P2X7 receptor upregulation in Huntington’s disease brains

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1 Abstract

Huntington’s Disease (HD) is a fatal degenerative disorder affecting the nervous system. It is characterized by motor, cognitive and psychiatric dysfunctions, with a late onset and an autosomal dominant pattern of inheritance. HD causing mutation consists on an expansion of repeated CAG triplets in the huntingtin gene (HTT), encoding for an expanded polyglutamine (polyQ) stretch in the huntingtin protein (htt). The mutation causes neuronal dysfunction and loss through multiple mechanisms, affecting both nucleus and cytoplasm. P2X7 receptor (P2X7R) emerged as a major player in neuroinflammation, since ATP -its endogenous ligand- is massively released under this condition. Indeed, P2X7R stimulation in the central nervous system (CNS) is known to enhance the release of pro-inflammatory cytokines from microglia and of neurotransmitters from neuronal presynaptic terminals, as well as to promote apoptosis. Previous experiments performed with neurons expressing the mutant huntingtin and exploiting HD mouse models demonstrated a role of P2X7R in HD. On the basis of those results, here we explore for the first time the status of P2X7R in HD patients’ brain. We report that in HD post-mortem striatum, as earlier observed in HD mice, the protein levels of the full-length form of P2X7R, also named P2X7R-A, are upregulated. In addition, the exclusively human naturally occurring variant lacking the C-terminus region, P2X7R-B, is upregulated as well. As we show here, this augmented protein levels can be explained by elevated mRNA levels. Furthermore, in HD patients’ striatum, P2X7R shows not only an augmented total transcript level, but also an alteration of its splicing. Remarkably, P2X7R intron 10-11 is more retained in HD patients when compared with controls. Taken together, our data confirm that P2X7R is altered in brains of HD subjects and strengthen the notion that P2X7R may represent a potential therapeutic target for HD.

2 Introduction
Huntington’s Disease (HD) is a neurodegenerative disorder characterized by motor dysfunction, cognitive decline and psychiatric symptoms (1). The HD causing mutation lies in the Huntingtin (HTT) gene. In normal population, HTT exon 1 includes from 6 to 35 repeats of the CAG triplet, while HD patients have 40 or more repeats (2). This mutation has an autosomal dominant inheritance, is highly penetrant and initiates the disease through various mechanisms (3). On one hand, the expanded CAG repeats of the HTT mRNA are able to trap several RNA-binding proteins (4), thus likely provoking their loss of function. Interestingly, splicing factors and spliceosome components are among the sequestered proteins (5). As a consequence, at least two pathogenic mis-splicing events, affecting HTT and MAPT, have been reported (6);(7). On the other hand, the expanded CAG triplets encode for an abnormally long poly-glutamine (polyQ) trait in the N-terminus of huntingtin protein (htt), which accounts for a toxic gain-of-function, as well (2). Both exon 1 and exon 1-like fragments contain the expanded polyQ trait and have been reported to trigger HD through toxic protein-protein interactions. Besides, htt fragments are prone to aggregate into micro-aggregates, fibrils and inclusion bodies, that in turn may sequester other proteins, both in the nucleus and in the cytoplasm (8). All this leads to global neuronal impairment and death. Medium spiny neurons of the striatum are the most vulnerable cell type to mutant htt, which triggers striatal atrophy, the main hallmark in HD patients (1). Nevertheless, mutant htt is expressed throughout the whole brain, provoking degeneration in many other regions of the CNS.

