Phosphoproteomic dysregulation in Huntington’s disease mice is rescued by environmental enrichment

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Huntington’s disease is a fatal autosomal-dominant neurodegenerative disorder, characterized by neuronal cell dysfunction and loss, primarily in the striatum, cortex and hippocampus, causing motor, cognitive and psychiatric impairments. Unfortunately, no treatments are yet available to modify the progression of the disease. Recent evidence from Huntington’s disease mouse models suggests that protein phosphorylation (catalysed by kinases and hydrolysed by phosphatases) might be dysregulated, making this major post-translational modification a potential area of interest to find novel therapeutic targets. Furthermore, environmental enrichment, used to model an active lifestyle in preclinical models, has been shown to alleviate Huntington’s disease-related motor and cognitive symptoms. However, the molecular mechanisms leading to these therapeutic effects are still largely unknown. In this study, we applied a phosphoproteomics approach combined with proteomic analyses on brain samples from pre-motor symptomatic R6/1 Huntington’s disease male mice and their wild-type littermates, after being housed either in environmental enrichment conditions, or in standard housing conditions from 4 to 8 weeks of age (n = 6 per group). We hypothesized that protein phosphorylation dysregulations occur prior to motor onset in this mouse model, in two highly affected brain regions, the striatum and hippocampus. Furthermore, we hypothesized that these phosphoproteome alterations are rescued by environmental enrichment. When comparing 8-week-old Huntington’s disease mice and wild-type mice in standard housing conditions, our analysis revealed 229 differentially phosphorylated peptides in the striatum, compared with only 15 differentially phosphorylated peptides in the hippocampus (statistical thresholds fold discovery rate 0.05, fold change 1.5). At the same disease stage, minor differences were found in protein levels, with 24 and 22 proteins dysregulated in the striatum and hippocampus, respectively. Notably, we found no differences in striatal protein phosphorylation and protein expression when comparing Huntington’s disease mice and their wild-type littermates in environmentally enriched conditions. In the hippocampus, only four peptides were differentially phosphorylated between the two genotypes under environmentally enriched conditions, and 22 proteins were differentially expressed. Together, our data indicates that protein phosphorylation dysregulations occur in the striatum of Huntington’s disease mice, prior to motor symptoms, and that the kinases and phosphatases leading to these changes in protein phosphorylation might be viable drug targets to consider for this disorder. Furthermore, we show that an early environmental intervention was able to rescue the changes observed in protein expression and phosphorylation in the striatum of Huntington’s disease mice and might underlie the beneficial effects of environmental enrichment, thus identifying novel therapeutic targets.
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**Abbreviations:**  
CAG = cytosine-adenine-guanine; EE = environmental enrichment; FC = fold change; FDR = false discovery rate; HD = Huntington’s disease; IPA = ingenuity pathway analysis; LC-MS/MS = liquid chromatography with tandem mass spectrometry; MSNs = medium spiny neurons; SH = standard housing; WT = wild-type

### Introduction

Huntington’s disease is a fatal autosomal-dominant neurodegenerative disorder caused by the expansion of a cytosine-adenine-guanine (CAG) repeat in exon 1 of the huntingtin gene. The tandem-repeat mutation leads to the formation of a mutant huntingtin protein, which is cleaved, misfolded and forms aggregates in all cells of the body. Although the
huntingtin protein is ubiquitously expressed, the disease is characterized by an exacerbated degeneration of the medium spiny neurons (MSNs) in the striatum, leading to a movement disorder often referred to as ‘Huntington’s chorea’. Other brain regions are also affected in Huntington’s disease, primarily the cortex and hippocampus, and dysfunction of associated neural circuits may lead to cognitive and psychiatric impairments. Unfortunately, there is currently no treatment available that can alter the course of the disease, highlighting the need to identify new therapeutic targets.

Transgenic R6/1 mice have been widely used to model Huntington’s disease in preclinical research, recapitulating the disease symptoms, including motor, cognitive and affective impairments. We have recently reported a protein phosphorylation dysregulation in the cortex of R6/1 Huntington’s disease male mice in standard-housing conditions, occurring primarily prior to motor-symptom onset. Our findings were supported by previous published evidence that protein phosphorylation is dysregulated in other Huntington’s disease mouse models. Protein phosphorylation, catalysed by kinases and hydrolysed by phosphatases, plays a crucial role in cellular signalling, making this molecular process a promising target for the development of new Huntington’s disease treatments. Here, we investigated whether protein phosphorylation dysregulation also occurs in other brain regions affected in Huntington’s disease (i.e. the striatum and hippocampus) and whether this can be modulated by environmental factors, via experimental manipulations of the housing conditions.

Indeed, previous studies have shown that the phenotype of Huntington’s disease mice can be ameliorated in enriched environment conditions. More specifically, EE [when compared with standard housing (SH) conditions] has been shown to delay the onset of striatal-dependent motor impairments and the onset of hippocampal-dependent long-term memory and spatial learning deficits. But despite these successful interventions, there is still no clear molecular mechanism of action.

In the present study, we therefore hypothesized that protein phosphorylation dysregulations occur in the striatum and hippocampus of pre-symptomatic Huntington’s disease mice compared with their wild-type (WT) littermates. Moreover, we hypothesized that the beneficial effects of EE drive changes in protein phosphorylation in these key brain regions. We aimed to use the acquired results to identify new drug targets for this devastating disorder.

A proteome analysis was conducted, to verify that the changes in phosphorylation observed were not due to protein expression changes. Here, we report the first proteomics study on R6/1 Huntington’s disease mice, identifying novel proteins differentially expressed (DE) prior to motor-symptom onset in this mouse model of Huntington’s disease.

Materials and methods

Animals and tissue collection

R6/1 (Huntington’s disease) transgenic hemizygous males were originally obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and bred with CBB6 (CBA–C57/B6) F1 females to establish the R6/1 colony at the Florey Institute of Neuroscience and Mental Health. After weaning, Huntington’s disease male mice and their WT littermates were group housed (four mice per cage) with two of each genotype.

As previously described, SH cages were 31 × 10 × 16 cm in size, with a layer of sawdust and tissues as nesting material. The environmental enrichment (EE) cages were larger (38 × 28 × 15 cm) and contained more nesting material, shredded paper, a running wheel, and a set of different objects (ladder, shelter, tunnel and random objects). The objects were changed once a week. Mice were randomly assigned to one of these environments from 4 weeks of age to 8 weeks of age (n = 6 per group, 24 animals in total) (Fig. 1). Mice were maintained in a 12 h light/dark cycle with access to food and water ad libitum. Mice were culled at 8 weeks of age via cervical dislocation for tissue dissection. Striatum and hippocampus samples were snap-frozen in dry ice before being stored at −80°C. Sample preparation time was minimized to maintain phosphorylation. All experiments were approved and performed in accordance with the guidelines of the Florey Institute Animal Ethics Committee and the National Health and Medical Research Council.

Sample preparation and phosphopeptide enrichment

Striatum and hippocampus samples from 8-week-old male mice (n = 6 per group) were used for protein extraction with a RIPA buffer containing protease and phosphatase inhibitors (PhosSTOP, Roche). Protein concentration in each sample was determined from a BCA assay. We precipitated 500µg of proteins overnight at −20°C, using 5x volume ice cold acetone. The following day, the pellet was solubilized in 8 M Urea in 50 mM TEAB and incubated for 30 min at 37°C. The samples were then treated with 10 mM TCEP and 55 mM IAA. Thereafter, the samples were diluted to 1 M urea with 25 mM TEAB and digested overnight at 37°C with Trypsin/LysC (1:50 protein:enzyme). The next day, the samples were acidified to 1% (v/v) formic acid. We used Oasis HLB 60 mg cartridges for solid-phase extraction. The cartridges were first washed with 80% ACN containing 0.1% TFA. The proteins were eluted with 80% ACN containing 0.1% TFA. A final BCA assay was performed to allow equal loading across all samples. Twenty milligrams of protein from each sample were kept for the proteomics experiment and resuspended in a buffer containing 0.5% ACN, 0.05% TFA. The remaining samples were then freeze-dried until phospho-enrichment. All samples were prepared in two separate batches, with samples from the four experimental groups present in each batch, and were randomized before LC-MS/MS analysis.

