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# Somatostatin and cannabinoid receptors crosstalk in protection of huntingtin knock-in striatal neuronal cells in response to quinolinic acid

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ABSTRACT

In the present study, we describe the status of somatostatin receptor 2 and 5 (SSTR2 and SSTR5) as well as cannabinoid type 1 receptor (CB1R) in Huntingtin (Htt) knock-in striatal neuronal cells. In mutant Htt (mHtt) knock-in ($STHdh^{Q111/111}$) and wild type ($STHdh^{Q7/7}$) striatal neuronal cells, SSTRs and CB1R exhibit prominent cytoplasmic expression and respond to agonist in a receptor specific manner. In response to quinolinic acid (QUIN)-induced toxicity, $STHdh^{Q111/111}$ cells are more vulnerable and display suppressed cell survival signaling pathways. Receptor-specific agonists protect cells from QUIN-induced toxicity and activate ERK1/2 in both STHdh cells. Co-activation of SSTRs and CB1R resulted in loss of protective effects, delayed ERK1/2 phosphorylation and altered receptor complex composition. These results provide firsthand evidence in support of the protective role of SSTRs in STHdh cells and the possible crosstalk between SSTRs and CB1R in the modulation of excitotoxicity in Huntington's disease.

## 1. Introduction

Huntington's disease (HD) is an inherited progressive autosomal dominant neurodegenerative disorder caused by an abnormal expansion of the CAG repeat in exon 1 of *huntingtin* gene (Zuccato et al., 2010). This gene encodes a ubiquitously expressed cytoplasmic protein, Huntingtin (Htt), with highest expression in the central nervous system (CNS). The threshold of disease onset is above 35 repeats, with an inverse correlation between the length of repeats and the age of HD onset. The clinical symptoms of HD include chorea, dystonia, and impaired cognition. The neuropathological hallmark of HD is the severe atrophy of neostriatum with marked neuronal loss and gliosis (Zuccato et al., 2010). Striatal neuronal loss occurs preferentially in the medium sized spiny neurons (MSNs), which make up over 90% of all striatal neurons. A subclass of medium-sized aspiny interneurons, expressing somatostatin (SST), neuropeptide Y , and neuronal nitric oxide synthase, survive the early process of neurodegeneration (Ferrante et al., 1985). The mechanism underlying the selective neuronal susceptibility and survival is unclear. In HD, overactivation of N-methyl-D-aspartate receptors (NMDARs) is proposed to be one of the prominent pathogenic mechanisms for the neuronal loss (Doble, 1999; Zuccato et al., 2010). This is further supported by the observations that the concentration of quinolinic acid (QUIN), an endogenous NMDA agonist, is elevated in the brain of HD patients and NMDARs are overactivated in HD models (Guidetti et al., 2004; Fan and Raymond, 2007). Local high expression of NMDAR in MSNs has been linked to their selective susceptibility in HD (Landwehrmeyer et al., 1995). Moreover, intrastriatal injections of NMDAR agonists such as QUIN, glutamate or NMDA to rodents *in vivo*, or the application of these agents to the neuronal cultures *in vitro* replicates the pattern of selective neurodegeneration as normally seen in HD (Beal et al., 1986, 1991; Kumar, 2004). The progression of HD is not only due excessive excitatory neurotransmission (Zuccato et al., 2010). Perturbed expression of dopamine, γ-aminobutyric acid (GABA), SST and endocannabinoids have also been associated with HD pathogenesis, although the detailed molecular mechanisms are not well elucidated yet.

CB1R is one of the most widespread members of G protein-coupled receptor (GPCR) family in the CNS, with high density seen in different brain regions, including hippocampus, cortex, cerebellum and basal ganglia (Mackie, 2005; Kano et al., 2009). Upon activation by endocannabinoids, CB1R is involved in a wide range of physiological, pharmacological and pathological activities, including learning, memory, motor behavior, appetite control, pain regulation, and neurodegenerative diseases (Pacher et al., 2006). In the CNS, CB1R expressed at GABAergic and glutamatergic terminals negatively regulates the release of GABA and glutamate through endocannabinoid-mediated retrograde signaling, contributing to the prevention of synaptic hyperactivation and attesting its neuroprotective role in several

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**Abbreviations**

| | |
|---|---|
| CB1R | cannabinoid receptor 1 |
| CNS | central nervous system |
| Co-IP | co-immunoprecipitation |
| Cy3 | cyanines 3 |
| D2R | dopamine receptor 2 |
| ERK1/2 | extracellular signal-regulated kinase 1/2 |
| FITC | fluorescein isothiocyanate |
| GPCR | G protein-coupled receptor |
| HD | Huntington's disease |
| Htt | Huntingtin |
| mHtt | mutant Huntingtin |
| MSN | medium-sized spiny neurons |
| NGS | normal goat serum |
| NMDAR | N-methyl-D-aspartate receptor |
| QUIN | quinolinic acid |
| RIPA | radioimmune precipitation assay |
| SE | standard error |
| SST | somatostatin |
| SSTR | somatostatin receptor |
| WIN | WIN 55212-2 |

neurodegenerative diseases (Katona et al., 1999; Gerdeman and Lovinger, 2001; Marsicano et al., 2003; Katona and Freund, 2008; Chiarlone et al., 2014). This concept is further strengthened by the fact that downregulation of CB1R at the levels of mRNA and protein has been observed in many neurological disorders including HD, whereas genetic rescue of CB1R improves striatal neuronal survival in HD transgenic R6/2 mice (Miller and Devi, 2011; Naydenov et al., 2014).

SST, also known as somatotropin release-inhibiting factor, is a growth hormone inhibitory peptide that suppresses the cell growth. In the CNS, SST serves as a neurotransmitter and neuromodulator and is involved in the regulation of a variety of neurological activities, including cognition, locomotor, sensory and autonomic functions (Schindler et al., 1996; Patel, 1999; Kumar and Grant, 2010). There is growing evidence suggesting that SST plays a critical role in various neurodegenerative diseases including Alzheimer's disease, HD, Parkinson's disease, multiple sclerosis, as well as neuropsychological disorders including major depression and schizophrenia (Aronin et al., 1983; Epelbaum et al., 1983; Sorensen, 1987; Beal et al., 1988; Burgos-Ramos et al., 2008; Tallent and Qiu, 2008; Lin and Sibille, 2013). SST exerts such profound effects via binding to five somatostatin receptors (SSTRs), namely SSTR1-5, which are expressed at various densities throughout the brain (Patel, 1999; Kumar and Grant, 2010). All five SSTRs inhibit cAMP in a $G_i$-dependent manner. SSTR subtypes have also been shown to regulate several downstream signal transduction pathways including mitogen-activated protein kinases and ion channels (Patel, 1999; Kumar and Grant, 2010).

