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TARGETING NEURONAL DOPAMINE D_1-HISTAMINE H_3 RECEPTOR HETEROMERS AS A NEW TREATMENT OF HUNTINGTON'S DISEASE

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1. Abstract

Huntington disease (HD) is characterized by an abnormal neurotransmission partly due to the aberrant activation of dopamine D_1 receptors (D_1R) in the striatum. It has been previously shown that there is an excess of dopamine production in HD and that overactivation of D_1R can produce not only an imbalance in neurotransmission but also can lead directly to signaling cascades that induce cell death. The main goal of this project was to find innovative approaches to reduce D_1R signaling in order to prevent HD damage. Our proposal was based on our previous results showing that D_1R function can be modulated by histamine through the activation of histamine H_3 receptors (H_3R) within the D_1R-H_3R protein-protein complex named receptor heteromer.

It has also been reported that alterations in striatal glutamatergic transmission due to polyglutamine huntingtin correlate with postsynaptic density rearrangement and with an abnormal NMDA glutamate receptor (NMDAR) signaling. Furthermore, NMDAR activation specifically increases dopamine-induced cell death via D_1R in striatal cells presenting polyglutamine huntingtin. Our previous results with BRET techniques demonstrated that NMDA and D_1 receptors can heteromerize in transfected cells. For these reasons, we also focused this project on the study of the NMDA receptor heteromerization with D_1R and H_3R, and the effect of H_3R ligands on D_1 and NMDA receptor affinity, signaling and neurotoxicity.

Thus, our hypothesis was that D_1R-H_3R and/or D_1R-H_3R-NMDAR heteromers could be new and promising therapeutic targets for HD and that H_3R ligands, by interacting with these heteromers, could prevent aberrant D_1R and/or NMDAR signaling including the D_1R-mediated cell death.

To prove our hypothesis, the main aims of our project were:
- To determine the expression and the pharmacological and functional characteristics of D_1R-H_3R heteromers in a cellular model of HD and in different brain regions of a mouse model of HD at different stages of the illness.
- To test different H_3R agonists and antagonists in order to test their capacity to provide neuronal protection.
- To explore the ability of D_1R-H_3R heteromers to form heterotrimers with NMDA receptors and its implication on neuronal protection.

- To explore the ability of the known human H_3R isoforms to form heteromers with D_1R and NMDAR with specific biochemical characteristics.

2. Results obtained

Functional D_1R-H_3R heteromers are expressed in wild type STHdHQ7 and HD model STHdHQ111 striatal cells

To test whether D_1R-H_3R heteromers could indeed be targets for controlling D_1R signaling in HD, we first analyzed the expression of both receptors in immortalized striatal cells expressing endogenous levels of full-length wild-type STHdHQ7 or mutant STHdHQ111 huntingtin. Ligand binding determined that both STHdHQ7 and STHdHQ111 cells endogenously express similar levels of D_1R and H_3R. By proximity ligation assays (PLA), D_1R-H_3R heteromers were detected as red spots surrounding the blue-stained nuclei in both cell types and in cells treated with control lentivirus vector, but not in cells depleted of H_3R by shRNA, as shown by RT-PCR and functionality. To ensure that D_1R-H_3R heteromers were functional in STHdH cells, cell signaling experiments were performed. Using both STHdHQ7 and STHdHQ111 cells and concentrations of ligands previously shown to be optimal for receptor activation of the ERK1/2 pathway, we observed that the D_1R agonist SKF 81297 was able to increase ERK1/2 phosphorylation whereas it was prevented by D_1R antagonist SCH 23390, and by the H_3R antagonist thioperamide via cross-antagonism. In addition, we tested a previously described alternative signaling pathway activated downstream of D_1R, Ca^{2+} mobilization. When cells were treated with the D_1R agonist SKF 81297 a robust and rapid increase in cytosolic Ca^{2+} was detected in both STHdHQ7 and STHdHQ111 cells. Importantly, this calcium release could also be dampened with the H_3R antagonist thioperamide (cross-antagonism). The above signaling data strongly support the presence of functional D_1R-H_3R heteromers in STHdH cells. To further demonstrate that an H_3R antagonist is dampening D_1R activation involving D_1R-H_3R heteromers, we evaluated the effect of interfering peptides, which are synthetic peptides with the amino acid sequence of domains of the receptors involved in the heteromeric interface. We therefore investigated whether synthetic peptides with the sequence of TM5, and TM7 (as a negative control) of D_1R, fused to HIV-TAT, were also able to disrupt receptor D_1R-H_3R heteromers measured by PLA. In agreement with our hypothesis, there was a near complete loss in fluorescence when STHdHQ7 and STHdHQ111 cells

were incubated with TAT-TM5 peptide, but not for the negative control in which the TAT-TM7 peptide was used. Next, we evaluated whether TM5 or TM7 would interfere with the observed cross-antagonism in calcium mobilization assays. Clearly, pretreatment of both STHdHQ7 and STHdHQ111 cells with the TAT-TM5, but not TAT-TM7, peptide disrupts the ability of the H_3R antagonist thioperamide to dampen D_1R calcium signaling. These results support the idea that TM5 forms part of the interface of the D_1R-H_3R heteromer and demonstrate that the H_3R antagonist effect is driven through direct interaction between D_1R and H_3R.