For the phosphopeptide enrichment, TiO beads (6:1 TiO:peptides) were washed with 50% ACN, 5% TFA (washing
buffer) and incubated for 10 min with 2 M lactic acid in 5% TFA, 50% ACN (loading buffer). The TiO beads in loading buffer were added to the peptides samples and incubated for 1 h. Subsequently, the phosphopeptides were washed and eluted with 1% ammonia followed by elution with 30% ACN. The samples were then acidified with 1 ul of formic acid per 10 ul eluent and freeze-dried. The phosphopeptides were resuspended in a buffer containing 2% ACN, 0.05% TFA just before LC-MS/MS analysis.

**Mass spectrometry analyses**

Samples were analysed by nanoESI-LC-MS/MS using an Orbitrap Exploris 480 mass spectrometer (Thermo Scientific) equipped with a nanoflow reversed-phase-HPLC (Ultimate 3000 RSLC, Dionex). The LC system was equipped with an Acclaim Pepmap nano-trap column (Dinoex-C18, 100 Å, 75 µm x 2 cm) and an Acclaim Pepmap RSLC analytical column (Dinoex-C18, 100 Å, 75 µm x 50 cm). The tryptic peptides were injected (concentration of 1µg on column, for the proteomics and phosphoproteomics) to the enrichment column at an isocratic flow of 5 µL/min of 2% v/v CH₃CN containing 0.1% v/v formic acid for 5 min applied before the enrichment column was switched in-line with the analytical column. The eluents were 5% DMSO in 0.1% v/v formic acid (solvent A) and 5% DMSO in 100% v/v CH₃CN and 0.1% v/v formic acid (solvent B). The flow gradient was (i) 0–6 min at 3% B, (ii) 6–95 min, 3–22% B, (iii) 95–105 min 22–40% B, (iv) 105–110 min, 40–80% B, (v) 110–115 min, 80–80% B, (vi) 115–117 min, 80–3% and equilibrated at 3% B for 10 min before the next sample injection. All spectra were acquired in positive ionization mode with full scan MS acquired from m/z 300–1600 in the FT mode at a mass resolving power of 120,000, after accumulating to an AGC target value of 3.0E6, with a maximum accumulation time of 25 ms. The RunStart EASY-IC lock internal lockmass was used. Data-dependent HCD MS/MS of charge states > 1 was performed using a 3 s scan method, at a normalized AGC.
target of 100%, automatic injection, a normalized collision energy of 30%, and spectra acquired at a resolving power of 15,000. Dynamic exclusion was used for 20 s.

Raw files were processed using the MaxQuant proteomics software package (version 2.0.1.0) with the Andromeda search engine for protein and peptide identification. The results were searched against a *Mus Musculus* database (SwissProt, Taxonomy ID 10090, downloaded April 2021) and using the default search parameters. Trypsin was selected as the cleavage enzyme, cysteine carboxamidomethyl was selected as fixed modification and methionine oxidation, serine, threonine and tyrosine phosphorylation as variable modifications. The match between run option was selected. Protein and peptides groups were set to a maximum false discovery rate (FDR) of <0.01. Each raw file was considered as one experiment.

**Bioinformatics and statistical analyses**

**Phosphoproteomics analysis**

The processed data was analysed with Perseus (version 1.6.14.0). First, we removed contaminants and reverse peptides from the matrix. Peptides with a phosphate localization probability higher than 0.75 were kept for further analysis. After expanding the site table, phosphopeptides intensities were log2 transformed and samples were annotated with their condition (i.e. WT_SH, WT_EE, HD_SH and HD_EE). Phosphoproteome characterization is available in Supplementary Figure 1. Phosphopeptides with valid values in 100% of the samples in at least one group were kept for statistical analysis. Following this, we normalized the intensities of the phosphopeptides in each sample by subtracting the median and missing values from normal distribution (0.3 width, 1.8 down shift). We then verified that the imputed missing values necessary for the statistical analysis were indeed low in samples and did not skew the data in anyway. In addition, we also defined the list of proteins that are present or absent in all four replicates (Supplementary Tables 1 and 2).

For the striatum phosphoproteomics dataset, samples 32 (HD_EE, low detection) and 69 (WT_SH, outlier) were discarded. For the hippocampus phosphoproteomics, sample 68 was discarded from the analysis (HD_SH, low detection). In the striatum, 4228 phosphopeptides were detected with enough valid values (100% of valid values in at least one group) to allow differential expression analysis. In hippocampus samples, 4369 phosphopeptides were retained for differential expression analysis.

**Phosphoproteomics statistical analysis**

The reproducibility of the experiment was assessed with Pearson’s correlation between biological replicates in all groups. The mean Pearson’s correlation coefficient for striatum samples \( r \) was 0.9407 for WT_SH \( (n = 5) \), 0.9272 for HD_SH \( (n = 6) \), 0.9332 for WT_EE \( (n = 6) \) and 0.9107 for HD_EE \( (n = 5) \). For hippocampus samples, we report a mean Pearson’s correlation of 0.948 for WT_SH \( (n = 6) \), 0.929 for HD_SH \( (n = 5) \), 0.951 for WT_EE \( (n = 6) \) and 0.927 for HD_EE \( (n = 6) \).

Significant changes in the phosphoproteome between genotypes or housing conditions were defined by Student’s \( t \)-tests, truncated by permutation-based FDR significance threshold of 0.05 with 250 randomizations, and 1.5 absolute fold change (FC).

**Proteomics and statistical analysis**

The processed data was analysed with Perseus (version 1.6.14.0). First, we removed contaminants, reverse, and only identified by site peptides from the matrix. The data was then log2 transformed and filtered by valid values, with a minimum of four valid values in each group. The intensities of peptides in each sample were normalized by subtracting the median and missing values were imputed from normal distribution (0.3 width, 1.8 down shift). We then verified that the imputed missing values necessary for the statistical analysis were indeed low in samples and did not skew the data in anyway. In addition, we also defined a list of proteins that are present or absent in all four replicates (Supplementary Tables 3 and 4).

The reproducibility of the experiment was assessed with Pearson’s correlation \( r \), which was high between samples within each group in the striatum (WT_SH: 0.9763, WT_EE: 0.9759, HD_SH: 0.9779, HD_EE: 0.9783, \( n = 6 \) per group) and hippocampus (WT_SH: 0.9933, WT_EE: 0.9935, HD_SH: 0.9938, HD_EE: 0.9924, \( n = 6 \) per group). In the striatum, 1769 proteins had at least four valid measurements in each group and were retained for statistical analysis. In the hippocampus samples, 2597 proteins were retained for further analysis. Significant changes in the proteome between genotypes or housing conditions were defined by Student’s \( t \)-tests, truncated by permutation-based FDR significance threshold of 0.05% with 250 randomizations, and 1.5 absolute FC.

**Gene ontology and pathway analysis**

The DE and phosphorylated proteins were entered into Panther (Pantherdb.org) and IPA (Qiagen) to retrieve protein class, canonical pathways and upstream regulators.