CB1R is highly expressed in MSNs. As a result of extensive degeneration of MSNs, CB1R is largely downregulated during the progression of HD (Herkenham et al., 1990; Glass et al., 1993, 2000). The loss of striatal CB1R contributes to the pathogenesis of HD, whereas knock-in of CB1R in MSNs enhances striatal neuronal survival in R6/2 mice (Blazquez et al., 2011; Naydenov et al., 2014). In addition, CB1R-mediated neuroprotective effect has been observed in various models of excitotoxicity (Kim et al., 2006; Pacher et al., 2006; Zoppi et al., 2011). In comparison, medium-sized aspiny interneurons expressing SST are selectively preserved at the early stage of HD and expression level of SST is increased in the basal ganglia in HD (Aronin et al., 1983; Dawbarn et al., 1985; Ferrante et al., 1985). The most profound connection between HD and SST has been established using SSTR1 and 5 double knock-out mice, which reproduce a comparable neurochemical phenotype in the striatum as seen in R6/2 mice (Rajput et al., 2011). SST protects striatal neurons against NMDA- and QUIN-induced excitotoxicity in a pertussis toxin-sensitive manner, indicating the involvement of SSTR subtypes in the neuroprotective effect of SST (Kumar, 2008). We recently described the colocalization of CB1R and SST in rat brain hypothalamus and hippocampus (Zou and Kumar, 2015; Zou et al., 2015). In extension to the colocalization studies using rat brain, we have also described that CB1R and SSTR5 functionally interact with each other and exist as constitutive heterodimers that dissociate upon receptor activation in transfected human embryonic kidney 293 cells (Zou et al., 2017). Concurrent activation of both SSTR5 and CB1R in cotransfected cells leads to a SSTR5-mediated dominant role in the modulation of signaling pathways including inhibition of cAMP and activation of extracellular signal-regulated kinase 1/2 (ERK1/2) (Zou et al., 2017). These results imply the crucial role that SSTRs and CB1R might play in the pathogenesis of HD. Till today, whether these receptor subtypes functionally interact with each other in HD remains elusive. Furthermore, the physiological significance and therapeutic implication of such crosstalk between SSTRs and CB1R are still unclear.

In addition to experimental models of HD including R6/1, R6/2 and YAC128 transgenic mice, recently a conditionally immortalized striatal cell line with mutant ($STHdh^{Q111/111}$) and *wt* ($STHdh^{Q7/7}$) Htt knock-in has been developed that display biochemical and neurochemical features of HD (Trettel et al., 2000; Ruan et al., 2004; Gines et al., 2010). Accordingly, in the present study, we investigated the functional interactions between SSTR2/CB1R and SSTR5/CB1R in QUIN induced excitotoxicity using $STHdh^{Q7/7}$ and $STHdh^{Q111/111}$ cells. We observe prominent cytoplasmic expression and colocalization of SSTRs and CB1R in both $STHdh^{Q7/7}$ and $STHdh^{Q111/111}$ cells. $STHdh^{Q111/111}$ cells with mutant Htt (mHtt) expression are more susceptible to QUIN-induced toxicity than $STHdh^{Q7/7}$ cells. Activation of either SSTRs or CB1R leads to neuroprotective effects through modulating cell survival pathways, specifically ERK1/2, against QUIN-induced excitotoxicity. Concurrent activation of SSTR and CB1R diminishes such pro-survival effect, suggesting negative impact on receptor crosstalk in STHdh cells.

## 2. Materials and methods

### 2.1. Materials

SST was procured from Bachem (Torrance, CA) and non-peptide SSTR2 agonist L-779976 and SSTR5 agonist L-817818 were kindly provided by Dr. S. P. Rohrer from Merck & Co. WIN 55212-2 (WIN) and ACEA were purchased from the Tocris Cookson Inc. (Ellisville, MO). All drugs were constituted in either dimethyl sulfoxide or ethanol at a final concentration < 0.1% (v/v), according to suppliers' instructions. Normal goat serum (NGS) was purchased from Vector Laboratories (Burlingame, CA). SSTR2 and 5 antibodies were produced in our laboratory. CB1R anti-goat polyclonal antibody was purchased from Santa Cruz Biotechnology (Dallas, TX). Fluorescein isothiocyanate (FITC) and cyanines 3 (Cy3) conjugated secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA). Rabbit polyclonal antibodies for p-and t-ERK1/2 were purchased from Cell Signaling Technology (Danvers, MA). Other chemicals and reagents were of AR grade and obtained from various sources.

### 2.2. Cell cultures

Conditionally immortalized striatal cells from E14 mHtt knock-in mice ($STHdh^{Q111/111}$) and their *wt* littermates ($STHdh^{Q7/7}$) were originally developed by Dr. M. E. McDonald and purchased from Coriell Institute (Camden, NJ)(Trettel et al., 2000). Cells were maintained at a permissive temperature of 33 °C in Dulbecco's modified Eagle medium

supplemented with 10% fetal bovine serum, 1% streptomycin-penicillin, and 400 μg/ml Geneticin in a humidified atmosphere containing 5% $CO_2$.

### 2.3. Double-labeled fluorescence immunocytochemistry

STHdh$^{Q7/7}$ and STHdh$^{Q111/111}$ cells were grown on poly-D-lysine coated coverslips in 24-well plate to 50–70% confluency. Cells were fixed with 4% paraformaldehyde on ice for 20 min, and permeabilized at room temperature for 15 min with 0.2% Triton X-100 before proceeding to blocking with 5% NGS for 1 h. Permeabilized (P) and non-permeabilized (NP) cells were probed with primary antibodies against CB1R (1:500) in combination with primary anti-SSTR2 (1:500) or anti-SSTR5 (1:500) antibodies at 4 °C overnight. After three washes in PBS, cells were incubated with FITC-conjugated goat anti-rabbit (1:400) and Cy3-conjugated donkey anti-goat (1:800) secondary antibodies for 2 h at room temperature. Cells were washed with PBS once and then incubated with 5 μg/ml Hoechst 33258 for 30 min for nuclear staining. Following three subsequent washes with PBS, cells were mounted and observed under Carl Zeiss confocal microscope.

Specificity of antibodies and immunoreactivity was validated as previously described (Kumar et al., 1999; Kumar, 2007; Sanford et al., 2008; Kano et al., 2009; Zou et al., 2015, 2017).

### 2.4. MTT assay

STHdh$^{Q7/7}$ and STHdh$^{Q111/111}$ cells were seeded at a density of 5,000 cells/well in 96-well plate and allowed to grow overnight. Cells were starved in serum-free medium for 24 h, followed by incubation with Locke's solution containing (154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 3.6 mM $NaHCO_3$, 5 mM HEPES, 20 mM glucose and 10 μM glycine) with SST, SSTR2 specific agonist L-779976, SSTR5 specific agonist L-817818, and CB1R agonists WIN and ACEA (1 μM each) in the presence or absence of 3 mM QUIN for 5 h (unless otherwise indicated). The Locke's solution was replaced with serum-free medium supplemented with the corresponding drugs as indicated earlier. After 24 h, Posttreatment cells were washed twice with PBS and then incubated with 20 μl of 5 mg/ml MTT solution for 2 h. The medium was then aspirated and replaced by 200 μl isopropanol. The plate was read on spectrophotometer at 570 nm.

### 2.5. Co-immunoprecipitation (Co-IP)

Cells were grown in 6-well plate until 80% confluency. To assess the effect of agonist on receptor interaction, cells were treated with L-779976 (1 μM), L-817818 (1 μM), ACEA (1 μM) alone or in combination for 30 min at 33 °C. After one wash with cold PBS, cells were lysed in radioimmune precipitation assay (RIPA) buffer. 250 μg of protein from whole cell lysates was immunoprecipitated with primary anti-SSTR2 or anti-SSTR5 antibody (1:500) overnight at 4 °C. Samples were then incubated with protein A/G agarose beads for 2 h at 4 °C to allow sufficient binding. The beads were then washed with cold PBS and solubilized in Laemmli sample buffer containing 5% β-mercaptoethanol. Samples were fractionated by SDS-PAGE and transferred to nitrocellulose membrane. Membranes were then incubated with anti-CB1R primary antibody (1:500) overnight at 4 °C and probed with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. Chemiluminescence reagents were used for developing and images were captured using Alpha Innotech Fluorchem 800 gel box imager. Specificity of immunoprecipitation was validated as previously described (Zou et al., 2017).