H_3R ligands prevent the D_1R-induced cell death in STHdHQ7 and STHdQ111 cells

It has been previously reported that upon activation of D_1R, STHdH cell viability is reduced. To explore whether H_3R ligands could impair D_1R through D_1R-H_3R heteromers in a pathologically relevant readout, we used D_1R-induced cell death as an output of D_1R activation in STHdH cells. As expected, STHdH cell viability decreased when treated with the D_1R agonist SKF 81297 in a concentration-dependent manner. Significant cell death did not occur until 30 μM SKF 81297 was used, an effect prevented by the D_1R antagonist SCH 23390. Pre-treatment with the H_3R antagonist thioperamide, which did not modify cell viability when administered alone, increased the number of surviving cells in the presence of the D_1R agonist SKF 81297 in both cell types. Importantly, the effect of the H_3R antagonist thioperamide was specific since no protection from D_1R agonist-induced cell death was observed in cells depleted of H_3R with shRNA lentiviral infection, but was observed in cells transfected with the control lentivirus. In addition, we also demonstrated that recovery of viability induced by the H_3R antagonist thioperamide was mediated by D_1R-H_3R heteromers since preincubation with D_1R TM5 peptide, but not D_1R TM7, impaired the H_3R antagonist protection from D_1R agonist-induced cell death. A correlation between the intensity of calcium responses and the activation of apoptotic pathways, such as p38, has been previously demonstrated. Thus, we measured changes in p38 phosphorylation levels using both concentrations of the D_1R agonist SKF 81297. Interestingly, we found that increased phosphorylation of p38 only occurred at the cytotoxic concentration of SKF 81297. Treatment with the H_3R antagonist thioperamide reduced p38 phosphorylation upon D_1R activation in both cell types. Moreover, the p38 inhibitor SB 203580 blocked p38 phosphorylation and protected against the cytotoxic effect of the D_1R agonist SKF 81297 in a dose-dependent manner, confirming that p38 is a key pathway involved in

D_1R-mediated cell death in these cells. Overstimulation of D_1R induces receptor internalization promoting rapid intracellular signaling, and D_1R expression is decreased in several models of HD. To test whether changes in receptor trafficking might be at play we analyzed whether 30 μM SKF 81297 can induce D_1R internalization in the striatal cells. We observed that 30 μM SKF 81297, that decreased cell viability, promoted D_1R internalization in both STHdh cells. Interestingly, the 30 μM SKF 81297-induced D_1R internalization correlated with D_1R-H_3R heteromer disruption evidenced by a lack of PLA staining in both STHdH cells treated with 30 μM SKF 81297. One potential way by which GPCRs can influence each other in a heteromer is by altering the trafficking of the partner receptor. Pretreatment with the H_3R antagonist thioperamide restored the number of punctate PLA spots decreased after overstimulation with the D_1R agonist SKF 81297. These results suggest that H_3R ligands are inhibiting D_1R internalization and D_1R-mediated cell death by inhibiting p38 phosphorylation and calcium signaling.

Functional D_1R-H_3R heteromers are expressed in wild-type HdhQ7/Q7 and in mutant knock-in HdhQ7/Q111 mice at early but not late HD stages

To test whether D_1R-H_3R heteromers can indeed be targets for treating HD, we investigated their expression and function in the striatum, cerebral cortex and hippocampus of a widely accepted preclinical model of HD, the heterozygous mutant knock-in HdhQ7/Q111 mice, and their wild-type HdhQ7/Q7 littermates. By PLA we confirmed that both HdhQ7/Q7 and HdhQ7/Q111 mice display D_1R-H_3R heteromers at 2 and 4 months of age in all brain regions tested. Heteromer expression was similar in all brain areas and no differences were observed between genotypes at 4 months of age. Surprisingly, an almost complete loss of D_1R-H_3R heteromers was found in 6 and 8 months of HdhQ7/Q111 mice but not in HdhQ7/Q7 mice, indicating that at more advanced disease stages the D_1R-H_3R heteromer is lost. The loss of heteromer expression is not due to a complete loss of receptor expression since by radioligand binding and mRNA expression analysis both receptors continue to be expressed. To test the role of D_1R-H_3R heteromers, organotypic mouse striatal, cortical and hippocampal cultures were obtained. Cell death was induced by the D1R agonist SKF 81297 (50 μM), and analysis of DAPI and propidium iodide staining was performed. As expected, D_1R agonist SKF 81297 treatment increased the percentage of cell death in all three regions compared to vehicle-treated organotypic cultures without significant differences between genotypes at 4 months of age. Importantly, slices pre-treated with

