CB1R by limiting association or catalyzing dissociation.

Compared to WT CB1R, WIN application to CB1R_6A produced a significantly lower BRET signal (Fig. 9 C), while CB1R_8A did not induce BRET signal increase at all, pointing to an impaired ability to recruit GRK3 (Fig. 9 D). Both aspartic acid mutants CB1R_6D and CB1R_8D exhibited similar GRK3 recruitment dynamics as WT CB1R (Fig. 9 E). The outcomes of these experiments imply that the phosphorylatable serines/threonines of the long motif of ^{460}TMSVSTDTS^{468} are essential for proper GRK3–CB1R interaction.

As SGIP1 has a profound impact on CB1R interactions, the recruitment dynamics of GRK3 in the presence of SGIP1 was also studied. Co-expression of SGIP1 strengthened and prolonged GRK3 recruitment only in the receptors that interact with GRK3, CB1R, CB1R_2A (Fig. 9 A & B). SGIP1 alone was insufficient to rescue this interaction in mutant receptors, CB1R_6A and CB1R_8A, that could not recruit GRK3 regardless of SGIP1 presence (Fig. 9 C & D).

The crucial role of the ^{425}SMGDS^{429} and ^{460}TMSVSTDTS^{468} regions in regulating CB1R is also illustrated by the results obtained with the CB1R-β-arrestin2 interaction assays. Upon WIN-stimulation of WT CB1R, β-arrestin2

was rapidly recruited to the receptor (Fig. 10 A). Moreover, the activation of CB1R_2A showed decreased recruitment of β-arrestin2 (Fig. 10 B). This interaction was completely abrogated in CB1R_6A and CB1R_8A mutants, as their activation did not produce an increase in BRET signal (Fig. 10 C & D). These observations show that ^{425}SMGDS^{429} region is not imperative for the β-arrestin2 recruitment. Nevertheless, it plays an important role as its alanine mutation clearly decreases recruitment efficiency. On the contrary, the serine/threonine residues in ^{460}TMSVSTDTS^{468} motif are essential for β-arrestin2 recruitment to the activated CB1R, as their alanine-mutation completely inhibits β-arrestin2-CB1R interaction.

Interestingly, all aspartic acid mutants exhibited similar diminished β-arrestin2-recruitment dynamics compared to WT CB1R (Fig. 10 E). Co-expression of SGIP1 strengthened and prolonged CB1R-β-arrestin2 complex formation upon WIN treatment (Fig. 10 A). This effect was also observed when serine/threonine residues in ^{425}SMGDS^{429} motif are mutated (Fig. 10 B). On the contrary, we did not detect any effect of SGIP1 on β-arrestin2 recruitment in CB1R_6A or CB1R_8A mutants (Fig. 10 C & D).

Although SGIP1 augments β-arrestin2 association with the receptors that interact with β-arrestin2 (WT CB1R, CB1R_2A), the presence of SGIP1 alone is incapable of rescuing this interaction in mutants (CB1R_6A, CB1R_8A), that cannot recruit β-arrestin2.