### 2.6. Western Blot analysis

STHdh$^{Q7/7}$ and STHdh$^{Q111/111}$ cells were grown to 80% confluency in 6-well plate. After treatment as indicated, cells were washed with

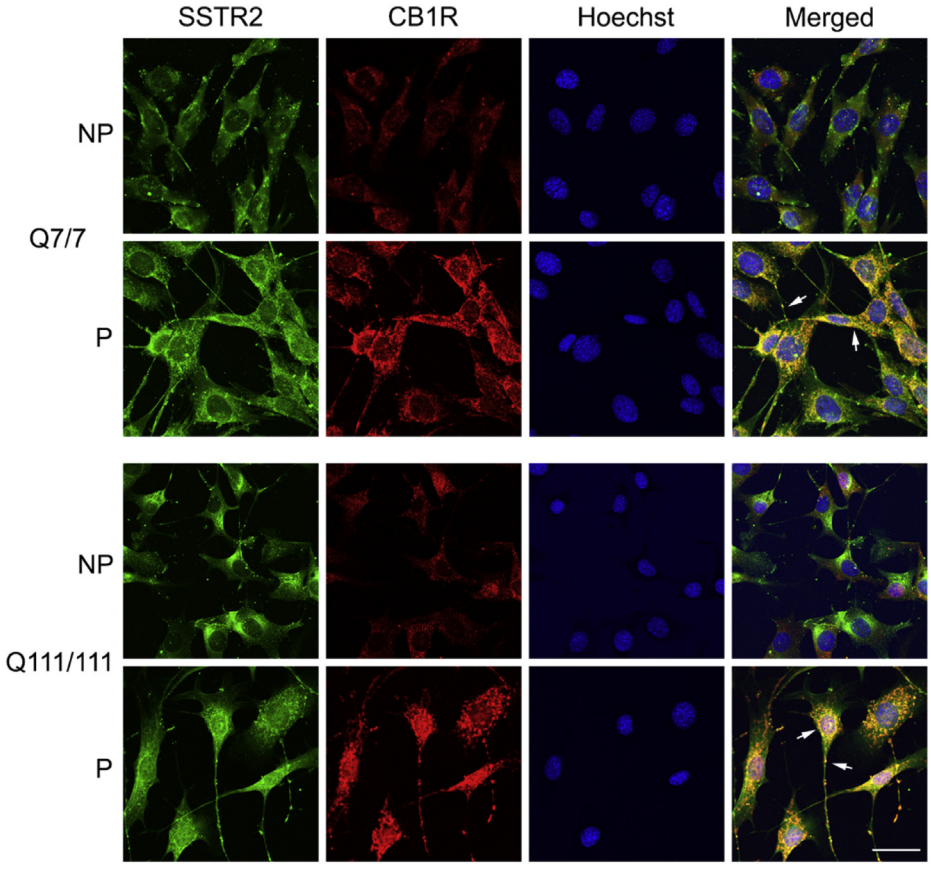


**Fig. 1. Colocalization of SSTR2 and CB1R is mainly confined intracellularly in STHdh$^{Q7/7}$ and STHdh$^{Q111/111}$cells.** Permeabilized (P) and non-permeabilized (NP) cells were incubated with rabbit anti-SSTR2 and goat anti-CB1R primary antibodies, followed by FITC-conjugated goat anti-rabbit and Cy3-conjugated donkey anti-goat secondary antibodies for final color development. SSTR2 (green) displayed higher membrane expression in STHdh$^{Q111/111}$ cells than in STHdh$^{Q7/7}$ cells, whereas cytosolic expression was comparable. In both *wt* (STHdh$^{Q7/7}$) and mutant (STHdh$^{Q111/111}$) striatal neuronal cells, CB1R-like immunoreactivity (red) was detected predominantly intracellular. Note in merged images, the neuronal cells lacking membrane colocalization but displaying strong intracellular colocalization (yellow, indicated by arrows). Scale bar = 40 μm.

cold PBS and lysed with RIPA buffer. Membrane and cytosolic portion were separated by centrifugation at 12,000 g at 4 °C for 1 h. 15 μg of protein was solubilized in Laemmli sample buffer containing 5% β-mercaptoethanol and then fractionated by SDS-PAGE. Samples were transferred to nitrocellulose membrane, followed by blocking with 5% non-fat milk for 1 h at room temperature. Primary antibodies against CB1R (1:500), SSTR2 (1:500), and SSTR5 (1:500) were used respectively. For downstream signaling pathways, 15 μg of protein from whole cell lysates were fractioned using SDS-PAGE. Primary antibodies raised against p- and t-ERK1/2 (1:1000) were used. After overnight incubation with primary antibodies, membranes were further incubated with corresponding secondary antibodies for 2 h at room temperature. Membranes were developed using chemiluminescence reagents and images were captured using Alpha Innotech Fluorchem 800 gel box imager. β-actin was used as a loading control for whole cell lysates.

### 2.7. Statistical analysis

Results were presented as mean ± standard error (SE). Statistical significance was determined either by one-way ANOVA or by two-way ANOVA and followed by post hoc Dunnett's test to compare against control or Bonferroni's multiple comparison test, as indicated in respective figure legend. Statistical analysis was carried out using Graph Pad Prism 5.0 and statistical differences were taken at $p < 0.05$. Results presented here represent at least three independent experiments.

## 3. Results

### 3.1. Expression and colocalization of SSTR2, SSTR5 and CB1R in STHdh striatal neuronal cells

Previous studies have shown the endogenous expression of CB1R in STHdh$^{Q7/7}$ and STHdh$^{Q111/111}$ cells (Blazquez et al., 2011; Laprairie et al., 2013). Whether SSTRs are expressed in these cells endogenously and colocalized with CB1R is not known. SSTRs and CB1R are well expressed in both *wt* and mutant striatal neuronal cells in a receptor-specific manner (Figs. 1 and 2). Unlike the well-known membrane localization of GPCRs in homologous or heterologous system, CB1R-like immunoreactivity (red) was prominently seen intracellularly rather than at the cell surface in both STHdh$^{Q7/7}$ and STHdh$^{Q111/111}$ cells. Moreover, the intracellular expression of CB1R is seen as punctated staining, suggesting a possible compartmentalization of intracellular CB1R immunoreactivity. In comparison to CB1R, SSTR2 (green) displayed higher membrane expression and comparable intracellular expression in both STHdh neuronal cells (Fig. 1). Like CB1R, the expression of SSTR5 (green) was confined predominantly to cytoplasm with relatively higher immunoreactivity in comparison to SSTR2 (Fig. 2). Most importantly, strong colocalization between SSTR2/CB1R and SSTR5/CB1R was observed in both cell lines intracellularly. In addition, dispersed staining was also seen in the processes (Figs. 1 and 2).

### 3.2. Agonist-induced changes in receptor expression in STHdh$^{Q7/7}$ and STHdh$^{Q111/111}$ cells

To support our immunostaining, receptor expression was also determined in whole cell lysates, cytosolic and membrane fractions prepared from STHdh$^{Q7/7}$ and STHdh$^{Q111/111}$ neuronal cells using Western blot analysis. As shown in Fig. 3A, SSTR2, SSTR5 and CB1R were well expressed in whole cell lysates and cytosolic fraction at the expected molecular sizes of 57, 58, and 53 kDa respectively. The receptors expression in membrane fractions was significantly lower than in cytosolic fractions. We also noted relatively higher expression of CB1R in membrane fraction prepared from STHdh$^{Q111/111}$ in comparison to STHdh$^{Q7/7}$ cells. In contrast, the expression of SSTR subtypes in whole cell lysate and cytosolic fractions was comparable in both cells but

