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# Pridopidine activates neuroprotective pathways impaired in Huntington Disease

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## Abstract

Pridopidine has demonstrated improvement in Huntington Disease (HD) motor symptoms as measured by secondary endpoints in clinical trials. Originally described as a dopamine stabilizer, this mechanism is insufficient to explain the clinical and preclinical effects of pridopidine. This study therefore explored pridopidine's potential mechanisms of action. The effect of pridopidine versus sham treatment on genome-wide expression profiling in the rat striatum was analysed and compared to the pathological expression profile in Q175 knock-in (Q175 KI) vs Q25 WT mouse models. A broad, unbiased pathway analysis was conducted, followed by testing the enrichment of relevant pathways. Pridopidine upregulated the BDNF pathway ($P = 1.73E-10$), and its effect on BDNF secretion was sigma 1 receptor (S1R) dependent. Many of the same genes were independently found to be downregulated in Q175 KI mice compared to WT ($5.2e-7 < P < 0.04$). In addition, pridopidine treatment upregulated the glucocorticoid receptor (GR) response, D1R-associated genes and the AKT/PI3K pathway ($P = 1E-10$, $P = 0.001$, $P = 0.004$, respectively). Pridopidine upregulates expression of BDNF, D1R, GR and AKT/PI3K pathways, known to promote neuronal plasticity and survival, as well as reported to demonstrate therapeutic benefit in HD animal models. Activation of S1R, necessary for its effect on the BDNF pathway, represents a core component of the mode of action of pridopidine. Since the newly identified pathways are downregulated in neurodegenerative diseases, including HD, these findings suggest that pridopidine may exert neuroprotective effects beyond its role in alleviating some symptoms of HD.

## Introduction

Huntington disease (HD) is a neurodegenerative disease caused by a dominant trinucleotide repeat expansion in the huntingtin gene (1) and is estimated to affect 43,000 individuals in the US alone (2). The severe impact of the disease on motor function and cognition, compounded by the lack of approved disease modifying treatments, drives a pressing need for novel therapeutic solutions.

Pridopidine is a small molecule in development for treatment of HD (PRIDE-HD, NCT02006472, Teva Pharmaceuticals). Originally described as a dopamine stabilizer, it was shown to act as a low-affinity antagonist at the dopamine D2 receptor

(D2R)(3). Behaviourally pridopidine was shown to either suppress d-amphetamine-induced hyperactivity, or stimulate hypoactivity in environmentally habituated rats (3). In two clinical trials (MermaiHD (4) and HART (5)), while pridopidine did not meet the primary endpoints (changes from baseline to week 12 in the Modified Motor Score, a subset of the Unified HD Rating Scale Total Motor Score), it did demonstrate significant improvement in the Unified HD Rating Scale Total Motor Score (TMS), one of the secondary endpoints.

Several recent lines of evidence suggest that pridopidine's activity may be attributed to binding the sigma 1 receptor (S1R), an endoplasmic reticulum (ER) chaperone protein. It was demonstrated that pridopidine exerts high affinity towards S1R with Ki between 70 and 80 nM, which is ∼100x higher than its affinity toward D2R (6). Indeed, at doses known to elicit behavioural effects, a rat PET brain imaging study demonstrates that pridopidine occupies S1R, but not D2R (7). This discovery precipitates the question of whether some of pridopidine's activities are mediated through S1R.

Much remains to be elucidated concerning S1R's function, as it lacks a precise mechanistic description and shares no homology to other proteins. S1R is an ER/mitochondria membrane resident ligand operated chaperone, which is implicated in cellular differentiation, neuroplasticity, neuroprotection and cognitive function in the brain (8). In HD, S1R is abnormally accumulated in neuronal nuclear inclusions (NNIs) and causes degradation of abnormally folded proteins in NNIs (9).

Multiple sources of genetic evidence implicate S1R mutations in neurodegenerative disease, including studies of amyotrophic lateral sclerosis (ALS), frontotemporal lobar degeneration (FTLD), and Alzheimer's disease (AD) (10–12). For example, a S1R mutation carried by a consanguineous family with juvenile ALS induces abnormal S1R localization to lower-density membranes in motor neurons (10). Similarly, several causative mutations in S1R were reported in familial FTLD (11). Moreover, the S1R SNP rs1799729 reduces S1R mRNA/protein expression level (13) and is associated with a modest increased risk for late onset AD (12), however, this finding remains controversial (14). In addition, S1R 2P variant (amino acid substitution) was reported as associated with a moderate increase in cognitive dysfunction in non-APOE-e4 carriers with AD (15). Taken together, these studies offer significant genetic evidence for the role of S1R in the neurodegenerative disease process.

The mechanisms beyond dopamine stabilization by which pridopidine protects and promotes neuronal activity in general, and specifically in the striatum, have yet to be elucidated. This study addresses this gap by utilizing an unbiased, hypothesis-free, genome-wide approach to assess a broad range of functional effects induced *in vivo* by chronic pridopidine treatment. Furthermore, the hypothesis that pridopidine may act similarly to other S1R ligands is specifically examined.

## Results

In order to elucidate the functional effects of pridopidine in an unbiased fashion, the genome-wide expression profiles of rat striatum, treated with either pridopidine or vehicle for a total of 10 days, were compared. Quality control analysis including review of array quality statistics and principle component analysis (PCA) did not detect any batch effects or outlier samples. The expression of multiple probesets was observed to be modulated by treatment (unadjusted $P < 0.05$: 2,239 probesets; the false discovery rate (FDR) adjusted $P < 0.05$: 28 probesets; fold change $(FC) > 1.5$ and $FC < -1.5$: 97 probesets).

### Genome-wide analysis identifies BDNF signalling as a key mechanistic effect of pridopidine

Examination of the most differentially expressed genes by significance level revealed that pridopidine robustly enhanced the expression of described transcriptional targets in the BDNF signalling pathway (16); expression was statistically significant for genes including EGR2, FOS, EGR1, ARC, KLF5, CDKN1A, HOMER1A, and CEBPB (Table 1, Figure 1, Supplementary Material, Table S1). Hypothesis testing analysis evaluated the specific changes induced by pridopidine in the BDNF pathway. A published list was used, which was comprised of a total of 69 probesets identified by microarray studies as being induced by BDNF treatment of cultured striatal neurons (17). Gene Set Enrichment Analysis (GSEA) revealed that a ranked list by FC of genes responding to pridopidine was highly positively enriched for genes induced by activation of the BDNF receptor ($P < 0.001$). The GSEA "leading edge" of enriched genes (i.e. the core of the gene set that accounts for the enrichment signal), as described in (18) was shown to include EGR2, KLF5, CEBPB, FOS, ARC, DUSP5, and EGR1

**Table 1.** BDNF GSEA leading edge genes not depicted in Fig 1., which were positively enriched by pridopidine treatment and also downregulated ($P < 0.05$) in HD mice ($n = 13$). Of note, GADD45G is also part of GR leading edge. EGR4, FOS, and NAB2 are also part of D1R leading edge.