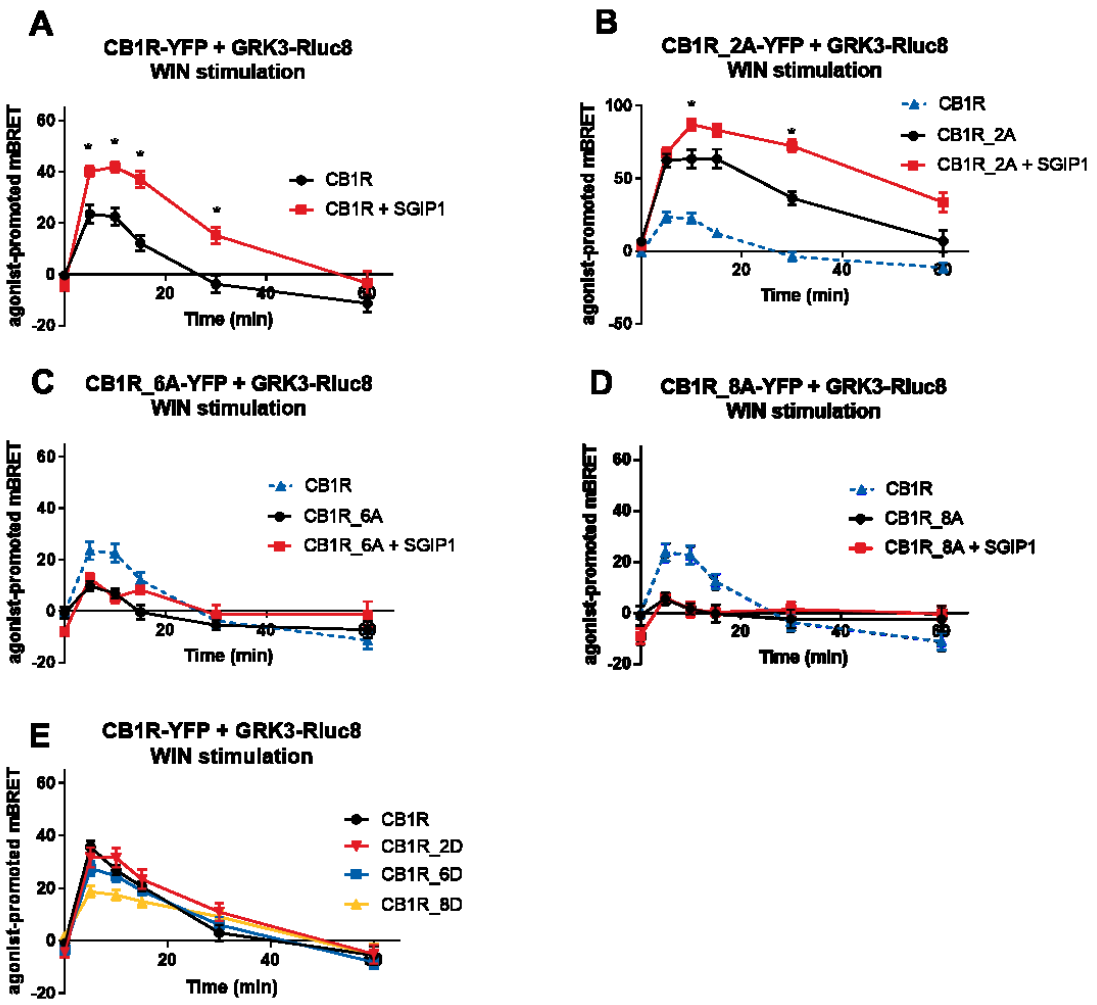

Figure 9. CB1R C tail multisite phosphorylation is pivotal for GRK3 recruitment and dissociation. SGIP1 increases the association of CB1R-GRK3 in CB1R mutants that interact with GRK3. A) Kinetics of GRK3 recruitment to CB1R in the presence and absence of SGIP1. **B)** Kinetics of GRK3 recruitment to CB1R, CB1R_2A, and CB1R_2A + SGIP1. **C)** Kinetic profile of GRK3 recruitment to CB1R and CB1R_6A in the presence or absence of SGIP1. **D)** Kinetics of GRK3 recruitment to CB1R, CB1R_8A, and CB1R_8A + SGIP1. **E)** Kinetic profiles of GRK3 recruitment to CB1R, CB1R_2D, CB1R_6D, CB1R_8D. Data represent the mean ± SEM of three experiments of independent cell preparations performed in three technical replicates. *$p \leq 0.05$.