P2X7 receptor (P2X7R) is a cation channel modulated by endogenous ATP. A single P2X7R subunit comprises a short intracellular N-terminus, two transmembrane motifs (TM1, TM2) separated by an extracellular loop, and an intracellular C-terminus tail. P2X7R subunits co-assemble in a trimeric chalice-like architecture provided of three ATP binding pockets (9);(10). When ATP binds to P2X7R, the channel is open, allowing the transit of small cations, e.g. Na+, Ca2+ and K+ (11). However, when the ATP activation is prolonged or repeated, P2X7R acts as a nonselective pore, which allows transit of large molecular weight molecules (11). It has been shown that both the TM2 domain (12) and the C-terminus (13) play a pivotal role in the pore formation. P2X7R is expressed in many cell types, including neurons and brain glial cells (microglia, astrocytes and Muller cells) (14);(15);(16). ATP is released as co-transmitter via synaptic vesicles and thus transient and localized increase in extracellular ATP can follow neuronal activity. Accordingly, a function of P2X7R in neuron-neuron communication and plasticity has been established (17). However, wider ATP release is observed in response to adverse events, including ischemia, hypoxia, mechanical stimuli, bacteria or toxins exposure (18). This kind of high levels of extracellular ATP lead to P2X7R-mediated neuron-glia cross-talk and glial activation, which in turn causes P2X7R upregulation. In general, P2X7R stimulation leads to a Ca2+ influx, which has different consequences depending on the cell type where it occurs. P2X7R provokes glutamate release from presynaptic nerve terminals and from astrocytes, responsible for an excitotoxic effect (19), while astrocyte and microglia are accountable for IL-1β, IL-6 and TNF-α release, triggering neuroinflammation (20). ROS production, combined with BDNF downregulation, are further mechanisms by which neuronal damage and reactive gliosis are achieved via P2X7R (21). Under
prolonged stimulation, P2X7R pore opens, prompting plasma membrane blebbing and lastly cell death (22). All this makes P2X7R a key player in neuroinflammation.

Notably, different P2X7R transcript variants deriving from alternative splicing exist both in human and mouse. In particular, ten isoforms (from A to J) have been described for the human receptor (23). P2X7R-A comprises 13 exons and corresponds to the canonical transcript (24). P2X7R-B differs from it for the retention of the 84 nucleotide-long intronic region between exon 10 and exon 11, while variants P2X7R-C/D/E/F/I lack either exon 4, 5, 7, 8 or 7 and 8 together. P2X7R-G and H have an extra exon named N3 between exon 2 and exon 3. P2X7R-I lacks both exon 2 and N3. Transcripts E, G and I also present the intron 10-11 retention. Of such transcripts, four have been extensively studied since they originate proteins. Therefore, not a unique P2X7R exists. Rather, four P2X7Rs have been described based on alternative splicing: P2X7R-A, B, H and J (23);(25).

P2X7R-A encodes the well characterized full-length P2X7R-A. It includes 595 aa constituting the N-terminus, TM1 and TM2 separated by an extracellular loop, and the intracellular C-terminus of the protein. The N-terminus can form intracellular complexes with many substrates including heat shock proteins, β2-integrin, α-actin and several protein kinases and phosphatases (26). The extracellular loop owns the ligand-binding sites and a number of N-glycosylation sites (27). The C-terminus of P2X7R-A, due to multiple protein-protein and protein-lipid interaction motifs (28), contributes to its communication with cytoskeletal and intracellular proteins (26), and is required for the formation of a pore, hence eliciting many functions of the receptor. P2X7R-B is the transcript for P2X7R-B and lacks the C-terminus as a consequence of the premature stop codon introduced by the intron 10-11 retention. Accordingly, this protein comprises 364 aa, where the last 18 aa are different from P2X7R-A. Interestingly, P2X7R-B seems to be the predominant P2X7R transcript in multiple human tissues, including the brain (23);(29). Experiments in HEK293 cells expressing P2X7R-B demonstrate its ability to form homotrimers and maintain all the ATP-stimulated channel functions, although being unable to form a non-selective pore and trigger apoptosis. Thus, P2X7R-B is free of the cytotoxic activity linked to the C-terminal tail and is generally considered a less “dangerous” form of P2X7R. However, when co-expressed, P2X7R-A and B can heterotrimerize efficiently. In this case, P2X7R-B potentiates P2X7R-A functions, including the formation of a pore and pro-apoptotic activity. Therefore, cells could modulate ATP responses by P2X7R-A and B expression ratio and combination in trimers (29). A pathophysiological role of P2X7R-B has been described in multiple conditions, including bone cancer (30) and bone differentiation (31). Is has also been described in neural progenitors (32), neuroblastoma cells (33) and glioblastoma cells (34). P2X7R-H contains 505 aa and is also known as P2X7R-ΔTM1, since the TM1 is absent. Indeed, P2X7R-H contains the N3 exon, which creates a new start codon responsible for the absence of the first part of the protein. However, when transfected in HEK293 cells, P2X7R-H is an inactive receptor (23). P2X7R-J includes only 258 aa and lacks the C-terminus, the TM2 and part of the extracellular loop. Still,
P2X7R-J can form heterotrimers with P2X7R-A. It emerged to act as a dominant negative, since it antagonizes the function of P2X7R-A in cervical cancer cells (25). To date, the implication of such variety around P2X7Rs expression both in physiological and pathological backgrounds has never been explored in the nervous system.