the H_3R antagonist thioperamide, which does not modify cell death when administered alone, protected cells from D_1R elicited cell death, indicating that functional D_1R-H_3R heteromers are expressed in different brain areas of HdhQ7/Q7 and HdhQ7/Q111 mice at early disease stages. The dramatic change in heteromer expression in 8 months-old HdhQ7/Q111 mice was mirrored by the lack of protection of the H_3R antagonist thioperamide against SKF 81297-induced cell death in organotypic cultures, corroborating that the presence of D_1R-H_3R heteromers is needed for the H_3R antagonist to prevent D_1R-mediated cell death.

Treatment with thioperamide prevents cognitive and learning deficits at early disease stages

To test whether the H_3R antagonist thioperamide can exert beneficial effects in the initial stages of the disease we evaluated the effect of chronic thioperamide treatment on motor learning and memory deficits in mutant HdhQ7/Q111 mice. Since cognitive decline is observed in these HD mice from 6 months of age and the D_1R-H_3R heteromers are expressed and functional until the age of 5 months, we chose 5 month-old animals to start the thioperamide treatment. Corticostriatal function in saline and thioperamide-treated HdhQ7/Q7 and HdhQ7/Q111 mice was analyzed by using the accelerating rotarod task that evaluates the acquisition of new motor skills. Next, recognition long term memory (LTM) and spatial LTM was analyzed using the T-maze spontaneous alternation task (T-SAT). Overall, these data demonstrate the effectiveness of thioperamide treatment in restoring motor learning and preventing spatial and recognition LTM deficits in HdhQ7/Q111 mice. We next tested if the reversion of the HD phenotype in mutant HdhQ7/Q111 mice induced by thioperamide treatment correlated with the preservation of D_1R-H_3R heteromer expression. By PLA we observed that in saline-treated 6-mo-old HdhQ7/Q111 mice the heteromer expression was significantly diminished with respect to the age-matched HdhQ7/Q7 mice. Notably, treatment with thioperamide significantly prevented the loss of D_1R-H_3R heteromers in all brain regions analyzed in HdhQ7/Q111 mice at both 6 and 8 months of age, suggesting that the altered trafficking observed in cells may potentially also occur in vivo.

D_1R-H_3R heteromer expression changes occur in other rodent HD models and in HD patients

The fact that thioperamide treatment prevents cognitive and motor learning deficits and prevents the loss of D_1R-H_3R heteromers at 6 and 8 months of age in a mouse model of HD is suggestive that thioperamide, or a future pharmacologically improved H_3R antagonist specifically targeting D_1R-H_3R heteromers, can be used to treat HD symptoms. To test this, we investigated D_1R-H_3R heteromer expression in other transgenic HD mouse models and in human caudate-putamen slices using PLA. The loss of heteromer expression compared with wild-type littermates was also observed in other mouse models of HD, the R6/1 and R6/2 mice transgenic for the human huntingtin exon 1. Importantly, D_1R-H_3R heteromers were detected as green spots surrounding the blue stained nuclei in human caudate-putamen slices from control individuals and low-grade (grade 0, 1 and 2) HD patients. In contrast, green spots were almost absent in samples from high-grade (grade 3 or grade 4) HD patients. These results show that D_1R-H_3R heteromer formation changes during disease progression and, importantly, that humans express D_1R-H_3R heteromers at early disease stages.

D_1R-H_3R-NMDAR heteromers are targets to prevent neuronal death in Alzheimer's disease.