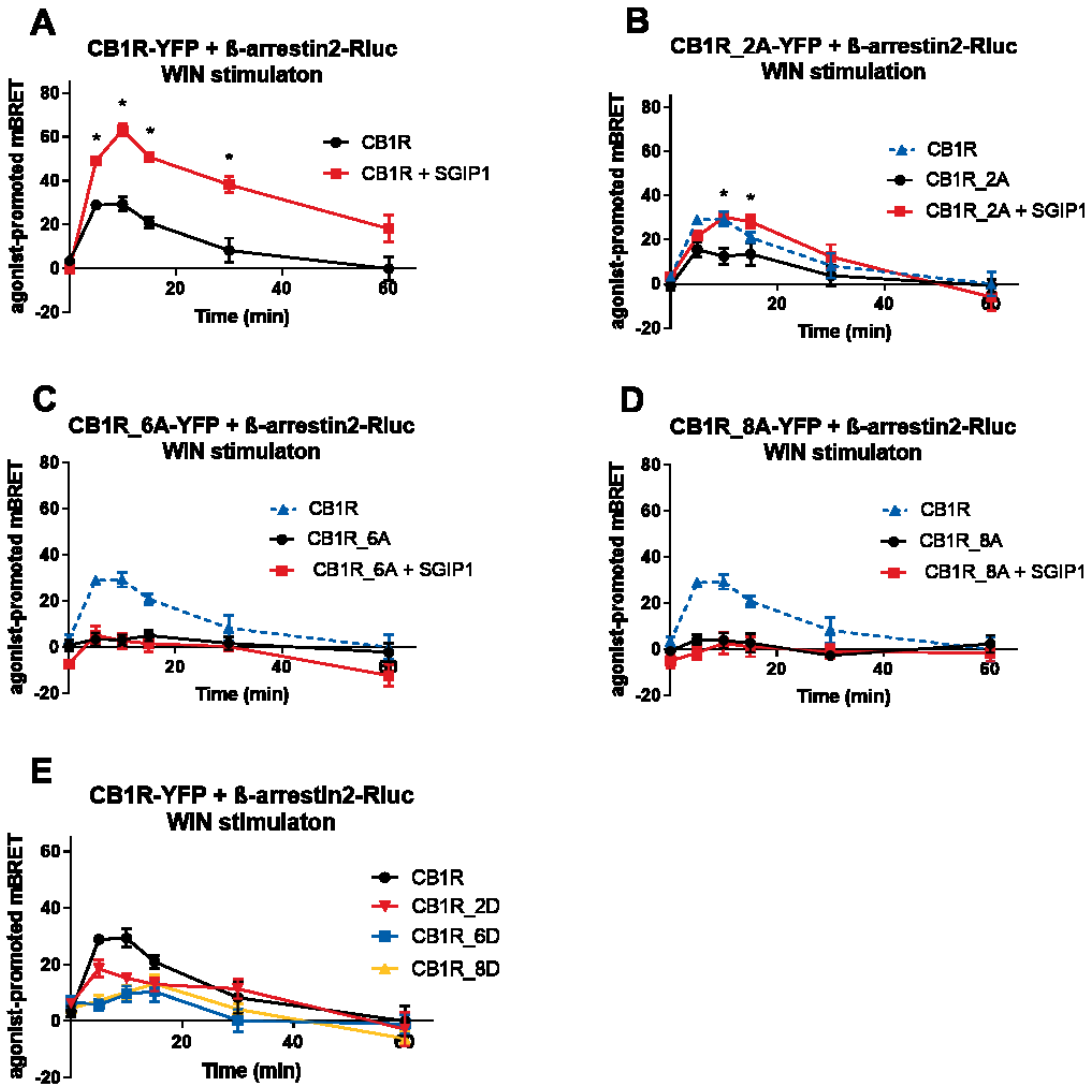

Figure 10. CB1R C tail multisite phosphorylation is important β-arrestin2 recruitment. SGIP1 strengthens the formation of CB1R-β– arrestin2 complexes in β-arrestin2-interacting receptors. A) Interaction dynamics of β-arrestin2 recruitment to CB1R in the presence/absence of SGIP1. **B)** Interaction dynamics of β-arrestin2 recruitment to CB1R, CB1R_2A, and CB1R_2A + SGIP1. **C)** Interaction dynamics of β-arrestin2 recruitment to CB1R and CB1R_6A in the presence/absence of SGIP1. **D)** Interaction dynamics of β-arrestin2 recruitment to CB1R, CB1R_8A, and CB1R_8A + SGIP1. **E)** β-arrestin2 recruitment to phosphomimetic CB1R mutants. Data represent the mean ± SEM from three experiments of independent cell preparations performed in triplicate. *$p \leq 0.05$.