Cell injury is a common feature of neuroinflammatory and neurodegenerative disorders, including traumatic brain injury, stroke, epilepsy, neuropathic pain, amyotrophic lateral sclerosis, multiple sclerosis, Alzheimer’s disease, Parkinson’s disease and HD. When damaged, cells release a great amount of ATP, which acts on P2X7R. Therefore, P2X7R has been proposed as a pivotal player in all these conditions and a potential common target for their treatment (35);(36);(37);(38);(39);(40);(41);(42). Besides, and regarding HD, P2X7R upregulation has been described in striatum of R6/1 and Tet/HD94 mouse models of HD (42). Moreover, cultures of primary neurons from such mice showed that P2X7R exhibits an altered permeability to calcium, suggesting a different functional state of the receptor and higher vulnerability to P2X7R-mediated apoptosis (42), thus indicating a direct link between P2X7R and HD. Remarkably, the report of lower rate of neuronal apoptosis and motor impairment recovery in HD mice following the administration of Brilliant Blue-G (BBG), a P2X7R antagonist, further suggested a contribution of P2X7R to this pathology (42), pinpointing P2X7R blockade as a possible therapeutic approach for HD. As, to date, P2X7R has not been explored in brains of HD subjects, here we aim to further validate the possible role of P2X7 in HD pathogenesis.

3 Materials and Methods

Human brain tissue samples

Striatal tissue, including caudate and putamen, from control and HD individuals, were provided by Banco de Tejidos Fundación Cien (BT-CIEN, Madrid, Spain; CTRL n=5, HD n=7), the Netherlands Brain Bank (Amsterdam, The Netherlands; CTRL n=4, HD n=5) and Banc de Teixits Neurològics (Barcelona, Spain; CTRL n=6, HD n=6). Controls and HD subjects are matched by age (CTRL=56 ± 3.8; HD=63 ±3.5) and sex (of the total, CTRL: M=70%, F=30%; in HD: M=60%, F=40%). Post-mortem interval (PMI) is lower than 24 h in all cases. HD subjects’ mean of CAG repetitions is 45.7 ±1.4. Written informed consent for brain removal after death for diagnostic and research purpose was obtained from brain donors and/or next of kin.

Antibodies

In order to explore P2X7Rs at protein level in brain samples, different antibodies have been employed:

| Host     | Brand        | Reference | Directed to | Epitope location | Epitope sequence       | Detects         |
|----------|--------------|-----------|-------------|------------------|------------------------|-----------------|
| Rabbit   | Alomone Lab  | APR-004   | C-terminal  | rat epitope 576-595 | KIRKEFPKTQGQYSGFKYPY  | P2X7R-A, P2X7R-H |