| Gene symbol | Rat microarray (pridopidine vs vehicle) | | | RNAseq Q175 vs Q25 HD mouse | | |
| --- | --- | --- | --- | --- | --- | --- |
| | probeset | FC | P | ENSG ID | FC | P |
| FOS | 1375043_at | 1.60 | 6.39E-03 | ENSMUSG00000021250 | −0.64 | 9.44E-03 |
| EGR4 | 1387442_at | 1.45 | 3.43E-04 | ENSMUSG00000071341 | −0.70 | 3.91E-04 |
| GADD45G | 1388792_at | 1.36 | 1.33E-02 | ENSMUSG00000021453 | −0.23 | 9.37E-03 |
| SRXN1 | 1372510_at | 1.26 | 8.52E-04 | ENSMUSG00000032802 | −0.08 | 3.49E-02 |
| DUSP14 | 1373324_at | 1.24 | 2.60E-02 | ENSMUSG00000018648 | −1.04 | 2.81E-06 |
| DCLK1 | 1387276_at | 1.23 | 4.46E-03 | ENSMUSG00000027797 | −0.41 | 4.14E-06 |
| DUSP6 | 1377064_at | 1.20 | 1.23E-01 | ENSMUSG00000019960 | −0.60 | 4.89E-05 |
| PDP1 | 1368910_at | 1.16 | 3.07E-01 | ENSMUSG00000049225 | −0.47 | 1.46E-04 |
| PLK2 | 1368106_at | 1.15 | 7.81E-02 | ENSMUSG00000021701 | −0.45 | 2.66E-06 |
| BAIAP2 | 1370074_at | 1.14 | 7.38E-03 | ENSMUSG00000025372 | −0.55 | 1.94E-07 |
| ARPP21 | 1373257_at | 1.12 | 2.38E-01 | ENSMUSG00000032503 | −0.96 | 4.91E-14 |
| GLS | 1382521_at | 1.11 | 2.69E-02 | ENSMUSG00000026103 | 0.11 | 5.75E-05 |
| NAB2 | 1374925_at | 1.11 | 5.85E-02 | ENSMUSG00000025402 | −0.33 | 5.49E-07 |

(all leading edge genes can be found in Figure 1, Table 1, or Supplementary Material, Table S1). To test the validity of this result, an independent analysis using a different statistical approach (the hypergeometric test) was employed to compare the list of 69 BDNF induced genes to the list of genes nominally changing in response to pridopidine treatment (2,239 genes). A highly significant overlap was found ($P = 1.73e-10$).

In order to validate these findings in gene expression, an independent profiling technology was employed. Six of the significant genes discussed above were selected for quantitative polymerase chain reaction (qPCR) validation. qPCR analysis confirmed that pridopidine upregulates the selected BDNF pathway

genes (Figure 1). Together, these results demonstrate that pridopidine upregulates the genes induced by BDNF.

Activation of the S1R has been reported to enhance BDNF secretion in rat hippocampi, primary rat astrocytes (19), mouse striatum (20) and in rat neuroblastoma cells (21). We tested the hypothesis that pridopidine, via activation of the S1R enhances secretion of BDNF protein, thereby activating the BDNF receptor and further downstream signalling. For that purpose the B104 neuroblastoma cell line was used, as it was reported to secrete more BDNF than other cell lines when grown in serum-free medium (21). Pridopidine at 100nM and at 1µM enhanced BDNF secretion as compared to control untreated cells. BDNF secretion
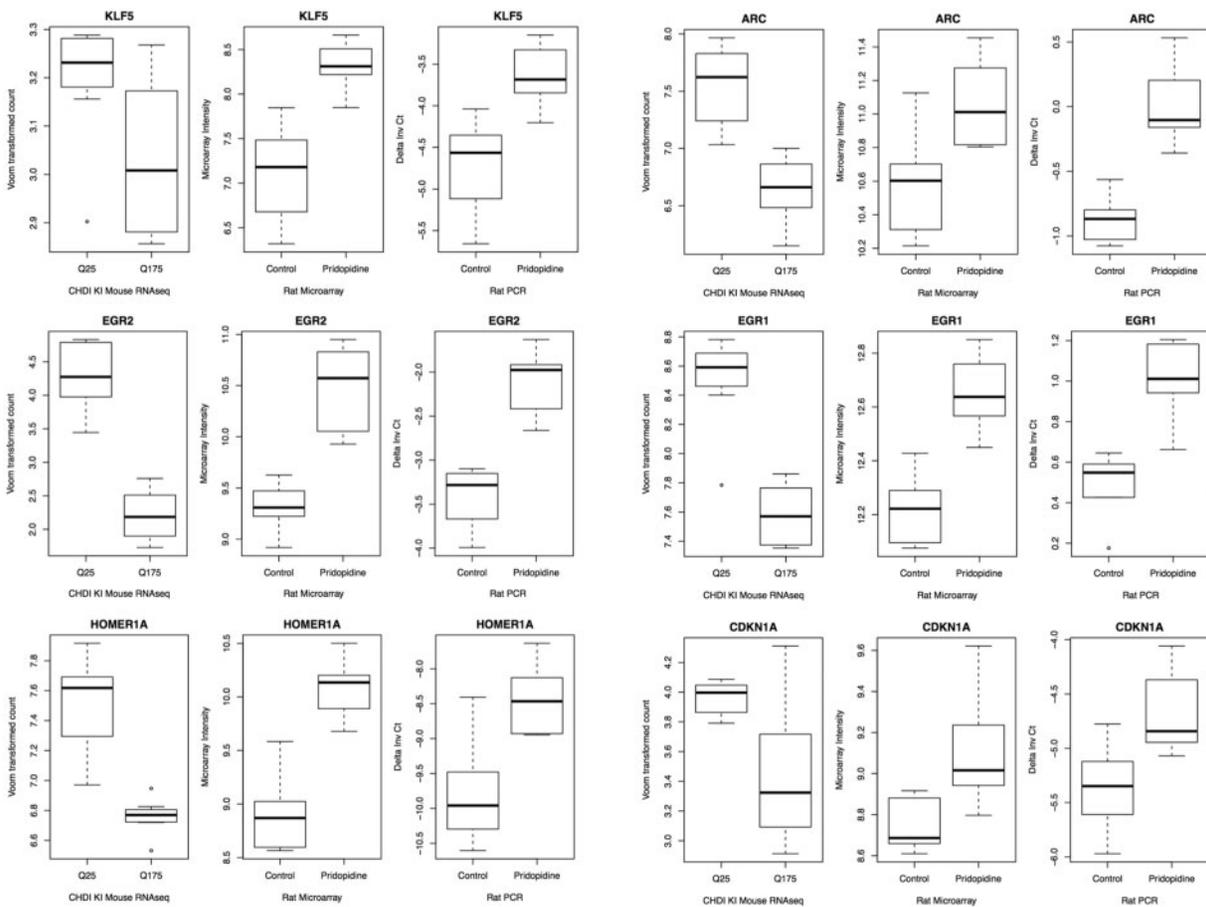


**Figure 1.** BDNF pathway genes modulated in HD CAG KI mice compared to WT and oppositely regulated by pridopidine treatment in rats.