Theoretically, the SGIP1 gene might produce as many as 20 SGIP1 variants due to alternative splicing. Nevertheless, only 4 splice variants have been identified so far. By isolating mRNA from three different mouse brain regions and subsequent transcription into cDNA by reverse transcriptase, we have identified 11 new, previously undescribed SGIP1 splice variants (Fig. 11 A). We chose variants that differ in the structure of the N-terminus (exons 4 and 5) and central part (exons 16 to 20) regions and cloned them into mammalian expression vectors (Fig. 11 A, marked by a green rectangle). Using the western blot technique and antibody against SGIP1 N-terminal region, which is conserved in all splice variants, I confirmed their expression in the heterologous expression system (HEK293 cells). When the length of this novel SGIP1 variants was compared to native SGIP1 of protein samples derived from the mouse brain's prefrontal cortex, the brain lysate's upper band migrated slower than the bands of tested splice variants (Fig. 11 B).

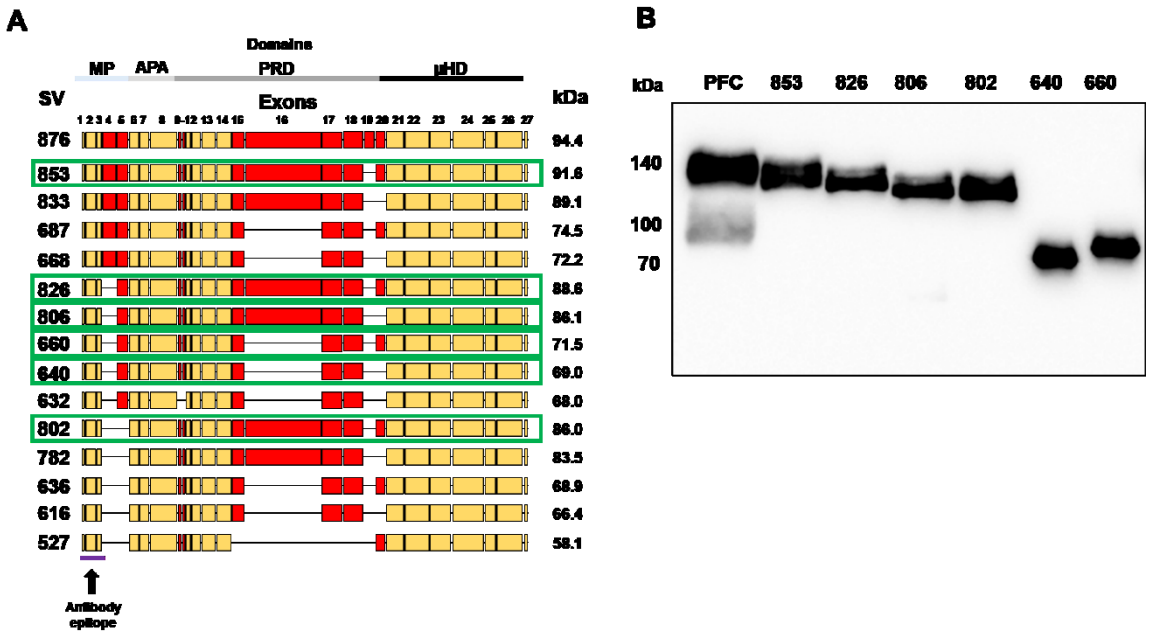

Fig. 11. List of identified SGIP1 splice variants. Tested SGIP1 variants are expressed in HEK293 cells. A) Schematic representation of SGIP1 variants, named according to the number of amino acids they are composed of. The SGIP1 variants selected for cloning into a mammalian expression vector and subsequently tested are marked by a green rectangle. Yellow color represents conserved exons, and red color depicts exons involved in the alternative splicing. MP – membrane binding domain, APA – AP2 activating domain, PRD – proline-rich domain, µHD – µ homology domain SV – splice variant. **B)** Western blot

analysis of selected SGIP1 variants and brain sample. Membranes were stained with anti-SGIP1 antibody that recognizes the conserved N-terminal region (marked by a purple line in A) section of this figure). SGIP1 splice variant samples were derived from HEK293 cells transfected with corresponding SGIP1, brain sample was derived from prefrontal cortex (PFC) of the mouse brain.