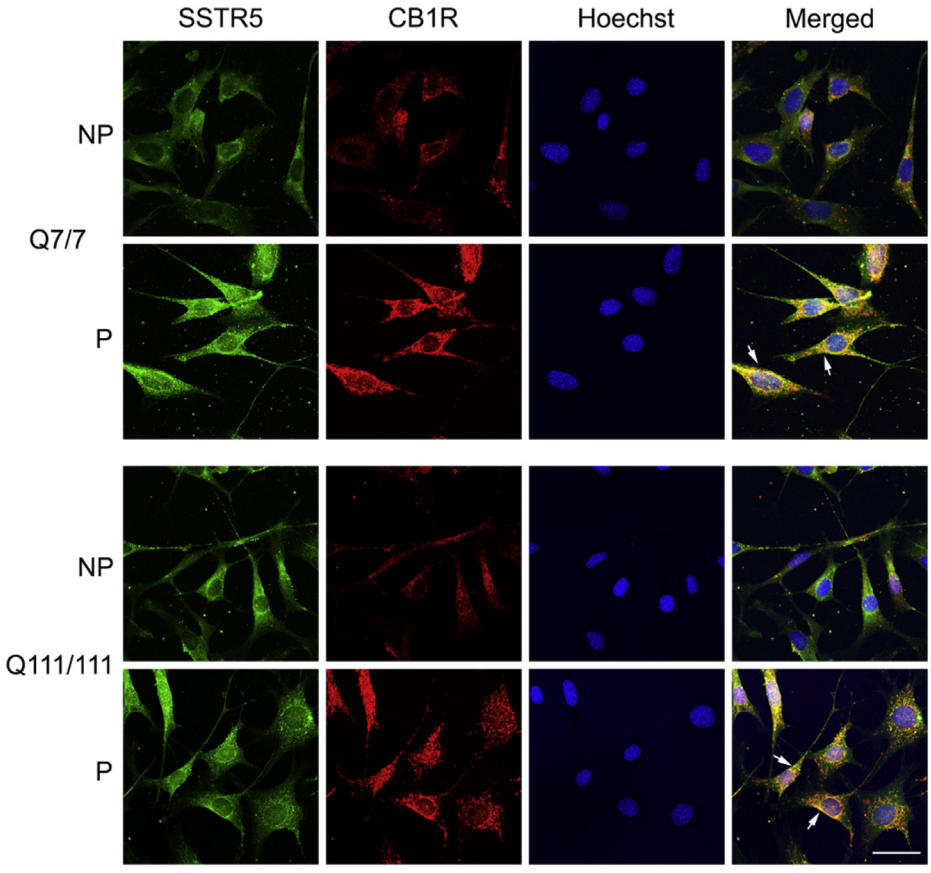


**Fig. 2. Intracellular colocalization of SSTR5 and CB1R in STHdh$^{Q7/7}$ and STHdh$^{Q111/111}$ cells.** Cells were grown and processed as described in legend to Fig. 1. SSTR5 (green), CB1R (red), and nucleus (blue) are shown in respective panels. Receptor colocalization is presented in merged images in yellow and indicated by arrows. Both SSTR5 and CB1R displayed predominant cytosolic expression in STHdhQ7/7 and STHdhQ111/111 cells. Colocalization was mostly seen as punctated staining in cytoplasm. Scale bar = 40 μm.

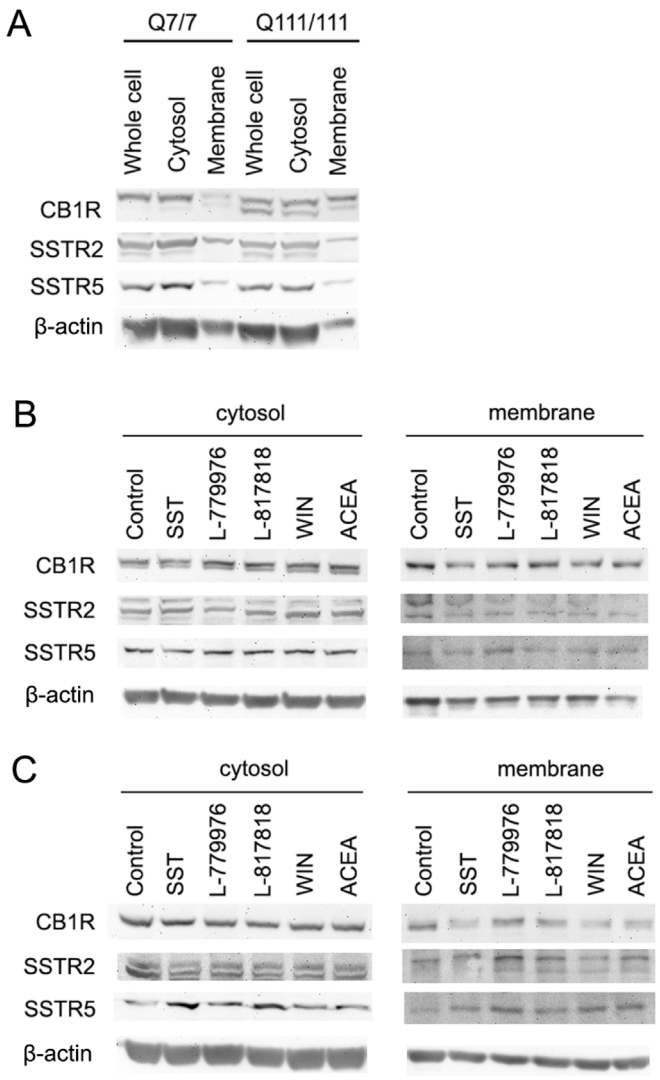


**Fig. 3. Agonist-induced changes of receptors expression in STHdh$^{Q7/7}$ and STHdh$^{Q111/111}$ cells.** 15 μg of total protein from whole cell lysate, membrane and cytosolic fractions was immunoblotted for the expression of CB1R, SSTR2 and SSTR5. (**A**) Membrane fraction prepared from STHdh$^{Q7/7}$ cells exhibited relatively higher expression of CB1R than from STHdh$^{Q111/111}$ cells, whereas cytosolic expression of CB1R was comparable between STHdh$^{Q7/7}$ cells and STHdh$^{Q111/111}$ cells. In contrast, SSTR2 showed higher membrane expression in STHdh$^{Q7/7}$ cells. Membrane expression of SSTR5 was low in both STHdh cells. (**B**) In SThdh$^{Q7/7}$ cells, CB1R expression was increased in cytosolic fraction in response to all agonist treatment except SST, whereas receptor membrane expression was decreased upon treatment of SST and WIN. The expression of either SSTR2 or 5 in cytosol was comparable to control upon agonist treatment. Membrane expression of SSTR2 was decreased upon all agonist treatment, whereas SSTR5 was increased in response to SSTR2 agonist treatment. (**C**) In STHdh$^{Q111/111}$ cells intracellular CB1R expression was unaltered whereas in membrane fraction, receptor expression was decreased in presence of receptor-specific agonists. Cytosolic expression of SSTR2 was decreased, while membrane expression was increased upon treatment of SSTR2 specific agonist. Expression of SSTR5 was increased in cytosol in the presence of SST and SSTR5-specific agonist. Note augmented SSTR5 expression in membrane fraction upon indicated treatment. β-actin was used as an internal loading control. Results are representative of three independent experiments.

relatively less in membrane fractions. These results suggest that irrespective of mHtt presence, receptor expression is primarily confined intracellularly.