**Results**

**Extensive dysregulation of the phosphoproteome in the striatum of Huntington’s disease mice in standard housing conditions**

When comparing Huntington’s disease and WT mice in SH conditions, we reported 229 DE phosphopeptides in the striatum (Fig. 2A). Of these, 101 phosphopeptides (83 phosphoproteins) were upregulated, and 128 (98 phosphoproteins) were downregulated in Huntington’s disease compared...
with WT mice. In proteomics, due to the stochastic nature of sampling by the mass spectrometer and/or detection limits, non-identification does not necessarily mean complete absence of a particular protein. However, a phosphopeptide being present/absent in all four replicates could suggest that phosphopeptide is indeed significant. These phosphopeptides are presented in Supplementary Table 1. We found that nine of the proteins whose phosphorylation was downregulated in Huntington’s disease compared with WT mice were also downregulated at the protein level (Supplementary Figure 2). For this reason, these nine phosphoproteins were excluded from the dataset for Panther and IPA analyses.

The Panther analysis revealed that the most prevalent protein classes for the differentially phosphorylated proteins between Huntington’s disease and WT mice in SH included transporters (15 proteins) and protein-modifying enzymes (15 proteins). The transporter class included multiple subunits of voltage-gated ion channels, including ones permeable for calcium (CACNA1C, E; CACNB2,4; CACNG2,4, CBARP), sodium (SCN2A) and potassium (KCND2 and KCNQ2). Several non-receptor serine/threonine protein kinases were also found differentially phosphorylated in the striatum of Huntington’s disease mice (Table 1). We found a high number of differentially phosphorylated cytoskeletal and scaffold proteins (13 proteins each). The cytoskeletal

![Figure 2](image-url)
proteins mainly involved actin and actin-binding proteins (SYNPO, ACTL6A, AFDN and ADD1,2,3). For some phosphosites, including the top 10 upregulated and downregulated (Table 2), as well as the ones mapping to kinases (Table 1), we mention if their upstream regulation and downstream functions are known (from phosphosite.org database).

Pathway analysis from IPA revealed that the Opioid Signalling Pathway (P-value 7.94E-14) was the most significant pathway for the changes occurring in the striatal phosphoproteome of Huntington’s disease mice prior to motor symptoms (Fig. 3). The analysis reported a negative Z-score (−1.213) for the Opioid Signalling Pathway, indicating an inactivation of this pathway in the striatum of Huntington’s disease mice, likely due to changes in the phosphorylation profiles of the proteins involved (ADCY5, ARRB1, CACN A1C; E; B1; B2; B4; G2; G4, Calm1, CAMK2A, GNAO1, GNG12, GRIN2A, ITPR1, PRKCB, PRKCE, RAF1, RASD2, RGS8 and RGS9, RPS6KA5, TH).

We also investigated which kinases and phosphatases are potentially driving the changes in phosphorylation observed in Huntington’s disease compared with WT mice. We performed the upstream analysis through IPA, selecting kinases and phosphatases as molecule types (Fig. 4). Four kinases had a calculated Z-score, based on the phosphorylation profiles of their targets. CAMK2A was the most significant of these, with a P-value of 3.0E-4 and a Z-score of −1.982, predicting an inhibition of the enzyme in HD_SH compared with WT_SH mice (with five of its targets being less phosphorylated). CAMK2A was itself less phosphorylated in Huntington’s disease mice at Serine 234 (Table 1).

MAPK1, also known as ERK2, also appeared with a negative Z-score (−0.922), with six of its targets down-phosphorylated in Huntington’s disease mice. However, MAPK1 expression and phosphorylation were unaltered in HD_SH compared with WT_SH mice in our dataset. CDK5 and PRKCG (gamma subunit of PKC) were also significant Upstream Regulators, with a positive Z-score (0.402 and 0.447, respectively): most of their protein targets were hyperphosphorylated in HD_SH compared with WT_SH mice. Phosphorylation of PRKCG and protein expression of both CDK5 and PRKCG was found unaltered in HD_SH compared with WT_SH.

To further understand the extensive phosphorylation changes in the striatum between WT and Huntington’s disease mice in SH conditions, we also extracted phosphopeptides corresponding to kinases using the Uniprot KB database. We detected 278 phosphopeptides belonging to

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**Table 1** Differentially phosphorylated kinases in the striatum of Huntington’s disease compared with WT mice in standard housing conditions

| Protein symbol | Protein name | Phosphorylation in Huntington’s disease | Residue | PhosphositePlus |
|----------------|--------------|----------------------------------------|---------|-----------------|
| CAMK2A; CAMK2B; CAMK2D; CAMK2G | Calcium/calmodulin-dependent protein kinase type II | ↓ | S234 | — |
| PRKCE | Protein kinase C epsilon type 9 | ↓ | S337 | — |
| MAP3K9 | Mitogen-activated protein kinase kinase 9 | ↓ | S622 | — |
| SPEG | Striated muscle-specific serine/threonine-protein kinase | ↓ | S493 | — |
| SPEG | Striated muscle-specific serine/threonine-protein kinase | ↓ | S2361 | — |
| MAP3K10 | Mitogen-activated protein kinase kinase 10 | ↓ | S583 | — |
| RP56KA5 | Ribosomal protein S6 kinase alpha-S | ↓ | S862 | Phosphorylation by cAMP protein kinase A (PKA): enzymatic inhibition, impairment of calcium-calmodulin activation |
| CAMKK2 | Calcium/calmodulin-dependent protein kinase type II subunit alpha | ↓ | S495 | — |
| MIN1 | Misshapen-like kinase 1 | ↓ | S644 | Blocks calcium-calmodulin binding, dephosphorylated by PTEN |
| CAMK2A | Calcium/calmodulin-dependent protein kinase type II subunit alpha | ↓ | T337 | — |
| MARK2 | Serine/threonine-protein kinase MARK2 | ↑ | S483 | — |
| SPEG | Striated muscle-specific serine/threonine-protein kinase | ↑ | S2135 | — |
| PI4KB | Phosphatidylinositol 4-kinase beta | ↑ | S511 | Phosphorylated by PKA, induces localization of enzyme in nucleus |
| RAF1 | Proto-oncogene serine/threonine-protein kinase | ↑ | S621 | Autophosphorylation at Ser621 necessary to avoid proteasome degradation |
| BAZ1B | Tyrosine-protein kinase | ↑ | S1464 | — |
| BAZ1B | Tyrosine-protein kinase | ↑ | S1468 | — |
Among these, 16 peptides were differentially phosphorylated (mapped to 12 kinases) between WT and Huntington’s disease mice in SH conditions (Table 1). Kinases differentially phosphorylated were mainly calcium/calmodulin regulated kinases and mitogen-activated protein kinases (Supplementary Figure 3).