Shown are six selected genes. qPCR was performed in the same samples as the microarray experiment, unless otherwise noted.

KLF5: RNAseq (ENSMUSG00000005148) FC = -0.17 P = 3.41E-02 adj P = 7.23E-02; microarray (1368363_at) FC = 2.29 P = 1.06E-04 adj P = 9.32E-02; PCR P < 0.004

EGR2: RNAseq (ENSMUSG00000037868) FC = -2.09 P = 6.54E-07 adj P = 3.15E-05; microarray (1387306_a_at) FC = 2.26 P = 9.91E-06 adj P = 2.09E-02; PCR P < 0.001

HOMER1A: RNAseq (ENSMUSG00000007617) FC = -0.75 P = 9.41E-06 adj P = 2.46E-04; microarray (1370997_at) FC = 2.25 P = 7.70E-06 adj P = 2.00E-02; PCR (result is from Table 2 dose response study 60mg treatment) P < 0.001

ARC: RNAseq (ENSMUSG00000022602) FC = -0.90 P = 4.04E-05 adj P = 7.79E-04; microarray (1387068_at) FC = 1.38 P = 7.55E-03 adj P = 4.79E-01; PCR P < 0.0002

EGR1: RNAseq (ENSMUSG00000038418) FC = -0.93 P = 3.88E-06 adj P = 1.26E-04; microarray (1368321_at) FC = 1.35 P = 6.44E-05 adj P = 6.47E-02; PCR P < 0.0007

CDKN1A: RNAseq (ENSMUSG00000023067) FC = -0.53 P = 4.28E-03 adj P = 1.71E-02; microarray (1388674_at) FC = 1.29 P = 7.81E-03 adj P = 4.86E-01; PCR P < 0.02.
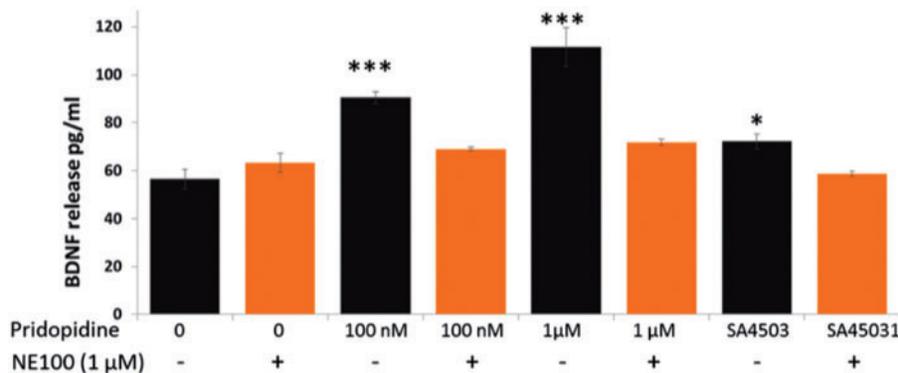
**Figure 2.** Effect of pridopidine treatment on BDNF release (pg/ml) as measured by in situ ELISA.

Data is expressed in pg/ml (mean ± SEM; *$P <$ 0.05; **$P <$ 0.01; ***$P <$ 0.001; one way ANOVA followed by Dunnett's test). SA4503 (1μM) was used as positive control. NE100, sigma-1 receptor antagonist (1μM).

**Table 2.** Pridopidine dose-response expression profiles of BDNF pathway genes. Presented is the fold change in mRNA expression level versus vehicle for each gene. Significance levels are marked based on unadjusted $P$-values from two-sample t test comparing delta Ct values versus vehicle, * $P <$ 0.05, **$P <$0.01, ***$P \leq$ 0.001

| Pridopidine dose | EGR1 | ARC | HOMER1A | EGR2 | KLF5 | CDKN1A | CEBPB |
|---|---|---|---|---|---|---|---|
| 0.3 mg/kg | 1.17 | 1.06 | *1.62 | 1.17 | 1.42 | 1.03 | 1.00 |
| 3 mg/kg | *1.32 | 1.30 | **1.86 | 1.53 | 1.33 | 1.34 | *1.29 |
| 15 mg/kg | **1.57 | ***1.69 | ***2.83 | **2.01 | *1.79 | 1.17 | 1.24 |
| 30 mg/kg | ***2.05 | ***2.25 | ***3.01 | ***3.63 | ***2.74 | **1.41 | ***1.57 |
| 60 mg/kg | **1.60 | ***1.55 | ***2.60 | ***2.60 | **1.80 | ***2.03 | ***1.43 |

was inhibited when the cells were co-incubated with NE100, a known S1R antagonist (Figure 2).

To strengthen the hypothesis that the BDNF pathway is upregulated due to activation of the S1R, we treated SD rats with lower doses of pridopidine (range 0.3–60 mg/kg) previously shown to reflect specific occupancy of the S1R versus D2R, and analysed the expression of seven selected genes in the BDNF pathway by qPCR. Pridopidine doses of 3 and 15 mg/kg in rats occupy 57 ± 2% and 85 ± 2% of S1R, respectively, and both do not show occupancy of the D2R, as determined by *in vivo* PET imaging (7). The significant occupancy proportion of the D2R (44–66%) was observed only at a dose of 60 mg/kg. This PET study supports the conclusion that the upregulation of genes in rats treated with 15 mg/kg pridopidine are a result of specific activation of the S1R. At 30 mg/kg, partial/low occupancy of the D2R is at levels of 22–33% (assuming linearity), and S1R is saturated. Indeed, qPCR analysis revealed that the upregulation of EGR1 (already up at 3 mg/kg), EGR2, HOMER1A, KLF5, and ARC expression were upregulated at the low 15 mg/kg dose and expression of CDNK1Aand CEBPB were significantly upregulated from a low dose of 30 mg/kg (CEBPB was significantly increased at 3 mg/kg but not at 15 mg/kg) (Table 2).

### Genome-wide analysis identifies the PI3K/AKT pathway, downstream of the BDNF receptor, as modulated by pridopidine

A hypothesis-free multi-pathway analysis using MsigDB's cellular pathways set revealed that genes upregulated in striatum by pridopidine treatment were positively enriched for the "Biocarta PI3K/AKT activation" pathway by GSEA ($P =$ 0.004). Genes in the leading edge included NR4A1, CDKN1A, IRS2, MDM2, and PIK3R2 (all leading edge genes can be found in Figures 1 and 3, or

Supplementary Material, Table S1). Differential expression of top fold change gene NR4A1 and overlapping BDNF pathway gene CDKN1A were then tested by qPCR and confirmed to be highly significantly increased (NR4A1 $P =$ 1E-6 Figure 3, CDKN1A $P =$ 0.017, Figure 1).