We next investigated the changes in receptor expression in cytosolic and membrane fractions prepared from STHdh$^{Q7/7}$ and STHdh$^{Q111/111}$ cells in response to agonist treatments. As shown in Fig. 3B, in STHdh$^{Q7/7}$ cells, intracellular expression of CB1R was increased in response to WIN and ACEA as well as SSTR2 and 5 specific agonist treatments, but not upon treatment with SST. The membrane expression of CB1R was decreased upon agonist treatment relatively to the greater extent in the presence of SST. The cytosolic expression of SSTR2 and 5 was comparable to control. In STHdh$^{Q7/7}$ membrane fraction, unlike CB1R, the expression level of SSTR2 and 5 was weak without any significant changes following agonist treatment. In contrast, the cytosolic expression of CB1R in STHdh$^{Q111/111}$ was unaltered whereas SSTR2 was decreased in response to all agonist treatments. SSTR5 expression in cytosolic fraction was increased only upon treatment with SST and SSTR5 specific agonist (Fig. 3C). In comparison to intracellular expression, the membrane expression of CB1R and SSTR subtypes in STHdh$^{Q111/111}$ cells was changed in an agonist-specific manner. As shown in Fig. 3B, CB1R expression in membrane fraction was decreased upon treatment with CB1R specific agonists and SST but not with SSTR specific agonists. SSTR2 expression in membrane fractions was increased in response to SSTR2 and 5 selective agonists, whereas SSTR5 expression level was increased in most cases when compared to control (Fig. 3C). Taken together, these results suggest that receptor trafficking in response to agonist may account for pharmacological response of cell.

### *3.3. Time-dependent changes in ERK1/2 phosphorylation in STHdh$^{Q7/7}$ and STHdh$^{Q111/111}$ cells in response to receptor activation*

As shown in Fig. 4, in STHdh$^{Q7/7}$ cells, the phosphorylation of ERK1/2 in response to L-779976, L-817818 or ACEA was increased significantly at 15 min when compared to control and other time points. ERK1/2 phosphorylation was decreased at 30 min and enhanced at extended time of 6 h upon agonist treatment. Conversely, STHdh$^{Q111/111}$ cells displayed significantly increased ERK1/2 activation at 5 min upon treatment with SSTR2 or CB1R specific agonist but not in presence of SSTR5 specific agonist. Furthermore, the status of pERK1/2 in STHdh$^{Q111/111}$ cells at 15 min was comparable to control upon all treatments while decreased at 30 min and 6 h in response to receptor specific agonist treatment. These results suggest a critical transition of ERK1/2 activation between 5 and 15 min. Therefore, 15 min incubation time was taken into consideration for the rest of the experiments on signaling pathways.

### *3.4. Enhanced ERK1/2 phosphorylation in the presence of receptor specific agonist and QUIN in striatal neuronal cells*

QUIN, an endogenous NMDAR agonist, has been used in experimental models of HD (Beal et al., 1986, 1991). As shown in Fig. 5A, basal pERK1/2 levels were decreased in both STHdh$^{Q7/7}$ and STHdh$^{Q111/111}$ cells upon treatment with QUIN. Next, we determined the changes in pERK1/2 in STHdh$^{Q7/7}$ and STHdh$^{Q111/111}$ cells upon treatment with receptor-specific agonist in the presence or absence of QUIN. As shown in Fig. 5B, STHdh$^{Q7/7}$ neuronal cells exhibit no significant changes in ERK1/2 phosphorylation in response to receptor agonists. In STHdh$^{Q111/111}$ cells, activation of SSTR2 and CB1R but not SSTR5 exhibited increased ERK1/2 phosphorylation. As shown in Fig. 5C, in the presence of QUIN, the phosphorylation ratio of ERK1/2 was significantly increased upon agonist treatment in both STHdh$^{Q7/7}$ and STHdh$^{Q111/111}$ cells, with the exception of SSTR5 specific agonist-treated STHdh$^{Q7/7}$ cell. Taken together, these results indicate a protective effect of SSTRs and CB1R agonists against QUIN-induced toxicity via activation of ERK1/2.

### *3.5. SSTRs and CB1R protect STHdh cells from QUIN-induced toxicity*

To determine whether changes in ERK1/2 relate to QUIN-induced cell death in the presence of receptor specific agonists, we performed

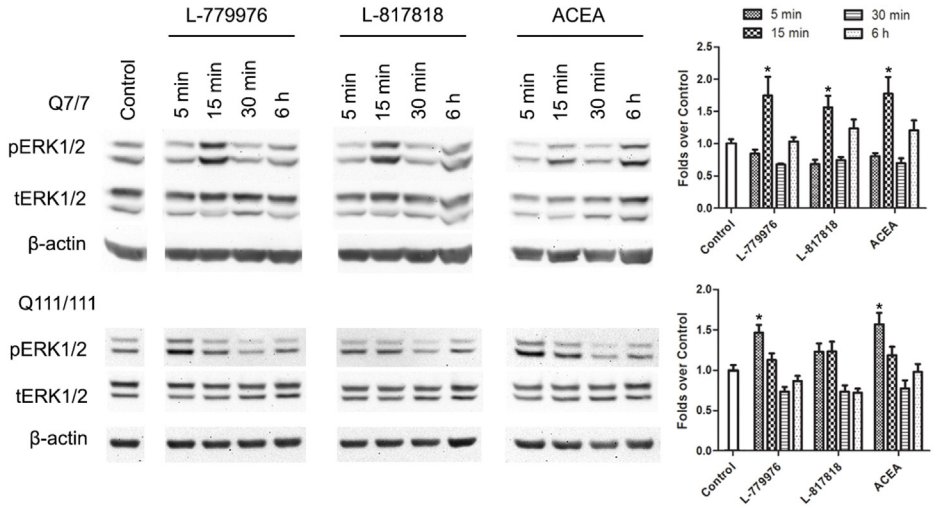


**Fig. 4. Time- and agonist-dependent changes in ERK1/2 phosphorylation in STHdh$^{Q7/7}$ and STHdh$^{Q111/111}$ cells.** To determine the status of ERK1/2 activity, STHdhQ7/7 and STHdhQ111/111 cells were treated with L-779976, L-817818 or ACEA (1 μM each) for different time points as indicated. In STHdhQ7/7 cells, maximum ERK1/2 phosphorylation was observed at 15 min upon treatment of all three selected agonists. In STHdhQ111/111 cells, pERK1/2 level was significantly increased at 5 min in response to L-779976 and ACEA. β-actin was used as an internal loading control. Data are presented as mean ± SE (n = 3). Statistical analysis was performed by using two-way ANOVA and Bonferroni posttest to compare against control. *, $p < 0.05$.

cell survival MTT assay. As shown in Fig. 6A&B, STHdh$^{Q7/7}$ and STHdh$^{Q111/111}$ cells exposed to 3 mM QUIN displayed 17.87 ± 1.18% and 29.21 ± 1.51% of cell death respectively. In the presence of receptor agonists, QUIN-induced cell death was blocked significantly in both STHdh$^{Q7/7}$ and STHdh$^{Q111/111}$ cells, suggesting that activation of either SSTR or CB1R improves cell survival against QUIN-induced excitotoxicity. Interestingly, treatment of receptor agonists exerted significant difference on cell death in STHdh$^{Q111/111}$ cells in comparison to STHdh$^{Q7/7}$ cells, with the exception of L-817818 and ACEA, suggesting a difference of receptor function influenced by genotype.

As shown in Fig. 6C, relative protection was comparable in STHdh$^{Q7/7}$ cells and STHdh$^{Q111/111}$ cells except in presence of WIN, which rescued 79.11 ± 9.43% and 28.71 ± 4.68% of QUIN-induced cell death in STHdh$^{Q7/7}$ and STHdh$^{Q111/111}$ cells respectively. These results indicate that WIN-mediated protection was perturbed in the presence of mHtt.

### *3.6. Combined agonist treatment alters QUIN-induced cell death, receptor interaction and ERK1/2 signaling*

#### *3.6.1. Receptor-mediated protective effect is diminished in cells treated with agonists in combination*

As shown in Fig. 7A and B, the neuroprotective effect seen in the presence of single receptor agonist was diminished in the presence of combined agonists. The only exception was STHdh$^{Q7/7}$ cells treated with the combination of SSTR2 and CB1R agonist, exhibiting 6.60 ± 1.20% cell death, which was comparable to single agonist treatment and significantly different from the effect in STHdh$^{Q111/111}$ cells.