### Table 2: Top 10 up- and downregulated phosphorylation residues in the striatum of Huntington’s disease compared with WT mice

| Protein symbol | Protein name            | Residue | −log(P-value) | log2(FC) | Biological process                                                                 | PhosphoSitePlus |
|---------------|-------------------------|---------|--------------|----------|------------------------------------------------------------------------------------|-----------------|
| TCF20         | Transcription factor 20 | S612    | 3.4725       | −4.4360  | DNA-binding transcription factor activity                                         | —               |
| STRN3         | Striatin-3              | S257    | 4.8191       | −3.5701  | Calmodulin binding, negative regulation of transcription                          | —               |
| JPH4          | Junctophilin-4          | T172    | 4.5153       | −3.2611  | Cross-talk between cell surface and intracellular calcium release                 | —               |
| ATG4B         | Cysteine protease ATG4B | S383    | 3.8025       | −3.0545  | Endopeptidase activity, autophagy                                                | Phosphorylated by MST4, induced enzymatic activity and autophagic flux<sup>23</sup> |
| TCF20         | Transcription factor 20 | S588    | 3.8777       | −2.8260  | DNA-binding transcription factor activity                                         | —               |
| PSD           | PH and SEC7 domain-containing protein 1 | S156 | 5.8585       | −2.7082  | Guanine nucleotide exchange factor, cytoskeleton remodelling                      | —               |
| CACNB1        | Voltage-dependent L-type calcium channel subunit beta-1 | T418 | 3.1459       | −2.6574  | Calcium ion transport                                                            | —               |
| FAM171B       | Protein FAM171B         | S752    | 2.6290       | −2.5435  | Unknown                                                                           | —               |
| SYNPO         | Synaptopodin            | S882    | 2.1970       | −2.1103  | Actin binding, cytoskeleton organization                                           | —               |
| CRKL          | Crk-like protein        | S107    | 2.3650       | −2.1083  | Activates Ras and Jun kinases signalling pathway                                   | —               |
| HCN2          | Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 2 | S795 | 3.5025       | 2.7518   | Cellular response to cAMP, ion transmembrane transport                           | —               |
| SRRM2         | Serine/arginine repetitive matrix protein 2 | S115 | 2.5783       | 2.7687   | Unknown                                                                           | —               |
| BASP1         | Brain acid soluble protein 1 | S131 | 2.6986       | 3.2284   | Negative regulation of transcription                                              | —               |
| EIF3B         | Eukaryotic translation initiation factor 3 subunit B | S68 | 6.7151       | 3.3981   | Translation initiation, protein synthesis                                         | —               |
| FAM122A       | Protein FAM122A         | S34     | 2.3730       | 3.5166   | Inhibitor of PP2A activity                                                       | Phosphorylated by CHK1, induces activity of PP2A<sup>13,14</sup> |
| MBOAT2        | Lysophospholipid acyltransferase 2 | S332 | 3.2901       | 3.6223   | Lipid modification                                                                | —               |
| HCN2          | Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 2 | S795 | 3.0927       | 5.0681   | Cellular response to cAMP, ion transmembrane transport                           | —               |
| PTPN4         | Tyrosine-protein phosphatase non-receptor type 4 | S899 | 2.9403       | 5.5266   | Tyrosine phosphatase activity                                                    | —               |
| SGIP1         | SH3-containing GRB2-like protein 3-interacting protein 1 | S491 | 8.3419       | 8.1776   | Clathrin-mediated endocytosis                                                   | —               |

Top differentially phosphorylated peptides in HD_SH compared with WT_SH in the striatum, sorted by log2FC, with description of the biological processes in which the phosphorylated protein is involved and if available, the function of the specific phosphosites (retrieved on phosphosite.org). FC, fold change; negative/positive Log2(FC) indicates the phosphorylated residue is downregulated/upregulated in Huntington’s disease mice; — indicates no known functional annotation or upstream regulator.
Differentially phosphorylated kinases in the striatum of HD_SH compared with WT_SH. The arrows indicate the phosphorylation state in the striatum of Huntington’s disease mice compared with WT mice (↑ increased phosphorylation in Huntington’s disease; ↓ decreased phosphorylation in Huntington’s disease). Functional annotations and up-stream regulator for each specific phosphorylation site were retrieved from phosphosite.org (— indicates no known functional annotation or upstream regulator).

We found that CAMKK2 phosphorylation at Serine 495 and CAMK2A phosphorylation at Threonine 337 impaired their calcium-calmodulin activation. Here, we observed a down-phosphorylation at these sites in Huntington’s disease compared with WT mice, which does not directly
translate into an increased activity of the enzymes but might increase their probability to be activated. Specific activation phosphosites, such as the autophosphorylation site Threonine 286 on CAMK2A, were not detected in our dataset. The increased phosphorylation of PI4KB at Serine 511 observed in Huntington’s disease mice, has shown to induce a localization of the kinase in the nucleus.21 Also, Serine 621 phosphorylation on RAF1, found upregulated in Huntington’s disease mice, has been shown to increase the protein’s stability and prevent it from being degraded in the proteasome (Table 1).

We also detected 25 phosphopeptides mapped to 15 phosphatases. Only one of these phosphopeptides was significantly different between WT and Huntington’s disease mice in SH conditions: tyrosine-protein phosphatase non-receptor type 4 (PTPN4), at Serine 899 (no known functional annotation or upstream regulator).

Minor changes in protein phosphorylation in the hippocampus of Huntington’s disease mice regardless of the housing conditions

In the hippocampus, we found 15 DE phosphopeptides between Huntington’s disease and WT mice in SH conditions: 12 of these were downregulated, and three were upregulated in Huntington’s disease compared with WT mice (Fig. 2C). While the number of differentially phosphorylated proteins was too low to perform a Pathway Analysis with IPA, we evaluated which protein classes these phosphoproteins belong to. We found three gene-specific transcriptional regulators: GPRASP1, TCF20 and BCL11A. Two proteins were guanine nucleotide exchange factors: PSD3 and ARHGEF17. We detected 311 phosphopeptides from 120 kinases. Only one was decreased in Huntington’s disease mice (PRKCG, Serine 373). We did not find any differentially phosphorylated phosphatases. A list of present/absent phosphopeptides in all four replicates is also available in Supplementary Table 2.

After the environmental intervention, we found fewer differences in protein phosphorylation between the two genotypes, where only four phosphopeptides were downregulated in Huntington’s disease compared with WT mice (Fig. 2D). Interestingly, in EE conditions, the microtubule-associated protein tau was found less phosphorylated at the 705 serine residue in Huntington’s disease compared with WT mice.

No changes in protein phosphorylation in the striatum of Huntington’s disease mice housed under environmental enrichment conditions

When comparing the striatal phosphoproteome of Huntington’s disease and WT mice in the EE condition (Fig. 2B), only one phosphopeptide (MAP1A, Threonine 1633) was upregulated in Huntington’s disease compared with WT mice.

Dysregulation in protein expression in the striatum and hippocampus of pre-motor symptomatic Huntington’s disease mice, rescued in the striatum exclusively by environmental enrichment

We also assessed whether Huntington’s disease mutation and/or EE affect protein expression in the striatum and hippocampus of 8-week-old mice.

In the striatum, we found 24 proteins whose expression was downregulated in Huntington’s disease compared with WT mice in SH conditions (Fig. 5A). As with the phosphoproteomics dataset, we have included a list of proteins that are present/absent in all four replicates in Supplementary Table 3. Interestingly, we did not find any differences in protein expression comparing Huntington’s disease and WT mice housed under EE conditions (Fig. 5B). Among the 24 proteins found DE between Huntington’s disease and WT mice in SH, 18 had been associated with Huntington’s disease previously (Table 3).

Some of the proteins we found downregulated in the striatum of Huntington’s disease mice are known to be involved in cAMP and cGMP metabolic processes, such as adenylyl cyclase type 5 (ADCY5), calcium/calmodulin-dependent 3’,5’-cyclic nucleotide phosphodiesterase 1B (PDE1B) and the cyclic nucleotide phosphodiesterase 10A (PDE10). Other proteins of interest were involved in calcium homeostasis: inositol 1,4,5-triphosphate receptor type 1 (ITPR1), calcium/calmodulin-dependent protein kinase IV (CAMK4), RAS guanyl-releasing protein 2 (RASGRP2) and voltage-dependent calcium channel subunit alpha-2/delta-3 (CACNA2D3). Lastly, we found a downregulation of proteins regulating cytoskeleton organization, such as alpha-actinin-2 (ACTN2), phosphatase and actin regulator 1 (PHACTR1) and microtubule-associated serine/threonine-protein kinase 3 (MAST3). Notably, we revealed six proteins downregulated in the striatum of Huntington’s disease mice, which have not previously been described as altered in Huntington’s disease pathogenesis (Table 3). Some of these proteins also play a role in cytoskeleton organization: tropomodulin-1 (TMOD1) and tripartite motif-containing protein 46 (TRIM46). We also found a downregulation of another protein involved in intracellular calcium homeostasis: N-terminal EF-hand calcium-binding protein 2 (NECAB2).