Since many studies have shown that BDNF signalling activates the PI3K/AKT pathway through PI3K (22), this result further supports the hypothesis that pridopidine may be neuroprotective through BDNF and/or PI3K/AKT pathway modulatory effects.

### Hypothesis-driven analysis corroborates impact of pridopidine on dopaminergic and glucocorticoid pathways

To further evaluate pridopidine's effect on additional pathways, a targeted pathway enrichment analysis was pursued for both dopaminergic transmission and glucocorticoid signalling.

Pridopidine is known to modulate dopaminergic transmission and was shown to stabilize dopamine-dependent motor activity (3). The hypothesis supporting pridopidine treatment impact on dopamine receptor signalling was tested using a published list of 33 genes with transcription levels that are changed in a dopamine D1 receptor (D1R)-dependent fashion in the striatum (23). Genes were ranked by pridopidine-induced fold-change, and then queried for enrichment of this D1R associated gene list using GSEA. Pridopidine significantly affected the expression of D1R associated genes ($P <$ 0.001, all leading edge genes listed in, Figures 1 and 4, Table 1 or Supplementary Material, Table S1). To summarize, pridopidine may also be impacting dopamine receptor signalling as evidenced by pathway enrichment as well as changes in individual pathway genes such as REM2 and PER1 (displayed in Figure 4).
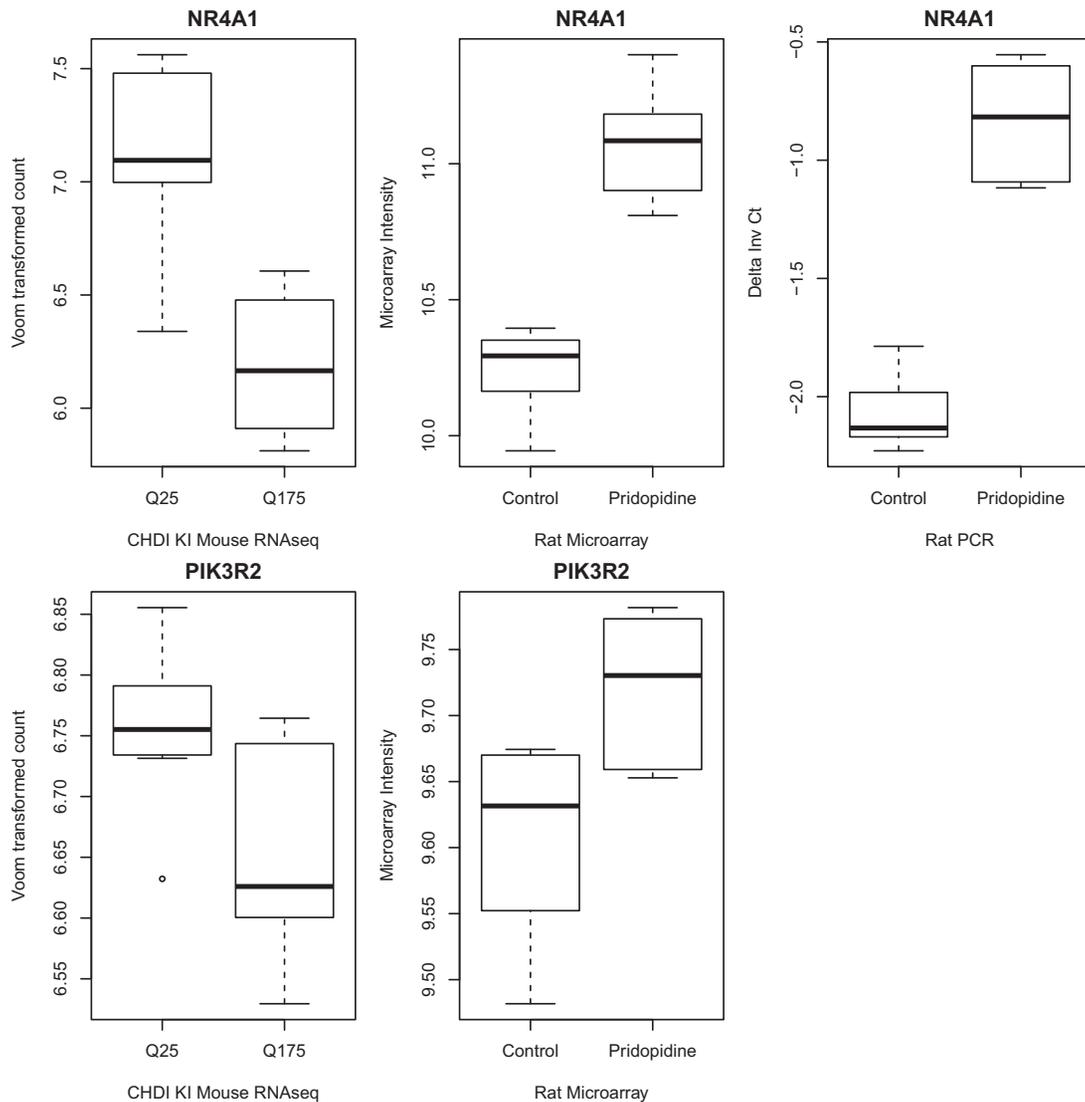
**Figure 3.** PI3K/AKT pathway genes modulated in HD CAG KI mice compared to WT and oppositely regulated by pridopidine treatment in rats.

CDKN1A is also part of the PI3K/AKT pathway lading edge and is shown in Figure 1. PCR is of same samples as microarray.

NR4A1: RNAseq (ENSMUSG00000023034) FC= -0.94 P = 8.06E-05, adj P = 1.36E-03; microarray (1386935_at) FC = 1.79 P = 1.18E-06, adj P = 4.25E-03; PCR P < 0.000001

PIK3R2: RNAseq (ENSMUSG00000031834) FC= -0.10 P = 1.40E-02 adj P = 3.25E-02; microarray (1370100_at) FC = 1.08 P = 2.57E-02 adj P = 6.30E-01; PCR not tested

There is a well-documented interaction between the glucocorticoid pathway and the BDNF pathway, which provided motivation to assess the transcriptomic profile of glucocorticoid therapies reported as effective in HD models (24). Twenty-two genes were previously reported to change significantly following dexamethasone (glucocorticoid) treatment in rat hypothalamus (25). GSEA of all genes using pridopidine FC-based ranking list revealed significant positive enrichment of genes compared to those upregulated post-dexamethasone-treatment (P < 0.001) and, consistently, significant negative enrichment of genes downregulated with dexamethasone treatment (P = 0.004, all up and down leading edge genes in Figure 5, or Supplementary Material, Table S1). Specific enriched glucocorticoid response genes with top upregulation statistics in pridopidine treated rat striatum, such as SGK1, NFKBIA, DDIT4, PDK4, DUSP1, and

TIPARP, are shown in Figure 5 (all remaining leading edge genes not in a figure are in Supplementary Material, Table S1). Upregulation in expression of SGK1, NFKBIA, DDIT4, DUSP1 and PDK4 was further confirmed by qPCR (Figure 5).