This is a provisional file, not the final typeset article
Western blot analysis

Different cohorts of samples from human brains were stored at -80°C and ground with a mortar in a frozen environment with liquid nitrogen to prevent thawing of the samples, resulting in tissue powder. Protein extracts were prepared by homogenizing tissue powder in ice-cold extraction buffer (20 mM HEPES pH 7.4, 100 mM NaCl, 20 mM NaF, 1% Triton X-100, 1 mM sodium orthovanadate, 1μM okadaic acid, 5 mM sodium pyrophosphate, 30 mM β-glycerophosphate, 5 mM EDTA, protease inhibitors (Complete, Roche, Cat. No 11697498001). Homogenates were centrifuged at 15000 rpm for 15 min at 4°C. The resulting supernatant was collected, and protein content determined by Quick Start Bradford Protein Assay (Bio-Rad, 500-0203). 10 μg of total protein were electrophoresed on 10% SDS-polyacrylamide gel, transferred to a nitrocellulose blotting membrane (Amersham Protran 0.45 μm, GE Healthcare Life Sciences, 10600002) and blocked in TBS-T (150 mM NaCl, 20 mM Tris–HCl, pH 7.5, 0.1% Tween 20) supplemented with 5% non-fat dry milk. Membranes were incubated overnight at 4°C with different anti-P2X7R antibodies directed to: C-terminus (rabbit, 1:1.000, Alomone Lab, APR-004), N-terminus (goat, 1:500, NovusBio, NBP1-37775) or extracellular domain (rabbit, 1:200, Alomone Lab, APR-008) in TBS-T supplemented with 5% non-fat dry milk, washed with TBS-T and next incubated with HRP-conjugated anti-rabbit IgG (1:2.000, DAKO, P0448) or anti-goat IgG (1:5.000, Bethyl, A50-101P) and developed using the ECL detection kit (PerkinElmer, NEL105001EA). As loading control, β-actin (1:50.000, Sigma, A2066), α-tubulin (1:20.000, Sigma, T9026) and vinculin (1:20.000, Abcam, ab129002) were used. Densitometric analysis was carried out by using a densitometer (BioRad GS800). Quantification was performed by using Image Lab 5.2 software (BioRad). In all cases, the average intensity value of the pixels in a background-selected region was calculated and was subtracted from each pixel in the samples. The densitometry values obtained in the linear range of detection with the antibody was normalized with respect to loading controls to correct for any deviation in loaded amounts of protein.

Immunohistochemistry

Formalin-fixed (4%, 24 h), paraffin-embedded tissue from striatum were used (CTRL n=3, HD n=3). Sections (5-μm thick) were mounted on superfrost-plus tissue slides (Menzel-Gläser) and deparaffinized. Brain sections were immersed in 0.3% H2O2 in methanol for 45 min to quench endogenous peroxidase activity. Sections were then immersed for 1 h in blocking solution (PBS containing 0.5% Fetal Bovine Serum, 0.3% Triton X-100 and 1% BSA) and incubated overnight at 4 °C with C-terminal directed anti-P2X7R (rabbit, 1:1.000, Alomone Lab, APR-004) or N-terminal directed anti-P2X7R (goat, 1:500, NovusBio, NBP1-37775), diluted in blocking solution. After washing, brain sections were incubated first with biotinylated anti-rabbit or anti-goat secondary
antibody and then with avidin–biotin complex using the Elite Vectastain kit (Vector Laboratories, PK-6101 and PK-6105). Chromogen reactions were performed with diaminobenzidine (SIGMAFAST DAB, Sigma, D4293) for 10 min. Sections where first dehydrated and then mounted with DePeX (SERVA). Images were captured using an Olympus BX41 microscope with an Olympus camera DP-70 (Olympus Denmark A/S).

**RNA extraction and cDNA synthesis**

Total tissue RNA was extracted from striatum of CTRL and HD patients using the Maxwell® 16 LEV simplyRNA Tissue Kit (Promega, AS1280). Quantification and quality of RNA was done on a Nanodrop ND-1000 spectrophotometer and Nanodrop 1000 v.3.7.1 (Thermo Scientific). Retrotranscription (RT) reactions were performed using the iScript cDNA Synthesis kit (Bio-Rad, PN170-8891) following manufacturer’s instructions. Briefly, 1μg of total RNA from each sample and 4x master mix (which includes all necessary reagents, a mixture of random primers and oligo-dT for priming) were brought to a final volume of 40 μl with DNAse/RNase free distilled water (Gibco, PN 10977). Thermal conditions consisted on: 5 min at 25°C; 30 min at 46°C and 5 min at 95°C.

**P2X7R quantitative and semi-quantitative PCR**

Quantitative RT-PCR was performed using gene-specific primers and TaqMan MGB probes for human P2X7R (Forward, 5’-GTGAACCAGCAGCTACTAGGGAG-3’; Reverse, 5’-TGAAGTCCATCGCAGGTCTTG-3’), β-actin and GAPDH. Fast thermal cycling was performed using a StepOnePlus Real-Time PCR System (Applied Biosystems) as follows: denaturation, one cycle of 95°C for 20 s, followed by 40 cycles each of 95°C for 1s and 60°C for 20s. The results were normalized as indicated by parallel amplification of the endogenous controls β-actin and GAPDH. Semi-quantitative RT-PCR was performed designing specific primers in P2X7R exon 10 and exon 11 (Forward, 5’-CATCGGCTCAACCCTCTCCTA-3’; Reverse, 5’-TTTGGCTCCACAATGGACTCG-3’) to amplify the intron 10-11 retaining isoforms in human brain cDNA. PCR amplification protocol used: 5 min 94°C + 30 cycles (30s at 94°C + 30s at 58°C + 2 min at 72°C) + 7 min at 72°C. PCR product was resolved on 1.5% agarose/gelgreen (Biotium, 41004) gels run at 120V for 1.5h. Images were taken in an UviDoc transilluminator (UviTec) and then scanned with densitometer (Bio-Rad, GS-900) and quantified with Image Lab 5.2 (Bio-Rad).