At the protein level, we quantified 79 kinases in the striatum. Three of these were downregulated in Huntington’s disease compared with WT mice under SH conditions (MAST3, PRKCB and CAMK4) and could therefore be responsible for changes in the phosphoproteome observed
between these two groups. We also measured the expression of 24 phosphatases in the striatal proteome, one of which was significantly downregulated in Huntington’s disease compared with WT mice in SH: PTPN5 (Table 3).

In the hippocampus, we found 22 proteins DE between Huntington’s disease and WT mice in SH conditions: two proteins were upregulated, and 20 proteins were downregulated in Huntington’s disease mice (Fig. 5C). We also found 22 proteins DE when comparing Huntington’s disease and WT mice under EE conditions: two proteins were upregulated in Huntington’s disease, and 20 proteins were downregulated in Huntington’s disease (Fig. 5D). Most of these differences were similar, with 15 proteins in common between the two comparisons, while seven proteins were specific to the differences between WT and Huntington’s disease mice in SH and seven were specific to the genotype differences in EE (Supplementary Figure 4). As with the striatal dataset, we have included a list of proteins that are present/absent in all four replicates in Supplementary Table 4.

Most of the proteins altered in the hippocampus between Huntington’s disease and WT mice in SH were calcium-dependent and/or involved in calcium homeostasis, including wolframin (WFS1), hippocalcin (HPCA), inositol-triphosphate kinase A (ITPKA), Ras guanyl-releasing protein 1 (RASGRP2) and protein kinase C (PRKCB). All of these were found downregulated in Huntington’s disease.

While 10 of the proteins DE in the hippocampus between Huntington’s disease and WT mice in SH had been associated with Huntington’s disease pathogenesis previously,
| Protein symbol | Full name | −log(P-value) | Log2(FC) | Biological process | Evidence altered in Huntington’s disease | Mouse model (age) brain region |
|----------------|-----------|--------------|----------|--------------------|------------------------------------------|--------------------------------|
| SCN4B          | Sodium channel subunit beta-4 | 4.1460      | −1.5547  | Positive regulation sodium transport | Yes, ↓ | R6/2 (4 weeks),25 R6/2 (12 weeks), striatum26 |
| RGS9           | Regulator of G-protein signalling 9 | 4.1450      | −1.4562  | G protein-coupled receptor signalling pathway | Yes, ↓ | R6/2 (12 weeks), striatum26 R6/2 (8, 12 weeks), striatum27 |
| PDE10A         | cAMP and cAMP-inhibited cGMP 3′,5′-cyclic phosphodiesterase 10A | 5.9402      | −1.3198  | Cyclic nucleotides phosphodiesterase activity | Yes, ↓ | R6/2 (8 weeks),28 R6/2 (12 weeks),26 striatum |
| RASGRP2        | RAS guanyl-releasing protein 2 | 3.4442      | −1.1674  | Cellular response to calcium ion | Yes, ↓ | R6/2 (12 weeks) striatum26 |
| ADCYS5         | Adenylate cyclase type 5 | 5.2938      | −1.0947  | cAMP synthesis process | Yes, ↓ | R6/2 (8 weeks),28 R6/2 (8, 12 weeks), striatum27 |
| ANKRD63        | Ankyrin repeat domain-containing protein 63 | 5.4243      | −1.0710  | Unknown | Yes, ↓ | R6/2 (12 weeks), cortex26 mRNA: R6/1 (24 weeks), R6/2 (6 weeks), striatum29 |
| PTPNS          | Tyrosine-protein phosphatase non-receptor type 5 | 3.8655      | −1.0510  | Negative regulation of MAP kinases activity | Yes (mRNA), ↓ | |
| ITPR1          | Inositol 1,4,5-triphosphate receptor type I | 8.1221      | −1.0456  | Endoplasmic reticulum calcium homeostasis | Yes, ↓ | R6/2 (8, 12 weeks), striatum27 |
| INF2           | Inverted formin-2 | 3.5387      | −1.0039  | Actin filaments organization | Yes, ↓ | R6/2 (8, 12 weeks), striatum27 |
| PDE1B          | Calcium/calmodulin-dependent 3′,5′-cyclic nucleotide phosphodiesterase 1B | 3.8514      | −0.8538  | Cyclic nucleotide phosphodiesterase activity | Yes, ↓ | R6/2 (8 weeks), striatum28 |
| FBXL16         | F-box/LRR-repeat protein 16 | 3.8527      | −0.8501  | Ubiquitin-dependent protein catabolic process | Yes, ↓ | R6/2 (8, 12 weeks), striatum27 |
| CACNA2D3       | Voltage-dependent calcium channel subunit alpha-2/delta-3 | 5.4635      | −0.8420  | Calcium ion transport | Yes, ↓ | R6/2 (8, 12 weeks), striatum27 |
| TMOD1          | Tropomodulin-1 | 3.2224      | −0.7501  | Actin filaments organization | No | |
| TRIM46         | Tripartite motif-containing protein 46 | 3.4297      | −0.7353  | Cytoskeleton organization, axonogenesis | No | |
| CAMK4          | Calcium/calmodulin-dependent protein kinase IV | 3.9480      | −0.7349  | Positive regulation of transcription | Yes, ↓ | R6/2 (6, 11 weeks), striatum30 R6/2 (8, 12 weeks), striatum27 |
| PHACTR1        | Phosphatase and actin regulator 1 | 5.9054      | −0.6941  | Actin filaments organization | Yes, ↓ | R6/2 (8, 12 weeks), striatum27 |
| SH2DS5         | SH2 domain-containing protein 5 | 3.0281      | −0.6853  | Rac-GTP levels regulation | No | |
| NECAB2         | N-terminal EF-hand calcium-binding protein 2 | 3.8746      | −0.6850  | Intracellular calcium homeostasis | No | |

(continued)
we report 12 novel proteins, whose involvement in Huntington’s disease has not been previously reported (Table 4). These involved proteins regulating calcium and diacylglycerol signalling (SYT7, CPNE7 and DGKZ). We also reported differential expression of proteins involved in glutamate signalling: protein shisa-6 (SHISA6) and neuronal pentraxin receptor (NPTXR) were both downregulated in Huntington’s disease mice and regulate AMPA immobilization and synaptic clustering. Moreover, subunit 1 of AMPA receptor (GRIA1) was also downregulated in Huntington’s disease mice.

We also assessed in the hippocampus whether we observed any changes in kinases and phosphatases, which might lead to differences in protein phosphorylation. In the hippocampus, we detected 102 kinases. Four of these were differently expressed in Huntington’s disease compared with WT in SH: IP3KA, KPCB, KPCA and DGKZ (Table 4). We also detected 27 phosphatases, but none were DE between the groups.

## Discussion

### Phosphoproteome dysregulation in Huntington’s disease mice in standard housing conditions

We report here, for the first time, differences in the striatal phosphoproteome of 8-week-old Huntington’s disease male mice in standard-housing conditions. Notably, these changes seem to precede the onset of motor symptoms (Supplementary Figure 5), which typically occur around 12 weeks of age in the R6/1 mouse model of Huntington’s disease used in this study. Therefore, underpinning the upstream molecular mechanisms, including the kinases and phosphatases involved, presents important therapeutic value.