An independent statistical analysis employing hypergeometric tests, in which genes changing in expression with pridopidine treatment were compared to genes changing in expression with dexamethasone, revealed highly significant overlap of upregulated (P = <1e-10) and downregulated (P < 0.0009) genes.

### Key pathways modulated by pridopidine are significantly and inversely expressed in an HD mouse model

In order to assess the relevance of these findings in a disease context, a reciprocity hypothesis was tested: gene expression
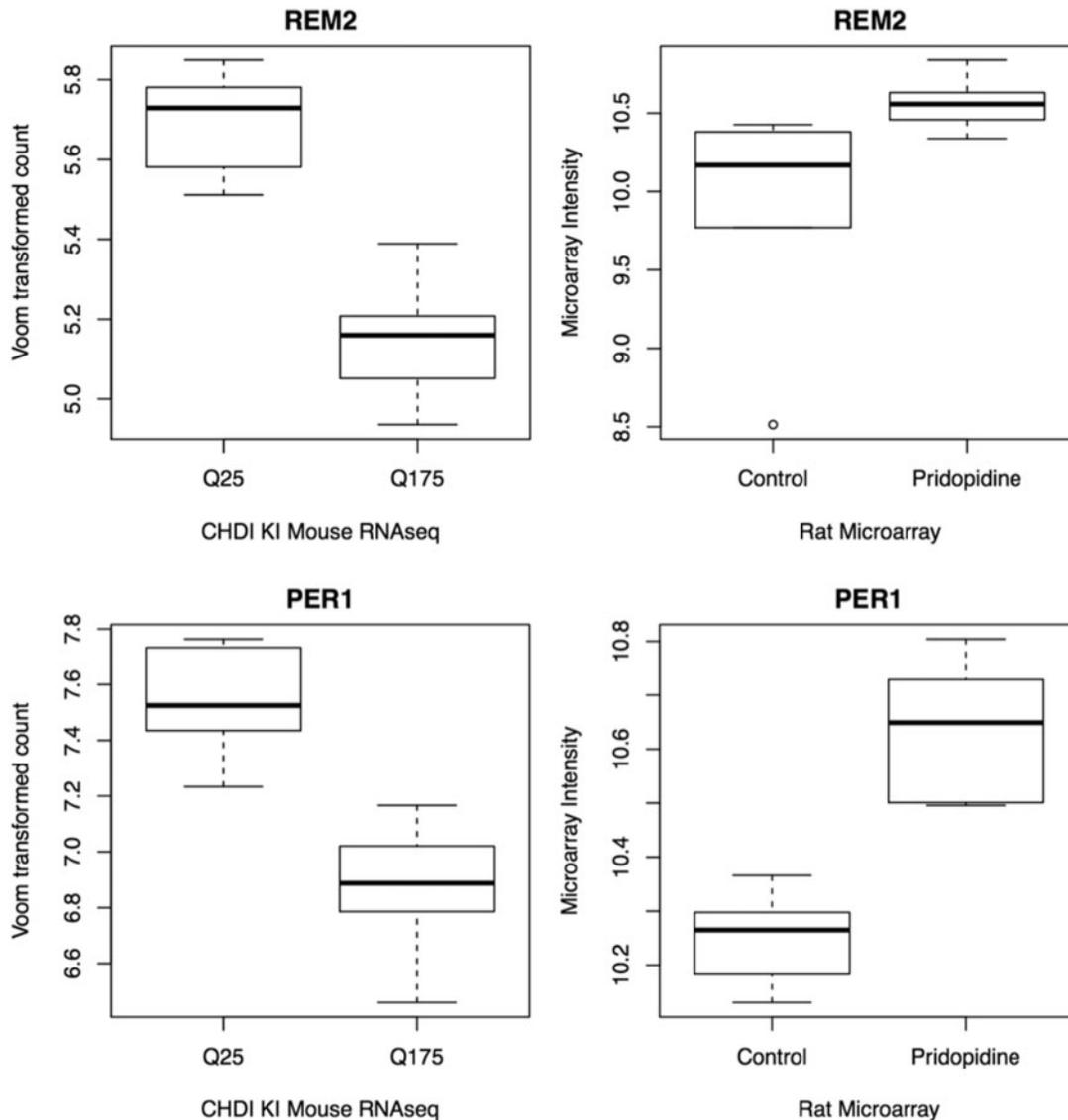
**Figure 4.** D1R pathway genes modulated in HD CAG KI mice compared to WT and oppositely regulated by pridopidine treatment in rats.

EGR1, EGR2, and KLF5 are also part of D1R leading edge and presented in Figure 1.

REM2: RNAseq (ENSMUSG00000022176) FC = -0.55 P = 2.99E-07, adj P = 1.71E-05; microarray (1374035_at) FC: 1.58, P = 2.63E-02, adj P = 6.30E-01; PCR not tested

PER1: RNAseq (ENSMUSG00000020893) FC = -0.67 P = 4.23E-06, adj P = 1.34E-04; microarray (1374855_at) FC: 1.31 P = 2.2E-05, adj P = 0.03; PCR not tested

changes associated with pridopidine treatment in the above-described pathways were tested in a mouse model of HD, assuming that a therapeutic benefit would be expected if the two profiles exhibit opposing directionality. The CHDI Foundation public RNAseq raw dataset, from Q175KI HD mice (compared to the healthy Q25KI control mice) were analysed against the rat pridopidine gene expression data. The BDNF pathway leading edge contains twenty-eight genes (Figure 1, Table 1 and Supplementary Material, Table S1) with gene symbols, out of which nineteen were conversely downregulated (P < 0.05) in the Q175KI mouse (nineteen genes listed in Figure 1, Table 1, and Supplementary Material, Table S1). Moreover, the PI3K/AKT pathway members NR4A1, PIK3R2 (Figure 3), and CDKN1A (Figure 1) that were found to be upregulated by pridopidine treatment, were also found to be downregulated in the Q175KI

mouse. Lastly, several GR and D1R pathway genes including REM2, PER1, TIPARP, and DUSP1 are upregulated by pridopidine treatment and downregulated in the Q175KI mouse (Figures 4 and 5, Supplementary Material, Table S1).