It was reported that SGIP1 significantly decreases WIN-induced CB1R internalization in HEK293 cells [10]. Thus, I investigated whether SGIP1 variants with differences in the MP and PRD domain structures exhibit the same internalization-impeding properties as previously studied SGIP1 (corresponding to 806 amino acid variant). WIN stimulation of CB1R in the absence of SGIP1 (mock) resulted in rapid receptor internalization with the maximum at 60 minutes (Fig. 12 A). Interestingly, the presence of all SGIP1 variants inhibited CB1R internalization to a comparable extent as previously described SGIP1 (806 aa) (Fig. 12 A). The expression of CB1Rs was not modified by the co-expression of SGIP1 variants (Fig. 12 B). Therefore, alterations in N-terminal (exons 4, 5) and central (exons 16, 20) regions of SGIP1 did not affect the ability to suppress CB1R internalization. Next, to show that SGIP1 is a specific inhibitor of CB1R internalization, we tested DAMGO-induced internalization of μ-opioid receptor (MOR) in the presence of SGIP1 isoforms. Neither of SGIP1 splice isoforms affected MOR endocytosis (Fig. 12 B).

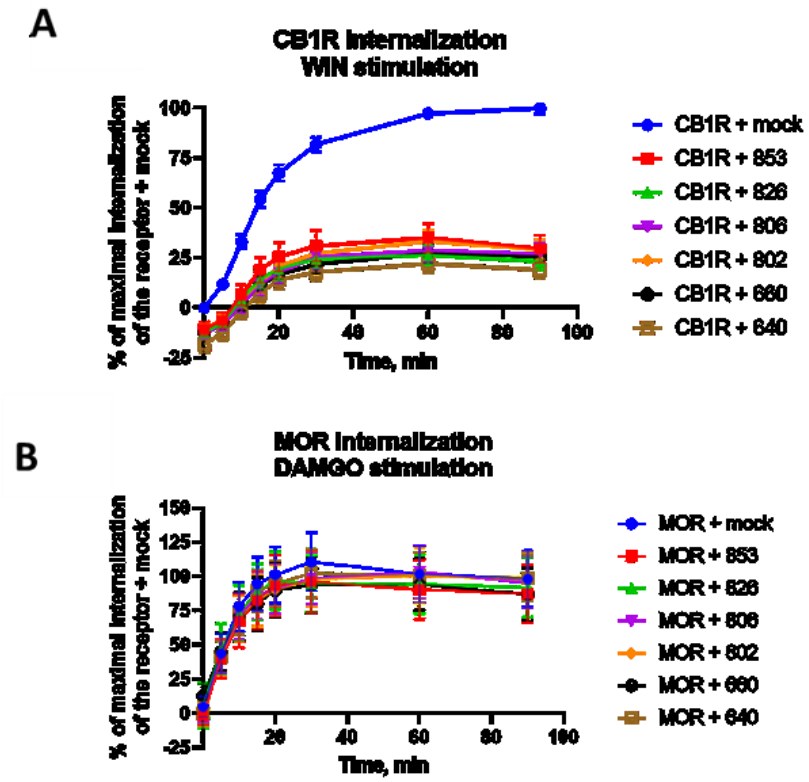

Fig. 12.SGIP1 splice variants decrease the internalization of activated CB1R. HEK293 cells were transiently co-transfected with the plasmids coding CB1R-SNAP/MOR-SNAP and SGIP1-Flag variant or empty plasmid pRK6 (1:2 ratio). Data were calculated from three experiments of independent cell preparations performed in three technical replicates. **A)** The kinetics of CB1R internalization in the presence of SGIP1 variants. Cells were stimulated by 1 μM WIN. Data represent the relative level of CB1R internalization calculated as the percentage of the maximal CB1R + pRK6 internalization after 1 μM WIN stimulation. **B)** The kinetics of MOR internalization in the presence of SGIP1 variants. Cells were stimulated by 5 μM DAMGO. Data represent the relative level of MOR internalization calculated as the percentage of the maximal MOR + pRK6 internalization after 5 μM DAMGO stimulation.