#### *3.6.2. Suppressed expression of CB1R in SSTR immunoprecipitate upon combined agonist treatment*

Our recent study on SSTR5 and CB1R heterodimerization suggests the possibility of interaction between SSTRs and CB1R in STHdh$^{Q7/7}$ and STHdh$^{Q111/111}$ cells (Zou et al., 2017). To determine whether such interaction exists in pathological condition, CB1R expression was determined in SSTR2 or SSTR5 immunoprecipitates using Co-IP in STHdh$^{Q7/7}$ and STHdh$^{Q111/111}$ cells following treatment with receptor specific agonist alone and in combination. SSTR2 and SSTR5 immunoprecipitates prepared from control and agonist-treated STHdh$^{Q7/7}$ and STHdh$^{Q111/111}$ striatal cells were probed for CB1R expression. As shown in Fig. 7C, a band at approximately 110 kDa was detected in both STHdh$^{Q7/7}$ and STHdh$^{Q111/111}$ cells, representing receptor complex formation between SSTR2/CB1R (left panel) and SSTR5/CB1R (right panel) respectively. In SSTR2 immunoprecipitates prepared from STHdh$^{Q7/7}$ cells, the intensity of the band showing SSTR2/CB1R complex was increased upon treatment with SSTR2 agonist and ACEA alone but remained comparable to control upon combined treatment. In comparison to SSTR2 immunoprecipitate, the expression of CB1R in SSTR5 immunoprecipitates from STHdh$^{Q7/7}$ striatal cells was increased to a greater extent upon combine treatment than single treatment. These results indicate a relatively stronger complex formation between CB1R and SSTR5 than SSTR2 in the presence of agonist treatment. In STHdh$^{Q111/111}$ cells, CB1R displayed a stable complex formation with SSTR2, irrespective of the treatments as indicated. However, the expression of CB1R in SSTR5 immunoprecipitates prepared from STHdh$^{Q111/111}$ cells was decreased following treatment with receptor agonist alone or in combination in comparison to control.

#### *3.6.3. Combined agonist treatment delayed ERK1/2 phosphorylation in STHdh$^{Q111/111}$ cells*

To determine whether the loss of neuroprotective effect and receptor complex formation is associated with the regulation of signal transduction pathways, next we examined the status of ERK1/2. As shown in Fig. 7D, in STHdh$^{Q7/7}$ cells, the time-dependent changes in ERK1/2 phosphorylation upon combined treatments were comparable to the treatment with ACEA alone but not SSTR2 or 5 agonists (refer to Fig. 4). Interestingly, in STHdh$^{Q111/111}$ cells, treatment of either combination of agonists delayed the activation of ERK1/2 from 5 min to 15 min, when compared to single agonist treatment. The phosphorylation ratio and expression level of ERK1/2 were not significantly altered at prolonged treatment (6 h) in comparison to single agonist treatment.

Taken together, these results indicate that receptor crosstalk upon concurrent activation differs from single receptor activation, which may account for the loss of protective effect as well as delayed ERK1/2 phosphorylation in cells treated with two agonists in combination when compared to single treatment.

## 4. Discussion

SSTR subtypes and CB1R exhibit overlapping structural and functional properties and colocalize in different brain regions. Whether CB1R and SSTR subtypes functionally interact in pathological conditions has not been studied yet. We have recently described that SSTR5 and CB1R form a constitutive heterodimers in heterologous expressing system and display novel and distinct properties in the regulation of downstream signaling pathways in comparison to native receptor (Zou et al., 2017). In the present study, using conditionally immortalized striatal *wt* (STHdh$^{Q7/7}$) and mutant (STHdh$^{Q111/111}$) neuronal cells, we describe their expression, colocalization, trafficking, pro-survival signaling pathways and cell viability under normal and QUIN-induced excitotoxicity in response to receptor-specific agonists. Our results

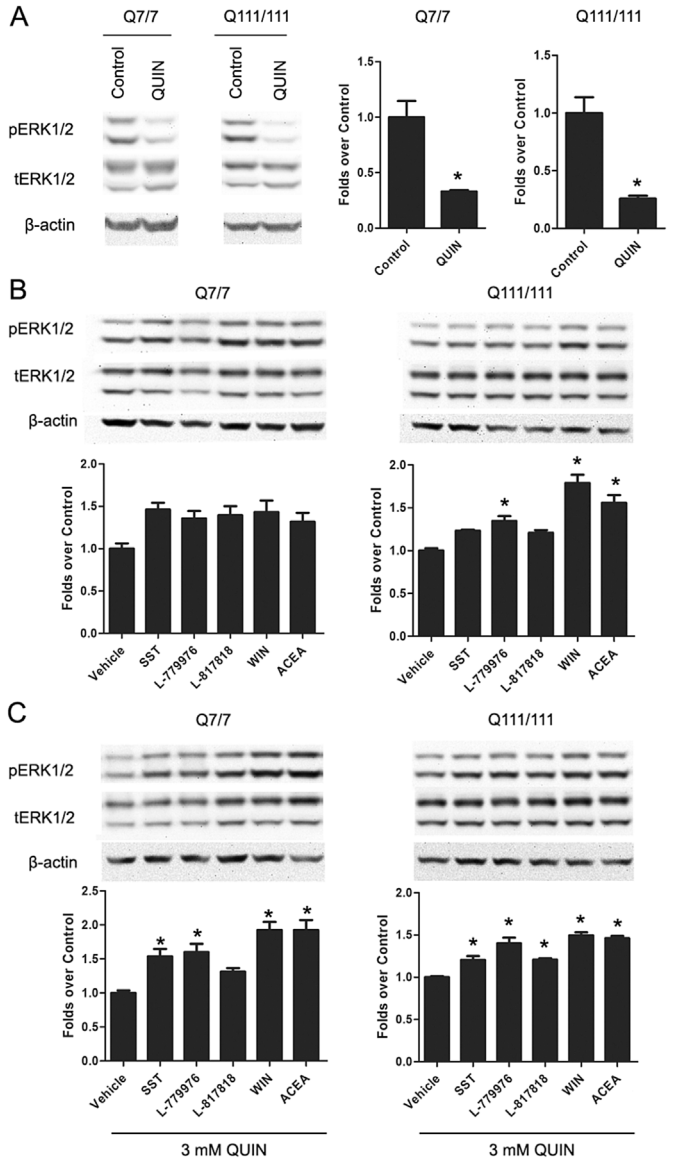


**Fig. 5. ERK1/2 phosphorylation in STHdh$^{Q7/7}$ and STHdh$^{Q111/111}$ cells is upregulated upon activation of SSTRs and CB1R in the presence of QUIN.** STHdhQ7/7 and STHdhQ111/111 cells grown to confluency were treated with 3 mM QUIN for 15 min in the absence or presence of SST, L-779976, L-817818, WIN-55212, or ACEA (1 μm each) and subjected to Western Blot analysis for the expression of ERK1/2. (A) Treatment with QUIN inhibited basal pERK1/2 level in both STHdhQ7/7 and STHdhQ111/111 cells. (B) In STHdhQ7/7 cells, ERK1/2 phosphorylation was not altered upon treatment with receptor-specific agonist. Whereas increased ERK1/2 phosphorylation was observed upon treatment of SSTR2 agonist, WIN and ACEA in STHdhQ111/111 cells. (C) In STHdhQ7/7 cells, all agonist treatments except SSTR5 agonist induced 1.5 folds increase of ERK1/2 phosphorylation over control. In STHdhQ111/111 cells, significant increase of ERK1/2 phosphorylation was observed in all agonist treatment. β-actin was used as an internal loading control. Data are presented as mean ± SE of three independent experiments. Statistical analysis was performed by using one-way ANOVA and post hoc Dunnett's test to compare against control. *, $p < 0.05$.

demonstrate that activation of either SSTRs or CB1R exerts protective effect against QUIN-induced excitotoxicity in both STHdh striatal cells, and changes in ERK1/2 phosphorylation. However, such protective effect was diminished upon concurrent activation of SSTRs and CB1R, which might be associated with delayed ERK1/2 phosphorylation and altered crosstalk between these two receptors. To our knowledge, this is the first comprehensive study characterizing SSTR subtypes and delineating their possible crosstalk with CB1R in *in vitro* models of HD.