Ultimately, the upstream regulation and downstream function of most of these phosphorylated sites remain unknown. Here, we investigated the functions of the proteins differentially modified, with the assumption that their activity, localization, and interactions might be affected by a change in their phosphorylation profile.

We found several differentially phosphorylated ion channels in the striatum of Huntington’s disease mice in SH conditions, including voltage-dependent ion channels, essential for the initiation and propagation of action potentials. These can be tightly regulated via phosphorylation, which can ultimately affect synaptic transmission and signal transduction. Additionally, we observed differential phosphorylation of scaffold proteins, crucial regulators of intracellular signal transduction. We also report differential phosphorylation of cytoskeleton proteins in the striatum of Huntington’s disease mice, mainly actin-binding proteins. Differential post-translational modification of actin-binding proteins, as seen in Huntington’s disease mice, can alter their interaction with actin, affecting overall actin cytoskeleton dynamics, axon growth, and neurite growth.

Numerous protein-modifying enzymes were found differentially phosphorylated in the striatum of Huntington’s disease mice, including non-receptor serine/threonine protein kinases. For some of these, we were able to predict the effect

| Protein symbol | Full name                                      | –log(P-value) | Log2(FC) | Biological process                   | Evidence altered in Huntington’s disease | Mouse model (age) brain region |
|----------------|-----------------------------------------------|---------------|----------|-------------------------------------|------------------------------------------|-------------------------------|
| ACTN2          | Alpha-actinin-2                                | 4.6819        | –0.6781  | F-actin cross-linking protein        | Yes, ↓                                   | R6/2 (8, 12 weeks), striatum27 |
| PLD3           | 5′-3′ exonuclease, phospholipase D3            | 3.6449        | –0.6711  | Inflammatory response                | No                                       |                               |
| LINGO1         | Leucine-rich repeat and immunoglobulin-like domain-containing nogo receptor-interacting protein 1 | 2.9775        | –0.6365  | Component of the reticulon 4 receptor | No                                       |                               |
| MAST3          | Microtubule-associated serine/threonine-protein kinase 3 | 2.9093        | –0.5997  | Cytoskeleton organization            | Yes, ↓                                   | R6/2 (8, 12 weeks), striatum27 |
| PRKCB          | Protein kinase C, beta type                    | 2.9380        | –0.5890  | Intracellular signal transduction    | Yes (mRNA), ↓                            | mRNA: R6/1 (24 weeks), R6/2 (6 weeks), striatum29 |
| SRM            | Spermidine synthase                            | 3.4001        | –0.5850  | Spermidine synthesis                | Yes (mRNA) ↓                             | mRNA: R6/1 (24 weeks), R6/2 (6 weeks), striatum29 |

List of proteins differentially expressed between HD and WT in SH in the striatum (FDR 5%, FC 1.5), sorted by log2FC. The last two columns highlight whether a downregulation in that protein’s expression has been published previously in Huntington’s disease mouse models (including at the mRNA level). FC, fold change; negative Log2(FC) indicates the protein is downregulated in Huntington’s disease mice.

### Table 3 (continued)
Table 4: Proteins differentially expressed in the hippocampus of Huntington’s disease compared with WT mice under standard housing conditions

| Protein symbol | Full name | $-\log(P\text{-value})$ | Log2(FC) | Biological process | Evidence altered in Huntington’s disease | mouse model (age), brain region |
|---------------|-----------|-------------------------|----------|--------------------|----------------------------------------|----------------------------------|
| WFS1 | Wolframin | 5.8006 | $-2.3572$ | Calcium ion homeostasis | Yes, ↓ | R6/2 (12 weeks), hippocampus<sup>26</sup> |
| RASGRPI | Ras guanyl-releasing protein 1 | 3.8749 | $-1.3971$ | Calcium and DAG-regulated nucleotide exchange factor | Yes, ↓ | R6/2 (12 weeks), hippocampus<sup>26</sup> |
| RIN1 | Ras and Rab interactor 1 | 2.7129 | $-1.0857$ | GTPase activity | Yes, ↓ | R6/2 (12 weeks), hippocampus<sup>26</sup> |
| ITPKA | Inositol-triphosphate 3-kinase A | 6.1320 | $-1.0517$ | Intracellular calcium signalling termination | Yes, ↓ | R6/2 (12 weeks), hippocampus<sup>26</sup> |
| PSD | PH and SEC7 domain-containing protein 1 | 4.4205 | $-0.9440$ | Guanine nucleotide exchange factor activity | No, (Psd3 yes, ↓) | R6/2 (2, 4, 8, 12 weeks), whole brain<sup>31</sup> |
| RGS14 | Regulator of G-protein signalling 14 | 5.8757 | $-0.7670$ | G protein-coupled receptor signalling pathway | Yes, (mRNA)↓ | mRNA: R6/2 (6, 24 weeks), striatum<sup>29</sup> |
| PRKCA | Protein kinase C | 5.8220 | $-0.7354$ | Intracellular signal transduction | No (found in mHtt inclusions<sup>23</sup>) | |
| CRACDL | Capping protein-inhibiting regulator of actin-like | 8.6673 | $-0.7245$ | Actin cytoskeleton maintenance | No | |
| CPNE7 | Copine-7 mRNA: R6/1 (24 weeks), striatum<sup>13</sup> | 4.0820 | $-0.6942$ | Cellular response to calcium ion | No, | |
| WIPF3 | WAS/WASL-interacting protein family member 3 | 5.8114 | $-0.6843$ | Actin filaments organization | Yes, ↓ | R6/2 (12 weeks), hippocampus<sup>26</sup> |
| NPTXR | Neuronal pentraxin receptor | 5.5566 | $-0.6751$ | Regulation of postsynaptic neurotransmitter receptor activity/assembly | No | |
| PRKCB | Protein kinase C, beta type | 7.5843 | $-0.6443$ | Intracellular signal transduction | Yes, ↓ | R6/2 (8, 12 weeks), striatum<sup>27</sup> |
| DGKZ | Diacylglycerol kinase zeta | 3.5668 | $-0.6390$ | Intracellular signal transduction | No | |
| SYT7 | Synaptotagmin-7 | 3.4900 | $-0.6338$ | Calcium-dependent exocytosis | No | |
| PEX5L | PEX5-related protein | 4.4986 | $-0.6204$ | Subunit of hyperpolarization-activated cyclic nucleotide-gated channels | No | |
| HPCA | Neuron-specific calcium-binding protein hippocalcin | 4.9671 | $-0.6201$ | Cellular response to calcium ion | Yes, (mRNA)↓ | mRNA: R6/2 (6, 24 weeks), striatum<sup>29,34</sup> |
| MLF2 | Myeloid leukaemia factor 2 | 3.5497 | $-0.6150$ | Unknown | Yes, (mRNA)↓ | mRNA: R6/2 (6, 24 weeks), striatum<sup>29</sup> |
| SHISA6 | Protein shisa-6 | 3.3054 | $-0.5980$ | AMPA receptor clustering and activity regulation | No | |
| GRIA1 | Glutamate ionotropic receptor AMPA type, subunit 1 | 6.7494 | $-0.5975$ | Ionotropic glutamate receptor activity | No | |
| ACTR3B | Actin-related protein 3B | 5.1705 | $-0.5922$ | Actin cytoskeleton organization | No | |
| PCSK1N | ProSAAS/proprotein convertase 1 inhibitor | 3.5691 | 0.6194 | Neuropeptide signalling pathway | Yes, ↑ | R6/2 (12 weeks), hippocampus<sup>26</sup> |
| H1-0 | Histone H1 | 2.4924 | 0.9376 | Chromosome condensation | No | |