**P2X7R transcript level and splicing alteration in HD by RNA-seq**

P2X7R expression levels in HD and CTRL post-mortem striatum samples was evaluated from our RNA-seq data (CTRL=3 vs HD=3) (43). For total mRNA transcript levels, reads were aligned against *H. sapiens* genome (GRCh38.p2 version) using TopHat2 aligner (44) and differentially expressed genes were obtained with Cuffdiff software (45). For the splicing analysis, percent spliced in (PSI) values were obtained running
Vast-tools (Vertebrate Alternative Splicing and Transcription Tools) (46). Alternatively, Tophat aligned reads were run with rMATS software for the detection of altered spliced events (47).

Statistics

All experiments were repeated at least three times, and the results are presented as the mean ± sem. Statistical analysis was performed with SPSS 21.0 (SPSS Statistic IBM). Where indicated, Student’s t test was applied and FDR was calculated in the case of RNA-seq data.

4 Results

4.1 P2X7R protein level is increased in striatum of HD patients

To further explore the potential role of P2X7R in HD pathogenesis, we first analyzed by Western blot the protein level of this receptor in the striatum of HD subjects, the most affected brain region in patients. As previously mentioned, human P2X7R mRNA can undergo differential splicing, thus originating multiple P2X7R variants. Of these, four are protein-encoding and generate the P2X7R-A, P2X7R-B, P2X7R-H and P2X7R-J isoforms, with expected molecular weights of 68.6, 41.8, 58.2 and 29.3 kDa, respectively. In order to detect P2X7R-A and P2X7R-H, which are provided of the canonical C-terminus, we used a C-terminus directed antibody. With this antibody we observed a unique band of about 70kDa, most likely corresponding to P2X7R-A, which was increased (2.8-fold, p=0.03) in brain striatal extracts of HD patients (Figure 1A). This P2X7R upregulation in HD specimens is in accordance with the one previously obtained in the same brain region in two mouse models of HD (42). P2X7R-H, which indeed presents the same C-terminus sequence of P2X7R-A, has not been detected with this antibody, even after a prolonged exposure, neither in normal nor in pathological conditions. Regarding the C-terminus truncated isoforms, P2X7R-B and P2X7R-J, we opted for a N-terminus directed antibody, which should also recognize the full-length form. First, after short exposure, we observed a doublet at 42-45 kDa, possibly corresponding to P2X7R-B. Such doublet results strongly increased (3.3-fold, p=0.01) in the striatum of HD patients (Figure 1B). Second, following long exposure, a band can be observed in the range of 30-38 kDa, possibly corresponding to P2X7R-J (Supplementary Figure S1). In HD, such band shows a non-statistically significant increase due to the great dispersion of data within the HD group (p=0.2). This antibody also detects a doublet at approximately 60 kDa. Such molecular weight could correspond to a homodimerization of P2X7R-J, however, no change occurs between control and HD specimens (p=0.5). Finally, a shade at approximately 70 kDa, possibly corresponding to P2X7R-A can be detected. It seems, therefore, that this antibody is somehow less efficient than the C-terminus directed one in detecting the full-length protein (Supplementary Figure S1). Since the extracellular loop represents a shared feature of all P2X7Rs isoforms, an extracellular loop-directed antibody has been used for the simultaneous detection of P2X7R-A and P2X7R-B (31);(34). Unfortunately, this antibody was not sensitive enough to
detect P2X7R in human postmortem brain samples (data not shown). Taken together, these results indicate that P2X7R is upregulated at protein level in HD, with an augmented level of both full-length (P2X7R-A) and C-terminus lacking (P2X7R-B) forms of the receptor.