We found that CB1R-like immunoreactivity is prominently confined intracellularly with weak expression at cell surface in *wt* and mutant STHdh cells, consistent with previous study showing that 4% of total CB1R is expressed at cell surface in STHdh$^{Q7/7}$ cells (Laprairie et al., 2013). The intracellular localization of CB1R has also been reported in other neuronal cell lines and cultured primary neurons, where the receptor is mostly found in endo/lysosomes and actively participates in the regulation of intracellular $Ca^{2+}$ storage (Rozenfeld, 2011). Recently, the presence of functional CB1R has also been observed in mitochondria that are associated with cellular respiration and energy metabolism (Benard et al., 2012). These studies support the intracellular-predominant yet fully-functional CB1R, which is not affected by mHtt. However, the exact location of intracellular CB1R in *wt* and mutant STHdh cells warrants future investigation.

Previous studies have shown that ACEA reduces CB1R membrane expression as a result of agonist-induced receptor endocytosis in STHdh$^{Q7/7}$ cells, supporting our observations on agonist-induced changes in CB1R expression in STHdh$^{Q7/7}$ cells (Laprairie et al., 2013). On the other hand, in STHdh$^{Q111/111}$ cells with endogenous expression of mHtt, the intracellular expression of CB1R is not altered upon agonist treatment, whereas membrane expression of CB1R is decreased as seen in STHdh$^{Q7/7}$ cells. It is highly possible that mHtt in STHdh$^{Q111/111}$ cells may interact with several transcriptional factors including cAMP response element-binding protein to interfere with the transcription of CB1R, as suggested earlier (Blazquez et al., 2011, 2015; Laprairie et al., 2013).

Similar to CB1R, both SSTR2 and 5 were mainly observed intracellularly in STHdh cells. However, what possible role intracellular SSTR2 and 5 might play in STHdh cells is not known. As shown in Fig. 3B&C, the expressions of SSTR2 and 5 is not only influenced by SSTR agonists, but also modulated by CB1R agonists, further supporting an interaction between SSTR and CB1R, which is consistent with our Co-IP result as described in Fig. 7C.

STHdh$^{Q111/111}$ cells exhibit relatively slow proliferative rate, increased susceptibility to stress and toxicity as well as impaired mitochondrial function in comparison to STHdh$^{Q7/7}$ cells (Trettel et al., 2000; Ruan et al., 2004). Consistent with these observations, we described increased neuronal cell death in STHdh$^{Q111/111}$ cells in response to QUIN-induced toxicity, in comparison to STHdh$^{Q7/7}$ cells. Activation of either SSTR or CB1R increases the cell viability in both STHdh$^{Q7/7}$ and STHdh$^{Q111/111}$ cells, which is in line with previous studies showing that CB1R exerts neuroprotective effect against NMDA toxicity in STHdh$^{Q7/7}$ cells (Blazquez et al., 2011, 2015). Interestingly, the protective effects of WIN and ACEA were affected differently by the presence of mHtt, as WIN, but not ACEA, significantly lost its protective effect in STHdh$^{Q111/111}$ cells. Considering WIN and ACEA both target CB1R, this difference may be due to their selective binding to other receptors, such as CB2R and TRPV1 (Pertwee et al., 2010).

Our results on ERK1/2 cell survival signaling pathways support the impaired ability of STHdh$^{Q111/111}$ cells to encounter stress. We also observed agonist-induced activation of ERK1/2, which is supported by previous studies on CB1R in STHdh cells, as well as on SSTRs in heterologous expression system (Patel, 1999; Kumar and Grant, 2010; Laprairie et al., 2013). Previous studies have also suggested a protective role of ERK1/2 against oxidative stress, emphasizing the beneficial role of ERK1/2 in cell survival, which is consistent with our results presented here (Gines et al., 2010). Considering the neuroprotective effect seen in *wt* and mutant striatal cells, we believe that ERK1/2 is the crucial determinant of SSTR and CB1R mediated protection. Although ERK1/2 has been proposed to be activated by β-arrestin 2, tyrosine kinase and cAMP, whether such mechanism is involved in our experimental condition is not known and warrants future studies. (Daigle et al., 2008; Dalton and Howlett, 2012).

The loss of receptor specific agonist-induced neuroprotective effect against QUIN-induced toxicity in combined agonist treatment is

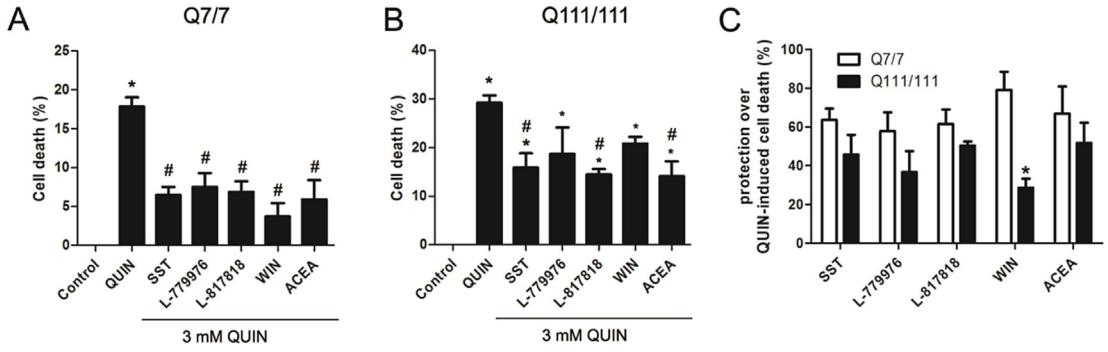


**Fig. 6. Receptor-mediated protection against QUIN is independent of the presence of mHtt.** Cells were grown in 96-well plate and serum-starved for 24 h, followed by incubation with Locke's solution containing SST, SSTR2 specific agonist L-779976, SSTR5 specific agonist L-817818, WIN and ACEA (1 μM each) for 5 h in the presence of 3 mM QUIN. Posttreatment culture medium was replaced with QUIN-free medium containing receptor agonist. Cells were incubated for additional 24 h and processed for cell viability using MTT assay. (A) In STHdhQ7/7 cells, QUIN induced 17.87 ± 1.18% cell death which was decreased to 3.73 ± 1.68%–7.53 ± 1.75% upon agonist treatment. (B) In STHdhQ111/111 cells, QUIN-induced cell death was relatively higher as 29.21 ± 1.51%, which was suppressed to 14.08 ± 3.08%–20.83 ± 1.37% with receptor specific agonist. Data are presented as percentage of cell death in comparison to control (considered as 0% cell death) and as mean ± SE of three independent experiments performed in triplicates. Statistical analysis was performed by using one-way ANOVA, followed by Bonferroni's multiple comparison test. *, $p < 0.05$ versus control; #, $p < 0.05$ versus QUIN-treated cells. (C) Protection exerted by receptor-specific agonist against QUIN in STHdhQ7/7 (white) and STHdhQ111/111 (black) cells. Protection against QUIN was calculated as protection (%) = ([Cell death]QUIN-[Cell death] agonist)/[Cell death]QUIN*100%. Data was presented as mean ± SE and analyzed using two-way ANOVA, followed by Bonferroni's posttest. *, $p < 0.05$ versus STHdhQ7/7 cells.