5. DISCUSSION

GRK3 together with β-arrestins execute a crucial role in the regulation and desensitization of numerous GPCRs, including CB1R [5-9]. Indeed, our experiments with GRK2/3 kinase family inhibitor cmpd101 revealed that the activity of these kinases is crucial for CB1R desensitization. In the performed experiments, we show that the application of inhibitor cmpd101 significantly decreased the formation of GRK3 with Gβγ subunits of G proteins as well as with the receptor itself, proving that catalytically active state of GRK3 is required for its proper interaction with Gβγ and CB1R. Furthermore, when the activity of GRK2/3 kinases was inhibited by cmpd101, stimulation of CB1R by WIN resulted in impaired β-arrestin2 recruitment, supporting the notion that the activity of these kinases is required for efficient and rapid β-arrestin2 recruitment to the activated CB1R.

Two clusters of serine/threonine residues in ^{425}SMGDS^{429} and ^{460}TMSVSTDTS^{468} of CB1R C-tail play a key role in CB1R desensitization [5-9]. Nevertheless, the relationship between these phosphorable regions and the binding of GRK3 or β-arrestins2 was not clear. To address this, a set of CB1R C-tail phosphorylation mutants was created by mutating serine and threonine residues in ^{425}SMGDS^{429} and ^{460}TMSVSTDTS^{468} to alanine, because it is chemically inert and it cannot be phosphorylated, thus mimics an unphosphorylated state of a protein. These mutant CB1Rs exhibited similar cellular localization, expression and G-protein signaling as wild type receptor. The results of experiments studying the recruitment of GRK3 to CB1R C-tail phosphorylation mutants revealed that serines of ^{425}SMGDS^{429} regulate the dynamics of GRK3-CB1R dissociation, presumably via GRK3 phosphorylation, while the serines/threonines within ^{460}TMSVSTDTS^{468} regions are essential for GRK3 recruitment. Interestingly, for the most part, GRK3 was able to associate with Gβγ regardless of CB1R C-tail phosphorylation patterns, albeit the interaction was marginally altered. It is

possible that in the absence of GRK3 binding to CB1R mutants, GRK3 acquires different conformational states with Gβγ. The importance of ^{425}SMGDS^{429} and ^{460}TMSVSTDTS^{468} phosphorylation in CB1R desensitization was further supported by showing that β-arrestins2 requires serines/threonines in the regions for proper binding to CB1R.

SGIP1, an interaction partner of CB1R, is a protein with profound physiological impact [10, 13-16]. As SGIP1 modifies CB1R properties like enhanced and prolonged CB1R-β-arrestin2 association upon the receptor activation or decreased ERK1/2 phosphorylation [10], we wondered if other CB1R-induced interactions are modified as well. Indeed, the results of our experiments show that the presence of SGIP1 augmented GRK3-Gβγ, CB1R-GRK3, and CB1R-β-arrestin2 interactions. When we looked at these interaction in CB1R C-tail phosphorylation mutants, we observed that interactions were augmented by SGIP1 only in receptors that maintained the ability to associate with the interaction partners. SGIP1 alone could not rescue these interactions in mutants that exhibited no recruitment of GRK3 and β-arrestin2. This observation implies that SGIP1 modifies CB1R properties via stalling the internalization of CB1R bound to interaction partners as a whole interactome complex and not via direct interaction of SGIP1 with GRK3 and β-arrestin2.

In our laboratory, the analysis of mRNA derived from a mouse brain resulted in identifying 12 unique previously undescribed SGIP1 splice variants, with the most splicing occurring in the PRD domain (between exons 16 and 20) and in the N-terminal MP domain (exons 4 and 5). Six SGIP1 splice variants were chosen for expression and functional testing to cover the sequence variability of regions affected by alternative splicing. When the expression of these splice variants in HEK293 cells was tested by immunoblot, the bands of tested SGIP1 splice variants differed from those derived from the mouse prefrontal cortex most likely due to different posttranslational modifications of

SGIP1 in HEK293 cells. Functional testing showed that all selected SGIP1 splice variants hindered CB1R endocytosis. This observation implies that the variations in the MP and PRD domain sequences do not alter the inhibitory effect on CB1R internalization. The fact that SGIP1 variants lacking exon 4, 5, or both maintained the internalization-inhibiting feature demonstrates that these exons are not vital for the SGIP1 effect on CB1R. Neither of SGIP1 splice variants altered the specificity of the SGIP1-CB1R interaction, and none of the isoforms affected MOR internalization. While this thesis establishes a basis for the characterization of SGIP1 splice variants, further studies are required to reason the existence of various SGIP1 forms.