## Discussion

Pridopidine therapy is being evaluated for motor benefits in patients with HD, having demonstrated positive effects in the secondary endpoints of two independent clinical trials, [4,5] which cannot be fully attributed to its dopamine stabilization effects. The current study sought to unravel additional mechanisms in an unbiased approach by profiling the effects of pridopidine treatment on rat striatal tissue. The striatum is the first brain region to manifest HD degeneration (26), and thus comprises a
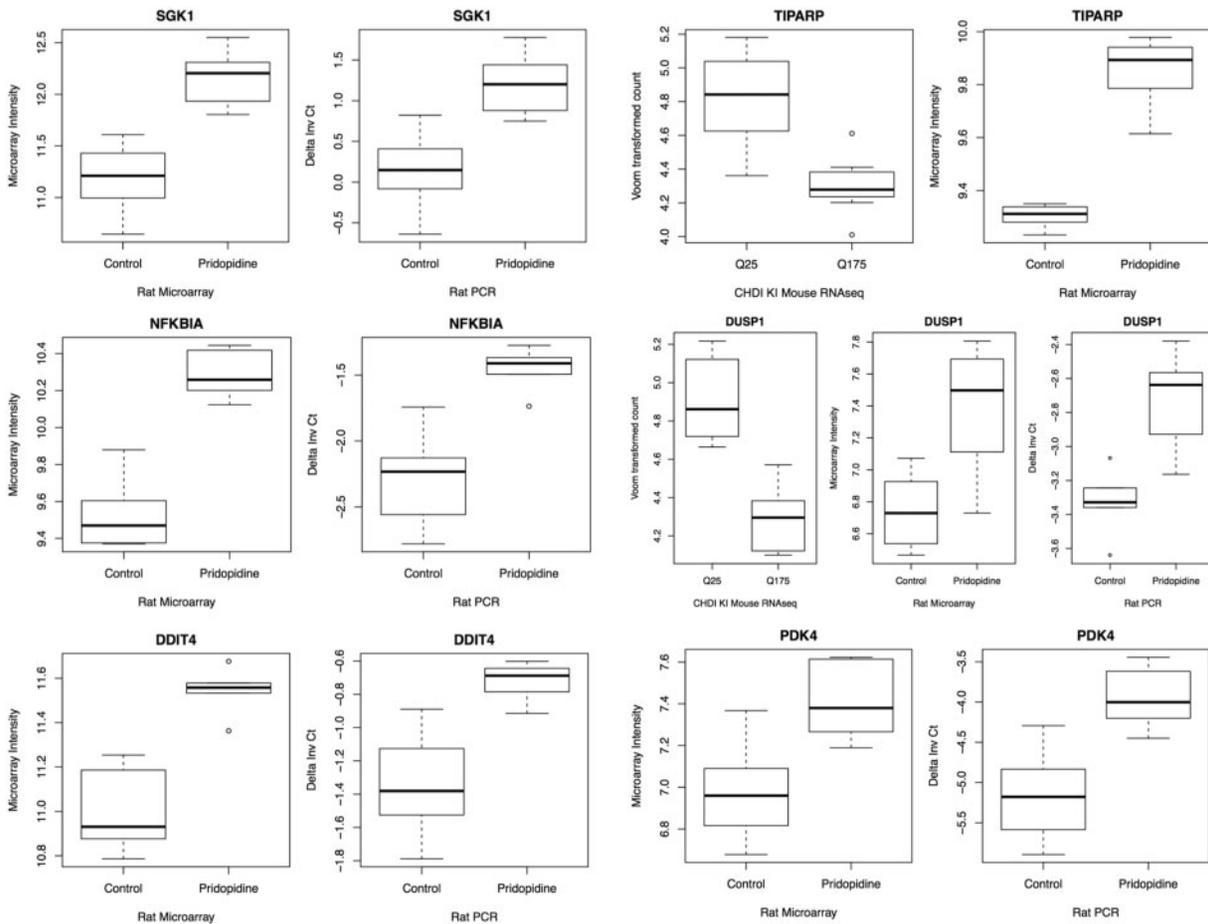
**Fig 5.** GR pathway genes modulated in HD CAG KI mice compared to WT and oppositely regulated by pridopidine treatment in rats.

pPCR was performed in the same samples as the microarray experiment.

SGK1: RNAseq not significant; microarray (1367802_at) FC = 1.98 $P$ = 2.28E-05 adj $P$ = 2.95E-02; PCR $P <$ 0.002

NFKBIA: RNAseq not significant; microarray (1389538_at) FC = 1.69 $P$ = 7.40E-07 adj $P$ = 3.84E-03; PCR $P <$ 0.0004

DDIT4: RNAseq not significant; microarray (1368025_at) FC = 1.46 $P$ = 1.01E-05 adj $P$ = 2.09E-02; PCR $P <$ 0.001

TIPARP: RNAseq (ENSMUSG00000034640) FC = -0.52 $P$ = 4.66E-04 adj $P$ = 5.58E-03; microarray (1374446_at) FC = 1.46 $P$ = 2.90E-07 adj $P$ = 2.16E-03; PCR not tested

DUSP1: RNAseq (ENSMUSG00000024190) FC = -0.63 $P$ = 8.71E-06 adj $P$ = 2.31E-04 0.000231857; microarray (1368146_at) FC = 1.42 $P$ = 1.98E-03 adj $P$ = 3.09E-01; PCR $P <$ 0.001

PDK4: RNAseq not significant; microarray (1369150_at) FC = 1.35 $P$ = 1.63E-03 adj $P$ = 2.86E-01; PCR $P <$ 0.002

key therapeutic target. The results reported herein indicate that pridopidine upregulates multiple neuroprotective pathways, which could translate into a long-term benefit in various motor and cognitive scales in clinical settings.

## BDNF pathway

Pridopidine significantly increased expression of genes known to be induced by the neuroprotective factor BDNF (17), including EGR1, EGR2, KLF5, CDKN1A, HOMER1A, and ARC as measured by microarray and confirmed by qPCR analysis (Figure 1, Table 2). BDNF plays a critical role in neuronal survival in HD. The BDNF receptor has been shown to be downregulated in brains from HD patients as compared to healthy people (27). BDNF overexpression in the YAC128 mouse model was reported to rescue HD features, including decreased motor function, cognitive

deficits, brain atrophy, and abnormal spine density (28). Cortical BDNF production has been shown to improve corticostriatal synaptic activation and enhance the survival of the GABAergic medium spiny neurons (MSNs) (29). Pridopidine induced gene expression in the BDNF pathway was compared to the expression of these genes in the striatum of the Q175KI HD mouse model. Of the pridopidine GSEA leading edge of BDNF enriched genes, 19 were nominally downregulated in the Q175KI mouse model (genes listed in Figure 1, Table 1).

## Pi3k/Akt pathway

Hypothesis-free multi-pathway analysis revealed enrichment of the PI3K/AKT pathway for genes upregulated by pridopidine treatment. AKT is a serine-threonine kinase also called protein kinase B (PKB), known to be a potent pro-survival kinase. AKT

exerts survival effects in neurons by phosphorylating several substrates, including apoptotic proteins (BAD), glycogen synthase 3 (GSK3) and the transcription factors of the forkhead family (FOXOs) (30).

AKT signalling is critical for neuronal function, and dysregulation of AKT activity is observed in neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease (31), as well as HD (32). In HD, direct involvement of impaired AKT signalling is supported by the observation that phosphorylation of the mutant polyQ-huntingtin by AKT abrogates its toxicity (33). Additional support comes from the finding that in HD post-mortem brains, AKT was found to be cleaved and inactivated by caspase-3 (34). In a rat model of HD, AKT downregulation preceded any signs of degeneration (35). The increased level and activity of AKT were also found in lymphoblasts and lymphocytes from HD individuals and in a mouse knock-in model of HD. This is likely a compensatory pro-survival response in early disease manifestation (35).