4.2 P2X7R immunoreactivity is augmented in HD brains

We further investigated P2X7R status in HD brains through immunohistochemistry, with the same two antibodies that were used for the Western blot analysis. Detection of P2X7R immunoreactivity with the C-term antibody in striatum of control subjects shows a weak punctate and cytoplasmic pattern mainly in neurons and neuropil; in striatal sections from HD patients we observe a more diffuse and intense reactivity, which in neurons appears to extend to their processes and a higher number of clearly immunoreactive cells was seen (Figure 2A). Since no P2X7R-H associated band has been detected by Western blot in this brain region, we can speculate that the signal detected here essentially corresponds to P2X7R-A.

Regarding the N-term antibody, the pattern in striatum of controls was also cytoplasmic and punctate mainly in neurons, but neuronal processes also get clearly stained. This pattern seems to be maintained in HD striatal sections, but with more abundant immunoreactive somata (Figure 2B). Since by Western blot this antibody identified mainly P2X7-B, while other bands emerged only after a prolonged exposure, we can speculate that such signal could essentially be related to P2X7R-B. This result, together with the increased protein level detected by Western blot, highlight an alteration of P2X7R-A and P2X7R-B in HD brains.

4.3 P2X7R transcript alteration in HD brains

Since P2X7Rs protein levels result augmented in HD striatum, we investigated whether this increase is associated with a higher amount of total P2X7R transcript in this brain area. To achieve such aim, we analyzed an RNA-seq study of the striatum of HD patients and controls that we have recently performed (43). We found a strong increase in total P2X7R transcript level (2.4-fold, q=0.005) in HD subjects (Figure 3A). We further analyzed P2X7R mRNA level in striatum by quantitative-PCR (Q-PCR) in an independent cohort of patients.

Coherently, we detected a tendency to a higher amount of P2X7R transcript (2.0-fold, p=0.07) in this region when comparing HD subjects with controls (Figure 3B). Taken together, these results suggest that the striatal increase in P2X7R protein levels detected in HD is likely associated with augmented P2X7R total transcript levels in the same brain region. This is consistent with the previously reported trend to an increase in P2X7R mRNA in the brain of R6/1 and Tet/HD94 mouse models of HD (42).

On one hand, previous data have shown the existence of naturally-occurring isoforms of P2X7R generated by differential splicing, which could play a role in physiological and/or pathological conditions (23);(29); (25). On the other hand, splicing alterations have been reported to contribute to HD pathogenesis (6); (7). Thus, we wondered whether P2X7R suffers splicing alterations in HD, as well. We therefore analyzed the RNA-seq
study performed in striatum with two different bioinformatics tools. By using Vast-tools, we found that the inclusion level of the 84 nucleotide-long intronic region located between exon 10 and exon 11 is increased by 39% in HD striatum (p=0.007, Figure 4A). We also found that exon 4 inclusion was decreased about 12% in patients (p=0.01, Supplementary Figure S2A). When we performed the equivalent analysis by using rMATS, we confirmed that intron 10-11 retention is increased by 41% (FDR=0.002) (Figure 4B) and that exon 4 inclusion is about 8% lower (FDR=0.04) in HD patients (Supplementary Figure S2B).

While no function has been annotated regarding exon 4, there is literature about intron 10-11 retention. As mentioned, it is involved in the production of P2X7R-B protein (23), which has been described to play a role in vitro (29). Therefore, we validated in an independent set of samples the increased retention of intron 10-11 by semi-quantitative RT-PCR with primers hybridizing in the flanking exons (10 and 11). This confirmed that, indeed, intron 10-11 is more retained in HD post-mortem brains, since the proportion of transcripts including this region respect to the non-intron containing ones is more abundant in our HD sample set (3.5-fold, p=0.008) (Figure 4C). Taken together, these results demonstrate that, in striatum of HD subjects, P2X7R undergoes mis-splicing with slightly decreased inclusion of exon 4 and markedly increased retention of intron 10-11, the latter explaining the increase in the P2X7R-B isoform observed by Western blot.