List of proteins differentially expressed between Huntington’s disease and WT in SH in the hippocampus (FDR 5%, FC 1.5), sorted by log2FC. The last two columns highlight if a downregulation/upregulation in that protein’s expression has been published previously in Huntington’s disease mouse models (including at the mRNA level). FC, fold change; negative/positive Log2(FC) indicates the protein is downregulated/upregulated in Huntington’s disease mice.
of a differential phosphorylation on their activity: the decreased phosphorylation in Huntington’s disease mice of CAMK2A and CAMKK2 allows for calcium/calmodulin binding. The increased phosphorylation of PI4KB, as seen in Huntington’s disease mice, induces its localization in nuclear speckles.23 PI4KB functions as a signalling molecule, responsible for the phosphorylation of phosphatidylinositol into the second messenger phosphatidylinositol bisphosphate, later phosphorylated into triphosphate. While the exact functions of PI4KB in the nucleus remain to be determined, phosphoinositides are known to be involved in gene transcription, mRNA processing and chromatin remodelling.37 RAF1, a MAPKKK acting upstream of ERK, was found to be hyperphosphorylated in Huntington’s disease mice, which translates into an increased stability of the enzyme.22 Concordant with our data, a previous study has found increased activation of RAF1, and its upstream regulators, in Huntington’s disease cells and mouse models.18

Several proteins differentially phosphorylated in Huntington’s disease were involved in the Opioid Signalling Pathway. The Opioid Signalling Pathway is not only involved in pain, but can also influence cognition and decision making,39 known to be impaired in clinical Huntington’s disease.40 More specifically, in the striatum, opioid signalling is known to interact with dopaminergic transmission, and is therefore critical for motor control in Huntington’s disease.41 The striatum expresses the highest levels of opioid receptors and its ligands in the brain.42 Although we do not expect striatal neuronal loss at this early stage of pathogenesis in these Huntington’s disease mice, a change in the phosphorylation profile of all these proteins potentially indicates a dysregulation in both the opioid and dopaminergic signalling pathways, prior to symptom onset.

Our upstream regulator analysis from IPA highlighted a potential interplay of multiple kinases responsible for the phosphoproteome shift in the striatum of Huntington’s disease mice. CAMK2A, the most significant kinase with a predicted inactivation, is a calcium/calmodulin-dependent serine/threonine protein kinase highly expressed in striatal MSNs.13 Once activated, it can phosphorylate and modulate targets such as NMDA and AMPA receptors,44,45 playing an essential role in striatal glutamatergic signalling. As such, its inhibition in the dorsal striatum leads to a loss of functional glutamatergic synapses.46 CAMK2A protein expression was previously found decreased in the hippocampus of fully symptomatic 10-week-old R6/2 Huntington’s disease mice.10 Here, while our phosphorylation data points towards an inactivation of the enzyme, we found no differences in CAMK2A protein expression between Huntington’s disease and WT mice.

Our analysis also predicted an inactivation of MAPK1 (ERK2) in the striatum of Huntington’s disease mice. The ERK pathway is involved in glutamatergic and brain-derived neurotrophic factor signalling, both of which are impaired in Huntington’s disease.47 Interestingly, activating ERK1/2 has shown beneficial effects in multiple cellular and animal models of Huntington’s disease.18,49 CDK5 and PRKCG were both predicted to be activated in the striatum of Huntington’s disease mice, concordant with previous studies, reporting aberrant activation of CDK5 in striatal cells and Huntington’s disease mouse models.50,51

We found few differences in the hippocampal phosphoproteome of Huntington’s disease mice (in both SH and EE conditions), compared with the extensive changes we revealed in the striatum. Our results might be explained by Huntington’s disease pathogenesis itself, characterized by a selective loss of striatal MSNs,3 whereby the striatum would be the first brain region to be affected. Fewer changes detected in the hippocampus might also be explained by a less homogeneous brain region, compared with the striatum. Indeed, the striatum is composed of nearly 95% of striatal projection neurons (GABAergic),32 while the hippocampus is more heterogeneous,21 and signals might cancel each other out.

In the hippocampus, we mainly observed differential phosphorylation of gene-specific transcriptional regulators, including TCF20, GRASPI and BCL11A. While the role of GRASPI on gene transcription is unknown, BCL11A haploinsufficiency has been associated with cognitive impairments.54

Proteome dysregulation in Huntington’s disease mice in standard housing conditions

We found limited protein expression differences when comparing the proteome of Huntington’s disease and WT mice under SH conditions in both brain regions. The extent of these changes was expected, considering the early timepoint in Huntington’s disease pathogenesis. However, as these occur before onset of motor symptoms, they are likely critical in the development of the phenotype. As expected, we found no changes in the huntingtin protein levels at this early stage.

In the striatum, 24 proteins were found downregulated in Huntington’s disease mice compared with WT mice in SH conditions, while no proteins were found upregulated in Huntington’s disease. As previously mentioned, our study is the first one characterizing the proteome of R6/1 Huntington’s disease mice. The lack of upregulated proteins might be explained by the early timepoint in the disease progression: the downregulated proteins might be an early indication of neurodegeneration or might be a sign of sequestration into huntingtin aggregates.

Our results aligned with previously published studies, as 18 of the 24 proteins found downregulated in Huntington’s disease mice in SH had been previously associated with Huntington’s disease pathogenesis. Furthermore, 14 of these proteins are known to interact with the huntingtin protein (Supplementary Figure 6). However, here, we revealed a downregulation of these proteins prior to motor onset in Huntington’s disease mice for the first time. Indeed, most proteomic studies have been performed on R6/2 Huntington’s disease mice, which
experience motor deficits from 6 to 8 weeks of age,\textsuperscript{55} and thus may be considered a juvenile-onset model. As 95% of Huntington’s disease patients exhibit adult onset, the R6/1 Huntington’s disease mice used in the present study provide a more accurate model of adult-onset Huntington’s disease.

As Huntington’s disease is associated with a reduced transcription of cyclic AMP early in the disease,\textsuperscript{56} we observed a downregulation in the expression of PDE10A and PDE1B, two cAMP/GMP phosphodiesterases highly expressed in MSNs. ADCY5 catalyses the formation of cAMP and was also downregulated in Huntington’s disease mice. A downregulation in ADCY5 has already been highlighted in the R6/2 mouse model of Huntington’s disease, later on in the disease.\textsuperscript{27,28}

In neurons, calcium signalling is critical for the propagation of the depolarizing signal, as well as the release of neurotransmitters at the synapse.\textsuperscript{57} We found a downregulation of proteins involved in calcium homeostasis in the striatum, including a voltage-gated calcium channel subunit (CACNA2D3), a neuronal calcium sensor (NECAB2), a regulator of calcium release from endoplasmic reticulum (ITPR1) and a calcium and diacylglycerol dependent protein kinase (PRKCB).

In the present study, we also revealed a downregulation in the striatum of Huntington’s disease mice of six novel proteins (Table 3). The phospholipase D3 (PLD3), found in neuronal lysosomes, was found reduced in the striatum of Huntington’s disease mice. Interestingly, a reduced function of the protein has been correlated with a faster rate of cognitive decline in Alzheimer’s disease patients.\textsuperscript{58} Further studies would be needed to underpin its involvement in Huntington’s disease-associated cognitive deficits. Tropomodulin 1 (TMOD1), an actin-binding protein essential for synapse formation,\textsuperscript{59} was found downregulated in the striatum of Huntington’s disease mice. Altered expression of TMOD1 has been found in several brain disorders, such as epilepsy\textsuperscript{60} and Down syndrome\textsuperscript{61} but not been described in Huntington’s disease before. Decreased levels of SH2 domain-containing protein 5, as seen in the striatum of Huntington’s disease mice, has been associated with impaired neurogenesis and synaptogenesis.\textsuperscript{62}

Together, the proteins found downregulated in the striatum of Huntington’s disease mice under SH conditions prior to motor symptoms indicate dysregulation in calcium signalling, destabilization of the cytoskeleton and neuronal cell loss. Our results are concordant with previous studies, and will guide further investigations exploring the involvement of associated molecular and cellular processes in Huntington’s disease pathogenesis.