### Dopamine D1R pathway

Pridopidine, a known modulator of the dopamine system has been shown to increase extracellular dopamine efflux in the striatum (23). Here we show that pridopidine increases expression of genes downstream of the D1R. As pridopidine is not known to directly bind and activate the D1R, this could be an indirect result of increased extracellular dopamine efflux. Of note, many of the genes induced by D1R, are common to the genes induced in the BDNF pathway (overlapping GSEA leading edge genes include CEBPB, EGR1, EGR2, EGR4, FOS, KLF5, NAB2 and NR4A3). As dopamine afferents to the striatum also express BDNF, which activates TRKB receptors (36), a possible interaction between DA and BDNF/TRKB signalling exists. For example CEBPB, EGR1, and FOS are activated by both D1R and TRKB receptors (37–39).

Dopamine actions in the striatum are mostly mediated by D1 and D2 receptors, which are coupled to different G proteins having opposite effects on the production of cyclic AMP (cAMP). D1Rs stimulates Adenylyl Cyclase (AC) activity, thus increasing intracellular cAMP levels (40,41), consequently activating the cAMP-dependent protein kinase A (PKA) (42). In contrast, D2Rs inhibit AC, thereby inhibiting cAMP increase and PKA activation. Pridopidine is known to act as a low affinity D2R antagonist (3). Since direct inhibition of D2R and indirect activation of D1R may thus result in similar downstream signal transduction, it is impossible to deduce in this study which of the two pathways is more pronouncedly affected by pridopidine.

### Glucocorticoid pathway

Gene expression results also support a role for pridopidine in the modulation of the glucocorticoid pathway (GSEA leading genes include PDK4, SGK1, DUSP1, DDIT4, and NFKBIA), mimicking the effects induced by the specific glucocorticoid receptor (GR) agonist dexamethasone. This is particularly noteworthy because dexamethasone has been shown to slow progression of HD in animal models (24), and dexamethasone-induced genes such as SGK1 have been shown to protect striatal neurons against HD-related toxicity (43). S1R ligands were previously reported to influence GR function in mouse fibroblast cells providing evidence for a possible functional interaction between S1R and GR (44). Pridopidine does not directly bind the GR (data on

file Teva Pharmaceutical Industries, not shown). It is therefore possible that pridopidine's effect on the GR pathway may be mediated by the S1R.

### Interplay between the key identified pathways

#### *AKT and BDNF*
The BDNF signalling pathway is connected to the downstream PI3K/AKT pathway activation (22). For example, BDNF binding to its receptor TRKB upregulates neuronal survival through an AKT phosphorylation-dependent mechanism (45). Furthermore PI3K/AKT signalling is needed for BDNF-induced dendrite formation in cortical neurons (46). Another report studying post-mortem brain tissues showed that BDNF treatment increases phosphorylation of AKT (47). Taken together, these findings suggest that the observed upregulation of the PI3K/AKT pathway by pridopidine may be mediated via upregulation of the upstream BDNF pathway, further substantiating pridopidine's effect on this neuroprotective pathway.

#### *AKT and dopamine*
Direct involvement of AKT with the dopaminergic transmission has also been observed. *In vitro* data from embryonic striatal neuron primary cultures revealed that D1R and D2R activation leads to increased phosphorylation of AKT (48). Additionally, *in vivo* studies have shown that in the mouse striatum, AKT is activated by cocaine (48). Moreover, AKT null mice demonstrated behavioural changes characteristic of dopaminergic transmission (49). On the other hand, elevation of dopamine in mice striatum was also reported to inhibit AKT, (and activate GSK3) but this involved activation of the D2R signalling cascade (50). Since pridopidine acts as an antagonist on the D2R, this may be an additional mechanism by which pridopidine increases activity of the PI3K/AKT pathway.

Several lines of evidence also reveal the important role of AKT signalling in dopamine-related behaviours. Hyperactivity in mice related to excessive dopamine tone (DAT-KO mice or amphetamine treated mice) is suppressed by inhibition of GSK3 (downstream to AKT activation) (51). Numerous additional studies have shown that alteration in dopamine-related functions and behaviours are caused by manipulation of the AKT pathway and conversely, genetic or pharmacological manipulation of dopamine receptors directly affect AKT signalling (52).

#### *Glucocorticoids and BDNF*
The GR and the BDNF receptor positively activate one another. The GR enhances BDNF-induced glutamate release (53) and dexamethasone induces phosphorylation of the BDNF receptor TRKB (54). Glucocorticoid receptors are also reported to enhance signalling by PI3K/AKT (55). Strong overlap between the GR and TRKB pathways was found during formation of long-term memory in rats, where activation of the GRs were coupled to the activation of CAMKIIα, TRKB, AKT, ERK, PLCγ and CREB (56). Interestingly, GR- and BDNF-related genes induced by pridopidine were partially overlapping with two genes in common: GADD45G and ERRFI1.

#### *Sigma-1 receptor*
Pridopidine treatment of rats, at doses known to specifically occupy the S1R but not the D2R, upregulated expression of BDNF pathway genes including EGR2, FOS, EGR1, ARC, KLF5, CDKN1A, HOMER1A and CEBPB. It is therefore suggested that upregulation of the BDNF pathway is driven by the interaction between

pridopidine and the S1R. Increases in BDNF secretion have been reported as a typical S1R agonist activity. For example, PRE084, a known S1R agonist, was shown to upregulate BDNF in the striatum of mice with 6-OHDA lesion (20). Similarly the high affinity S1R agonist SA4503 was reported to potentiate BDNF secretion in a cellular model via a S1R-dependent mechanism (21). Herein, we show that pridopidine enhances secretion of the BDNF protein in rat neuroblastoma cells, and that this increase is mediated via the S1R, as treatment with NE100, a known antagonist of the S1R, abolished the increase in BDNF. To summarize, this data shows that pridopidine activation of the BDNF pathway is likely mediated through S1R.

Genetic abnormalities in S1R have been implicated in a variety of neurodegenerative and neuropsychiatric diseases, and activation of the S1R is thought to exert protective therapeutic effects in some of these disease conditions (19). For example, the S1R agonist, igmesine was shown to have neuroprotective and anti-depressant effects in several animal models (57). Another S1R agonist, SA4503, inhibited deficits in a mouse long term memory test (58). Moreover, treatment of a mouse model of Parkinson's disease with the S1R agonist PRE-084 demonstrated positive modulation of dopaminergic fibre density and increased neurotrophic factors (20). Taken together, these results suggest S1R as a possible HD therapeutic target. Adding to the growing evidence of the protective effects of S1R is Donepezil (Aricept), an approved drug for treating AD, which also targets S1R in the brain, suggesting S1R stimulation could be contributing to Donepezil efficacy in AD (8).