**Discussion**

Here we perform, to our knowledge, the first analysis of P2X7R status in brains of HD subjects. By analyzing P2X7R proteins through Western blot and immunohistochemistry and P2X7R transcript isoforms through RNA-seq and subsequent validation by RT-PCR, here we report a clear increase in the total levels of P2X7R. Both the full length isoform P2X7R-A and the lower molecular weight isoform P2X7R-B show increased levels that can be explained by the changes observed at the transcript level, the latter affecting both the total transcript levels as well as the increased retention of intron 10-11 which originates the P2X7R-B isoform.

To date, no disease-modifying treatment is available for HD subjects, who only receive palliative care. Although a number of clinical trials have been completed during the past years, no compelling results emerged. Thus, the research of new therapeutic approaches, possibly acting on the multiple features of the disease, remains open. Previous data demonstrated that P2X7R participates in the modulation of neurotransmitter release (48) and also in microglial (49) and astroglial activation (50), thus highlighting a role of this receptor in neuroinflammation. Since neuroinflammation is a shared feature of many CNS diseases, including HD, P2X7R has been proposed to be a potential therapeutic target for their treatment. More compelling evidence of P2X7R as a likely therapeutic target specifically for HD arose from experiments performed in various HD cellular and mouse models, where the receptor was found to be upregulated and functionally altered (42), thus suggesting a role of P2X7R in the pathophysiology of HD. Interestingly, antagonizing P2X7R through the administration of BBG provokes less body weight loss and improves motor
Those results strongly supported the hypothesis of P2X7R as a target for the treatment of HD. However, evidence of P2X7R being in fact altered in the brains of HD subjects was missing. In order to address the status or P2X7R in HD brains here we focused on striatum, the most affected brain region in the disease.

In the present study, we investigated the protein level in control and HD striatal samples first by Western blot with an antibody raised against a C-terminal epitope. This antibody is expected to recognize the full length P2X7R-A isoform. In good agreement with the expected weight of 68.6 kDa, we detected a band at around 70 kDa, thus fitting P2X7R-A. Interestingly, experiments performed on a cervix cancer cell line with the same antibody showed that P2X7R-A can reach the weight of 85 kDa (27) due to the N-glycosylation at five different sites (Asn-187, -202, -213, -241, and -284) (11). Since a unique band was identified in our study, it seems that the N-glycosylated P2X7R is not detectable in striatal samples. Moreover, although the antibody could potentially detect P2X7R-H as well, we did not observe any signal at its expected molecular weight. We therefore believe that the P2X7R-H isoform is not expressed or it is expressed at a very low amount in human striatum. In summary, the Western blot experiments with the C-terminal antibody allowed us to conclude that P2X7R-A is expressed in human striatum and is upregulated in HD patients (Figure 1A). This result is in accordance with the previous one obtained in R6/1 mice in the same brain region (42). We have also explored at protein level the P2X7R isoforms lacking the C-terminus, which are generated following differential splicing: P2X7R-B and P2X7R-J (23). We wondered whether these variants were expressed in the striatum and whether changes occur in the pathological context of HD. To address these questions, we used an antibody that binds an N-terminal sequence. We observed a doublet at 42/45 kDa possibly corresponding to P2X7R-B which is upregulated in HD (Figure 1B). Regarding P2X7R-J, we observed a band between 30 and 38 kDa, which could correspond to such form, since the predicted molecular weight based on its sequence (UniProtKB - Q15G98) is 29.3 kDa. There is a non-significant tendency to an increase in HD regarding such band (p=0.2) (Supplementary Figure S1). It must be considered that P2X7R-J has been discovered in cervical cancer cells (25). There, the group describes a 42/45 kDa doublet as P2X7R-J, as this isoform maintains four out of the five N-glycosilation sites of the full length form, that, after post-translational modification, would increase its weight till 41.8 kDa. Since we did not obtain the 85 kDa band equivalent to the N-glycosylated form of P2X7R-A in our samples, we believe that the forms we are observing are the unglycosylated ones. In such condition and taking into consideration that the molecular weights of 42/45 kDa are closer to the one P2X7R-B is expected to show, 41.8 kDa, we consider that such doublet could correspond to P2X7R-B rather than to P2X7R-J.