Alzheimer's disease (AD) is a neurodegenerative disorder causing progressive memory loss and cognitive dysfunction. Anti-AD strategies targeting cell receptors consider them as isolated units. We have demonstrated the presence of D_1R-H_3R-NMDAR heteromers in heterologous systems and in rodent brain cortex. Heteromers were detected by co-immunoprecipitation and PLA in the rat cortex where H_3R agonists, via negative cross-talk, and H_3R antagonists, via cross-antagonism, decreased D_1R agonist signaling determined by ERK1/2 or Akt phosphorylation, and counteracted D_1R-mediated excitotoxic cell death. Both D_1R and H_3R antagonists also counteracted NMDA toxicity suggesting a complex interaction between NMDAR and D_1R-H_3R heteromer function. By bioluminescence resonance energy transfer (BRET), we demonstrated that D_1R or H_3R form heteromers with NR1A/NR2B NMDAR subunits. D_1R-H_3R-NMDAR complexes were confirmed by BRET combined with fluorescence complementation. The endogenous expression of complexes in mouse cortex was determined by PLA and similar expression was observed in wild-type and APP/PS1 mice. Consistent with allosteric receptor-receptor interactions within the complex, H_3 receptor antagonists

reduced NMDAR- or D_1R-mediated excitotoxic cell death in cortical organotypic cultures. Moreover, H_3R antagonists reverted the toxicity induced by ß1–42-amyloid peptide. Thus, H_3R in the D_1R-H_3R-NMDAR heteroreceptor complexes arise as a promising target to prevent neurodegeneration.

Different human H_3R isoforms also heteromerize with D_1R and NMDAR

At least 20 distinct isoforms of the H_3R have been identified as a result of gene splicing events, with distinct brain expression patterns and signaling properties, although many still have not been fully characterized. Here, we have described the activation profile of distinct $G\alpha_{i/o}$ protein subtypes ($G\alpha_{o1}$, $G\alpha_{o2}$, $G\alpha_{i1}$, $G\alpha_{i2}$ and $G\alpha_{i3}$) and of β-arrestin I recruitment of four H_3R isoforms (H_3R329, H_3R365, H_3R415 and H_3R445), which mainly differ in the length of their third intracellular loop, by bioluminescence resonance energy transfer (BRET) functional assays. Comparing efficacies among H_3R isoforms in both functional assays brings to light that they have different degrees of functionality in inverse proportion to the level of deletion of their sequence, in some cases even showing no activation, a phenomenon known as isoform-functional selectivity or isoform bias. BRET assays revealed that H_3R329, H_3R365, H_3R413 and H_3R415 can also form heteromers with D_1R, with the glutamate NMDAR and with both receptor simultaneously forming heterotrimeric complexes. These findings provide evidence of the importance of heteromerization between distinct H_3R isoforms and D_1R and enlarge the complexity level inherent to the histaminergic neurotransmission.

3. Relevance and future implications

We do believe that this project can have important and relevant benefits. From the perspective of scientific advances, the project provides new data about the existence of heteromers between D_1R and H_3R. Both receptors are of great relevance in the central nervous system. In addition, we have pharmacologically and functionally characterized this interaction. The knowledge about the specific role of these receptors and how they interact and modulate each other in different physiological processes is of high relevance to understand what is happening in pathological conditions. From a more applied perspective, in this project we have shown that striatal and cortical H_3R are crucial for the control of motor activity and that H_3R, forming heteromers with D_1R, not only modulate the motor activity induced by D_1R but also acts as a brake on the D_1R

signaling, especially when it is overactivated. Thus, H_3R can avoid excitotoxicity and neuronal death induced by the overactivation of D_1 receptors in neurodegenerative situations such as Huntington's disease and Alzheimer's disease. For all these reasons we consider that the scientific and social interest of this project is high.

From a pharmacological point of view, the concept of heteromeric receptors as therapeutic targets for mental and/or neurodegenerative diseases that involve G-protein coupled receptors (GPCRs) is of high interest. It is well known that GPCRs constitute the target for more than 25% of existing drugs, but the speed at which they appear in the market is much lower than would be expected given the huge amount of processes that these receptors control both peripherally and in the central nervous system. One reason for this delay may be that the strategies used for the development of new drugs are not considering the real target. In most cases, the developed drugs are directed to GPCRs considering them as individual entities and not as dimers or higher order oligomers. It has been extensively reported that receptor heteromers are, at least, dimeric entities that result from the combination of two or more receptors. These oligomeric complexes acquire unique biochemical and functional properties that are different from those of their individual components and that allow a fine and selective modulation of their signaling pathways. This ability to allosterically modulate each other should be taken into account when designing therapeutic strategies targeting these receptors. Therefore, the actual therapeutic target should be heteromers and oligomers of GPCRs and not individual or monomeric receptors.
An example of an oligomeric complex that must be considered as a target for the design of therapeutic strategies is the one described in this project, the dopamine-histamine receptor heteromer (D_1-H_3) and also the trimeric complex of these receptors with glutamate NMDA receptors (D_1-H_3-NMDA). Both complexes can be a promising target for the treatment of neurodegenerative disorders, such as Huntington and Alzheimer's.

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