6. CONCLUSIONS

The objective of this thesis was to describe and characterize molecular aspects that govern the regulation of cannabinoid receptor 1 (CB1R) signaling and desensitization.

Using biophysical methods in tandem with pharmacological tools, I show that G protein-coupled receptor kinase 3 (GRK3) is an important regulator of CB1R. GRK3 must be in the active state to couple to the activated CB1R or Gβγ subunits of G proteins. In addition, the recruitment of β-arrestin2 to CB1R is dependent on the activity of GRK2/3.

CB1R C-terminal tail features two serine/threonine clusters, ^{425}SMGDS^{429} and ^{460}TMSVSTDTS^{468}, that are crucial in facilitating CB1R interactions with GRK3 and β-arrestin2. ^{460}TMSVSTDTS^{468} motif favors GRK3 recruitment while ^{425}SMGDS^{429} region regulates the stability of GRK3-CB1R interaction. In the case of β-arrestin2, the ^{460}TMSVSTDTS^{468} motif serves as the primary initiation docking site, followed by the β-arrestin2 interaction with the ^{425}SMGDS^{429} region. In addition, distinct phosphorylation patterns or "bar codes" within the CB1R C-terminal region are required for GRK3 and β-arrestin2 binding to CB1R. GRK3 couples to Gβγ regardless of CB1R C-tail phosphorylation patterns, albeit the interaction is likely stabilized by CB1R-GRK3 coupling.

Src homology 3-domain growth factor receptor-bound 2-like (endophilin) interacting protein 1 (SGIP1) enhances CB1R interactions with GRK3, β-arrestin2 as well as GRK3-Gβγ coupling. SGIP1 cannot rescue interactions that are inhibited by CB1R phosphorylation patterns. Thereby SGIP1 regulates the dynamics of interactions between molecules that are part of the temporal CB1R signalosome established during desensitization. The alterations in SGIP1 proline-rich domain (PRD) and membrane phospholipid binding domain (MP) due to alternative splicing do not affect SGIP1 expression in HEK293 cells, nor the CB1R endocytosis hindrance.

In conclusion, this thesis's data and observations contribute to understanding the molecular mechanisms controlling CB1R signaling and desensitization. More profound knowledge of molecular events involved in these processes represents a crucial step in creating therapeutic approaches based on the modulation of endocannabinoid system.

7. SUMMARY

The endocannabinoid system is an important regulator of synaptic plasticity and plays a crucial role in many central nervous system (CNS) functions and its development. Cannabinoid receptor 1 (CB1R) is expressed most densely in CNS and is a target for endocannabinoids and phytocannabinoids, such as Δ^9-THC. Activity and signaling of CB1R is tightly regulated, mainly via receptor desensitization and internalization, as abnormal activity and dysregulation of CB1R results in a broad spectrum of pathological conditions.

This study describes molecules and events surrounding CB1R desensitization in more detail. Data and observations of presented research show that two serine/threonine-rich regions of CB1R, ^{425}SMGDS^{429} and ^{460}TMSVSTDTS^{468}, are involved in the recruitment of molecules involved in receptor desensitization: G protein-coupled receptor kinase 3 (GRK3) and β-arrestin2. I demonstrate that GRK3 has to be in active conformation to form complexes with CB1R or G protein subunits Gβγ. Furthermore, the recruitment of β-arrestin2 to CB1R depends on the activity of GRK2/3.

I show that the interaction partner of CB1R Src homology 3-domain growth factor receptor-bound 2-like (endophilin) interacting protein 1 (SGIP1) profoundly modifies the dynamics of signalosome interactions during CB1R desensitization. In addition, I characterize newly identified splice variants of SGIP1 and demonstrate that alternative splicing-based alteration in SGIP1 domains do not affect CB1R internalization hindering properties of SGIP1.