The immunohistochemical analysis of P2X7R in striatal sections shows increased staining mainly in neurons, opposite to Alzheimer’s disease tissue and mouse models which show increased P2X7R staing in glial cells (51), possibly indicating a major role of increased P2X7R in altered neurotransmission rather that to
neuroinflammation in HD. The immunohistochemical analysis has been carried out exploiting the same antibodies used for the Western blot analysis. Since the C-terminus directed antibody substantially recognizes P2X7R-A while the N-terminus directed one recognizes mostly the P2X7R-B associated bands, we can assume that these are the proteins accountable for the observed immunoreactivities. However, we cannot exclude that the signal observed in this brain sections could be associated with other P2X7Rs not clearly detectable by Western blot. Interestingly, the fact that the N-terminal antibody stains neuronal processes that are not detected with the C-terminus directed antibody in control tissue suggests that P2X7R A and B isoforms may have different spatial distributions in physiological conditions. Regardless of this, the increased number of somata with clear immunostaining detected with both antibodies is in accordance with the increased protein levels detected by Western blot in HD. To date, we do not know the meaning of the increased protein level of both P2X7R-A and P2X7R-B in the neurons of HD patients. In this regard, it has been reported that P2X7R-A and P2X7R-B play a role in undifferentiated and neural-differentiated embryonic stem cells (ESC) (32). The authors there demonstrated that while before differentiation both isoforms are well expressed by ESC, under neural differentiation the ratio between P2X7R-A and P2X7R-B favors the full length isoform (32). They speculate that P2X7R-B good expression in the undifferentiated condition ease cell proliferation and differentiation, avoiding cellular death (32). Indeed, P2X7R-B trophic activity had been previously described in HEK293 cells as well as in a human osteosarcoma cell line (29);(30). However, neurons in stratum are post-mitotic cells, which do not enter cell cycle, neither differentiate. On one hand, it is possible to speculate that P2X7R-A and P2X7R-B increase in HD could represent a consequence of the inflammatory mechanisms related to HD. On the other hand, it is possible that such increase, and especially of P2X7R-B, could be an adaptive mechanism to favor neuronal survival in HD. Further experiments are required to establish P2X7Rs associated pathophysiological mechanisms in neurons.

At the mRNA level, we observed increased total P2X7R mRNA levels in HD striatum, which in turn may explain the increase in total protein levels, but we do not know the precise underlying mechanisms. These might take place at the transcriptional level, as there are multiple transcription factors altered in HD and global transcriptomic alteration is well documented (52). Another possibility is that it is due to a post-transcriptional mechanism, for instance involving micro-RNAs, given their ability to control stability and translation of their target transcripts. In this regard, it is known that P2X7R transcript can be regulated by microRNA-22 (mir-22) (53). More precisely, experiments performed in a mouse model of status epilepticus demonstrated that P2X7R transcript is a target of mir-22, which normally inhibits its translation (53). On the other hand, it has been reported that many microRNAs are downregulated in HD mouse models, including mir-22 (54). Indeed, the overexpression of miR-22 inhibited neurodegeneration in rat primary striatal cultures exposed to a mutated human huntingtin fragment (Htt171-82Q) (55). Thus, P2X7R increase in HD could be, at least in part, explained via mir-22 diminution in this pathology, but further investigation regarding the status of mir-22 in samples from HD patients would be necessary to ascertain this. Finally, and specifically regarding the increase
in P2X7RB isoform at both mRNA and protein level, it can be explained by the here reported increased
retention of intron 10-11 in P2X7R transcript in HD. The likely underlying mechanism could be the already
reported alteration of many splicing factors in HD brain tissue and cell and mouse models such as MBLN1(4)
or SRSF6 (7);(56). Thus we here provide for the first time a connection between HD dysregulated splicing and
P2X7R-mediated mechanisms in HD. Nevertheless, further research is needed to identify the specific splicing
factors binding near or at P2X7R intron 10-11.

In conclusion, here we report an upregulation of P2X7R in HD brains that, together with the analogous
increase in HD mouse models and the preclinical studies -also in the mouse models- showing efficacy of
P2X7R manipulation, further provide evidence of a role of P2X7R in this pathology and strengthen its
potential as a drug target.

5 Author Contributions

J.J.L. directed the study and designed experiments. A.E. and I.O. performed the RNA-sequencing analysis.
I.O. performed q-RT–PCR and semi-q-RT-PCR analysis. M.S.G. carried out Western blotting and the
immunostaining. I.O. analyzed data and wrote the draft of the manuscript. J.J.L. performed the manuscript
revision, read and approved the submitted version.

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