In the hippocampus, we reported 22 proteins DE between WT and Huntington’s disease mice when housed in SH conditions. Several proteins we found dysregulated in the hippocampus were involved in calcium signalling, including wolframin (WFS1, which maintains ER calcium homeostasis), and hippocalcin (HPCA), a neuronal calcium sensor. We also report a downregulation of proteins involved in glutamatergic signalling in the hippocampus of Huntington’s disease mice: SHISA6, responsible for AMPA-type glutamate receptor immobilization at postsynaptic density, and glutamate receptor 1 (GRIA1, AMPA type). AMPA receptors are known to mediate most of the fast excitatory synaptic transmission, playing an important role in long-term potentiation and long-term depression of synaptic transmission.\textsuperscript{53}

### The effect of the environment on the proteome and phosphoproteome

Our analysis also investigated a potential effect of EE, from 4 to 8 weeks of age, on the phosphoproteome and proteome of Huntington’s disease mice and their WT littermates. EE has previously been shown to improve cognitive and motor performance in Huntington’s disease mice, as well as to alleviate Huntington’s disease-related motor, affective and cognitive symptoms.\textsuperscript{64} Our experiment was designed with an environmental intervention initiated in young mice, as the brain is more sensitive to an environmental modulation early in development,\textsuperscript{64} and the most striking therapeutic effects of EE on Huntington’s disease mice have involved delay in disease onset.\textsuperscript{13,64} The behavioural outcomes of such an early and short environmental intervention had not yet been investigated. Here, contrary to previously published studies,\textsuperscript{64} we found no effects of EE on the motor performance of 8-week-old mice (Supplementary Figure 5). These results on Huntington’s disease mice could be explained by the early timepoint in the disease progression, where an improvement in motor performance cannot be seen before onset of Huntington’s disease-associated motor symptoms.

Although not seen at the behavioural level (as the tissues were collected at 8 weeks of age which is prior to motor onset in these Huntington’s disease mice), it is possible that such early environmental intervention leads to long-lasting effects, including those acting on a molecular level. Indeed, when mice were housed in EE conditions, we found no significant differences in protein phosphorylation in the striatum between Huntington’s disease mice and their WT littermates (apart from one phosphopeptide). The lack of protein phosphorylation changes in the striatum of Huntington’s disease and WT mice after an EE intervention could be explained by the rescue of certain protein kinases and phosphatases, at the protein level. Indeed, while PKC, MAST3, CAMK4 and PTPN5 were found downregulated in SH conditions, their expression was not significantly changed between the two genotypes in EE conditions. Phosphorylation changes were also reduced in the hippocampus when both genotypes were housed in EE.

Interestingly, in the hippocampus, we revealed a reduced MAPT (tau protein) phosphorylation at amino-acid residue 705 when comparing Huntington’s disease versus WT mice housed in EE (Fig. 2D). This finding is in contrast to our recent study which found an increased tau phosphorylation in the cortex of 8-week-old R6/1 Huntington’s disease mice when housed in SH conditions.\textsuperscript{6} However, this could reflect a potential beneficial effect of EE in Huntington’s disease.
mice, whereby EE could have a different effect on WT versus Huntington’s disease mice.

At the protein level, we found no differences between the striatal proteome of Huntington’s disease and WT mice in EE conditions. In the hippocampus, the same number of DE proteins was found when comparing Huntington’s disease and WT in EE conditions and in SH conditions. However, the proteins DE were partly different in the two conditions, suggesting that EE might have a different effect on the hippocampus of Huntington’s disease and WT mice.

Previous EE interventions have been shown to reduce the size of polyglutamine-expanded huntingtin aggregates in Huntington’s disease mice.\(^65\) We can hypothesize that the reduced aggregate size correlates with a delay of protein sequestration into the inclusion bodies, therefore found with an unchanged concentration in the soluble fraction in enrichment conditions. Indeed, most of the proteins found downregulated in the striatum of Huntington’s disease mice in SH conditions, prior to motor symptoms, are known to interact with huntingtin protein (Supplementary Figure 6).

One limitation of the present study is that it is unknown which component of EE is driving these changes in protein phosphorylation and protein expression (i.e. physical activity, sensory simulation, cognitive activity, etc.). Furthermore, it could involve multiple components acting in synergy. This is beyond the scope of the current study, however further experiments investigating specific components of EE would help address the underlying mechanisms. Additionally, the environmental paradigm used in this study presents limitations regarding its translation to the clinic. The SH condition, in which we observed extensive dysregulations in protein phosphorylation, would refer to a deprivation state in humans, whereas EE is likely to be closely to a ‘normal’ (or at least average) level of sensorimotor stimulation and physical activity in humans.

When comparing WT in SH versus WT in EE, and Huntington’s disease in SH versus Huntington’s disease in EE, we did not find any significant proteins or phosphopeptides in both brain regions (Supplementary Figures 7–10). Previous studies have reported specific protein expression and protein phosphorylation changes in WT rodents and Huntington’s disease mouse models after a EE intervention.\(^{66–69}\) However, the enrichment protocol used in these studies, as well as the duration of the intervention and the age of the animals at the start of the intervention vary with our present study. These factors are known to affect the outcome of such interventions.\(^{70}\) The stringent statistical thresholds (i.e. FDR 5% and FC 1.5) could also help explain the results, as our study might have been underpowered to detect a smaller effect of environmental intervention, compared with the Huntington’s disease transgene effect. The environmental intervention could also have a different effect on WT and Huntington’s disease mice, leading to significant changes between the two genotypes only in standard-housing conditions. The correlation among samples was similar in each group (see Pearson’s correlation in methods), thus a potential higher variability in EE samples does not explain the lack of significant results.

In the current study, we found that a protein phosphorylation dysregulation occurs in the striatum of Huntington’s disease male mice at a very early stage of pathogenesis, prior to onset of motor deficits. This was associated with minimal changes in protein expression. Following up on our previous findings revealing a phosphoproteome dysregulation occurring in the cortex of pre-symptomatic Huntington’s disease male mice,\(^8\) the present study only investigated protein and phosphorylation changes in male mice. Additional proteomics and phosphoproteomics experiments using Huntington’s disease female brain samples remain to be conducted. Comparing male versus female data in a future study would be informative.

**Conclusion**

Here, we have identified new leads to develop novel therapeutics for Huntington’s disease, by targeting the phosphoproteome. We also report the outcome of a 4-week EE intervention, which was able to rescue these changes observed in the striatum of Huntington’s disease mice, both at the protein expression and protein phosphorylation levels. While the exact molecular mechanisms of EE are unclear, this intervention has previously shown beneficial effects on Huntington’s disease mice, on cognitive, affective and motor deficits, as well as neurodegeneration. Our current study reveals the effects of EE on protein phosphorylation in Huntington’s disease mice, which will inform the future development of novel therapeutic approaches, including enviromimetics.

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**Competing interests**

The authors have no conflict of interest to disclose.
Supplementary material

Supplementary material is available at Brain Communications online.

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD032205.

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