Characterizing the events that drive the interactions involved in CB1R desensitization represents a pivotal step in understanding cannabinoid signaling and tolerance development.

8. SHRNUTÍ

Endokanabinoidní systém (ECS) hraje klíčovou roli v mnoha funkcích a vývoji centrálního nervového systému (CNS), je stěžejní v procesu synaptické plasticity, řídí homeostázu synaptických spojů. Centrální molekulou nervového ECS je kanabinoidní receptor 1 (CB1R). tento receptor je v CNS hojně exprimován, aktivován je endokanabinoidy a fytokanabinoidy, nejznámější je tetrahydrokanbinol Δ^9-THC, jedna z aktivních složek marijuany. Signalizace CB1R podléhá sofistikované regulaci, především prostřednictvím desenzitizace a internalizace receptoru.

Tato práce podrobněji popisuje molekuly a děje provázející desenzitizaci CB1R. Údaje a pozorování z mého výzkumu naznačují, že dvě oblasti CB1R bohaté na serin/treonin, ^{425}SMGDS^{429} a ^{460}TMSVSTDTS^{468}, se podílejí na vazbě molekul zapojených do desenzitizace receptoru: G proteinem spřažené receptorové kinázy 3 (GRK3) a β-arrestinu2. V této práci jsem prokázal, že GRK3 musí být v aktivní konformaci, aby mohla vytvářet komplexy s CB1R nebo podjednotkami G proteinu Gβγ. Kromě toho je vazba β-arrestinu2 na CB1R závislá na aktivitě GRK2/3.

Interakční partner CB1R Src homology 3-domain growth factor receptor-bound 2-like (endophilin) interacting protein 1 (SGIP1) zásadně modifikuje dynamiku interakcí CB1R signalosomu během desenzitizace. Námi nově identifikované sestřihové varianty SGIP1 se změnami ve sekvencích stěžejních domén SGIP1 způsobují asociací s CB1R inhibici internalizace CB1R, avšak ani jedna varianta neovlivňuje mu- opioidní receptor.

Charakterizace interakcí, které se podílejí na desenzitizaci CB1R, představuje důležitý krok k pochopení kanabinoidní signalizace a vývoje tolerance.

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OVERVIEW OF AUTHOR'S PUBLICATIONS

Publications based on the data shown in the dissertation thesis

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Other publications

Sevcovicova, A., Plava, J., **Gazdarica, M**., Szabova, E., Huraiova, B., Gaplovska-Kysela, K., Cipakova, I., Cipak, L., Gregan, J. (2021). Mapping and Analysis of Swi5 and Sfr1 Phosphorylation Sites. *Genes*. doi.org/10.3390/genes12071014; IF 4.141

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OVERVIEW OF AUTHOR'S PRESENTATIONS

16th IMG PhD Conference, Czech Academy of Sciences (Prague, Czech Republic, 2023): Cannabinoid receptor 1 and the pharmacology of legal psychoactive hexahydrocannabinol (scientific presentation)

Gordon Research Conference - Cannabinoid Function in the CNS (Castelldefels, Spain, 2023): SGIP1 selectively inhibits internalization of desensitized cannabinoid receptor 1 (poster presentation)

The 33rd Annual International Cannabinoid Research Society Symposium on the Cannabinoids (Toronto, Canada, 2023): The internalization of cannabinoid receptor 1 is selectively inhibited by SGIP1 (poster presentation)

Scientific conference of Second Faculty of Medicine, Charles University (Prague, Czech Republic, 2022): SGIP1 modulates the kinetics and interaction of cannabinoid receptor 1 and G protein-coupled receptor kinase 3 (poster presentation)

Scientific conference of Second Faculty of Medicine, Charles University (Prague, Czech Republic, 2019): Cannabinoid receptor 1 multisite phosphorylation is crucial for desensitization and internalization (poster presentation)

4th Endocannabinoid Pharmacology Meeting (Bern, Switzerland, 2018): GRK3 and SGIP1 regulate CB1 receptor signaling (poster presentation)