possibly associated with the changes in receptor expression and functionality as well as delayed ERK1/2 phosphorylation. Indeed, co-activation of CB1R/CB2R heterodimers in SH-SY5Y cells abolishes neuritogenesis induced by either receptor agonist (Callen et al., 2012). This evidence suggests that the activation of two receptors at a time might mask the effect of one receptor. Moreover, dopamine receptor 2 (D2R) is endogenously expressed in STHdh$^{Q7/7}$ cells and forms heterodimer with CB1R (Bagher et al., 2016). Co-activation of D2R influences CB1R coupling to G protein and β-arrestin-mediated ERK1/2 signaling (Glass and Felder, 1997; Jarrahian et al., 2004; Kearn et al., 2005; Bagher et al., 2016). Similarly, we have reported earlier in recombinant system that CB1R forms heterodimer with SSTR5 which leads to a SSTR5-

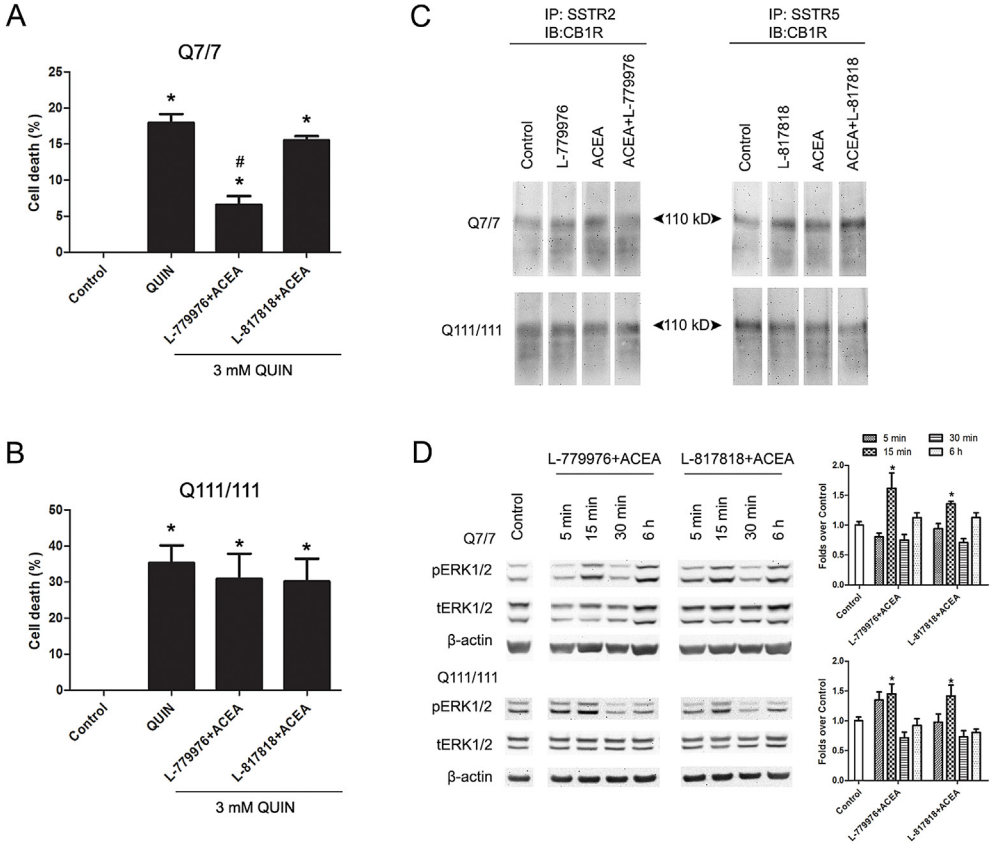


**Fig. 7. Concurrent activation of SSTR and CB1R altered the protective effects, ERK1/2 signaling, and receptor complex composition.** STHdhQ7/7 and STHdhQ111/111 cells were treated with receptor specific agonists in combination and processed for assessment of cell viability, receptor interaction and ERK1/2 activation. (A) In STHdhQ7/7 cells, QUIN-induced cell death was rescued by ACEA in the presence of L-779976 but not L-817818. (B) Combined agonist treatment failed to enhance cell viability in the presence of QUIN. Note the loss of protective effect in STHdhQ111/111 cells in comparison to single agonist treatment. Data are presented as percentage of cell death in comparison to control (taken as 0% cell death) and are as mean ± SE of three independent experiments performed in triplicates. Statistical analysis was performed by using one-way ANOVA, followed by Bonferroni's multiple comparison test. *, $p < 0.05$ versus control; #, $p < 0.05$ versus QUIN-treated cells. (C) In STHdhQ7/7 cells, SSTR2 and CB1R complex formation was increased in response to individual receptor activation but decreased upon co-activation. In contrast, cells with mHtt expression displayed stable complex formation in all condition as indicated. SSTR5 and CB1R exhibited strong complex formation upon agonist treatment in STHdhQ7/7 cells, whereas in STHdhQ111/111 cells, SSTR5/CB1R complex expression was lost upon treatment with receptor agonist in combination. (D) STHdhQ7/7 cells showed highest pERK1/2 activity at 15 min, comparable to that upon single agonist treatment. In STHdhQ111/111 cells, increased p-ERK1/2 was observed at 15 min in contrast to 5 min, as seen upon single agonist treatment. β-actin was used as an internal loading control. Data are presented as mean ± SE of three independent experiments. Statistical analysis was performed by using two-way ANOVA and Bonferroni posttest to compare against control. *, $p < 0.05$.

dominant signaling in cAMP/protein kinase A/ERK pathway (Zou et al., 2017). SSTR2 and 5 have also been shown to heterodimerize with each other as well as with D2R respectively (Rocheville et al., 2000a, 2000b; Baragli et al., 2007; Grant et al., 2008). In the present experimental condition, the potential involvement of D2R in the interaction between SSTR subtypes and CB1R via heterodimerization cannot be ruled out from discussion. This is further supported by the fact that D2R-antagonism modulates CB1R allosterically that D2R antagonist not only favors CB1R coupling to Gs protein, but also inhibits Gi-dependent ERK1/2 phosphorylation whereas enhances Gs-dependent CREB phosphorylation. (Bagher et al., 2016). Such interaction bears clinical significance, as evidence suggests that D2R antagonists might be beneficial in treating early HD symptoms [reviewed in (Pidgeon and Rickards, 2013)]. Whether altered expression of receptor complex upon combined agonist treatment is associated with the diminished protective effects is not known. To address how combined treatment-induced changes in receptor-receptor interaction and signaling pathways are involved in the loss of neuroprotection, further studies are in progress in this direction.

## 5. Conclusions

Taken together, results presented in the present study address the role of SSTR/CB1R in pathogenesis of HD. These observations include the changes in SSTR/CB1R complex formation and opposing effect on cell survival signaling pathways upon co-activation of both receptors. In $STHdh^{Q7/7}$ and $STHdh^{Q111/111}$ striatal cells, SSTR2/CB1R and SSTR5/CB1R interact with each other and respond in agonist dependent manner. Single receptor-mediated neuroprotection in QUIN-induced toxicity is diminished upon co-activation of SSTRs and CB1R, which is possibly attributed to the modulation of ERK1/2. These results provide novel insight for the role of SSTRs and CB1R in the pathogenesis of HD.

## Conflicts of interest

The authors declare that they have no conflicts of interest with the contents of this article.

## Author contributions

S.Z. and U.K. designed the study and wrote the manuscript. S.Z. performed the experiments and analyzed the data.

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