In conclusion, pridopidine upregulates gene expression in the BDNF, PI3K/AKT, D1R, and GR pathways (summarized in Figure 6) in opposite directionality to the dysregulation induced by HD pathology. These pathways share several gene targets and functional implications. For example, there are several genes common to the BDNF and dopamine signalling pathways, which are upregulated by pridopidine (CEBPB, EGR1, EGR2, FOS and KLF5). These tight interactions between pathways in the directionality predicted to alleviate disease further substantiates the potential role for pridopidine in promoting cognition and neuroprotection. Studying pridopidine's effects directly in HD and other neurodegenerative disease models may provide additional insights into its therapeutic potential. Overall, pridopidine presents a unique mode of action, beneficially stabilizing motor activity and in parallel upregulating neuroprotective pathways known to be impaired in neurodegenerative diseases, in particular HD.

## Materials and Methods

### Drugs

Pridopidine HCL (TV-7820, ACR16, Batch #150IN0758A) dissolved in sterile ddH$_2$O. Sterile ddH$_2$O was the vehicle control.
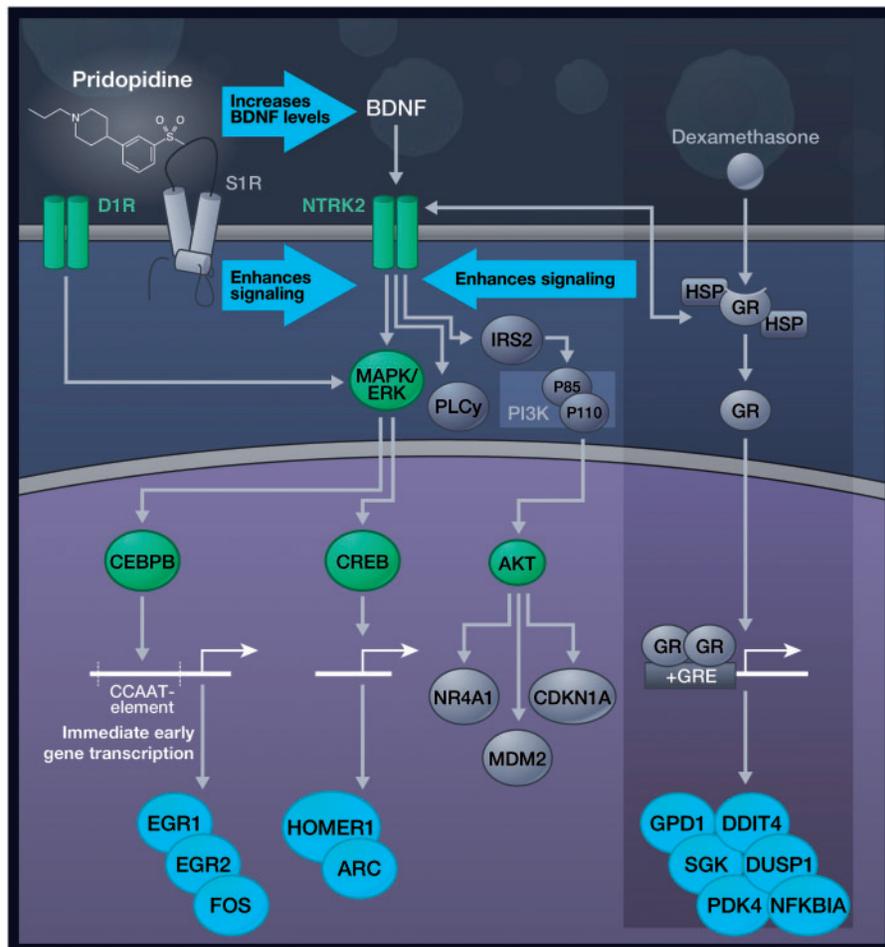


**Figure 6.** Impact of pridopidine treatment on BDNF signalling, AKT activation, and the glucocorticoid pathway.

## Experimental design of pridopidine versus control treatment

Sprague Dawley (SD) male rats ($n = 6$ per group) were treated daily by oral gavage with pridopidine at a dose of 60 mg/kg or vehicle (water) over the course of 10 days. On day 10, 90 min following last drug/water administration, brains were removed, and quickly rinsed with cold physiological saline. The striatum of the left hemisphere was gently extracted and immediately immersed in 1000 μl of RNAlater® Solution (AB-AM702, Life Technologies, Rhenium Israel) in pre-labelled polypropylene vials and stored at 4 °C overnight (to allow the solution to thoroughly penetrate the tissue), then moved to –20 °C until analysis. RNA was isolated from the striatum of each rat and analysed as described below. This study aimed to profile gene expression changes in a dose regimen that has been validated to induce a robust efficacy readout *in-vivo*. Pridopidine dose of 60 mg/kg administered by gavage was chosen as it was consistently efficacious *in vivo* in reducing motor activity in hyperactive rats. The nonclinical safety profile of pridopidine at 60 mg/kg, was separately evaluated in SD rats, in a 21 days repeat-dose oral toxicity studies. Plasma exposure levels of pridopidine tested at day 21 were below the level found to correlate with CNS side effects and was thus considered safe (data on file, Teva Pharmaceutical Industries, not shown).

## RNA extraction

Striatum tissue samples were homogenized in QIAzol Lysis Reagent and total and miRNA purified using the miRNeasy Mini Kit per manufacturer's instructions (Qiagen). RNA integrities were assayed using an Agilent Bioanalyzer system and quantitation was completed using a NanoDrop instrument. One μg of total RNA was converted into labelled target antisense RNA (cRNA) using the single round RNA amplification and Biotin Labelling system (Enzo). Resultant cRNA was purified by magnetic beads and quantified via spectrophotometry. A total of 11 μg of purified cRNA was fragmented using a 5X fragmentation buffer, then a hybridization mixture was prepared following the affymetrix hybrization, washed and stained using the Mini Kit methods. cRNA was applied to arrays, incubated at 45 °C for 16 h, washed and stained and subsequently scanned using the Affymetrix GeneChip Scanner.

## Gene expression microarray analysis

The gene expression data from the 12 striatum samples (6 pridopidine-dosed and 6 vehicle-dosed) was Robust Multichip Average (RMA) normalized (affy package v1.42.3) in R(v3.1.2). Probesets were annotated according to the Affymetrix Rat230_2 Release 22 annotation file and principal component analysis (PCA) was used to test for outlier samples and batch effects. Probesets without associated gene symbols in this release were identified using Rat230_2 Release 32 and 34. The limma package (v3.18.13) in R (v3.1.3) was used to identify probesets differentially expressed between the two groups of biological replicates, and all probesets were tested for differential expression. In order to query pathway biology, the probesets/genes were ranked by FC between pridopidine treatment and vehicle. This was used as a ranked list in gene set enrichment analysis (GSEA) v2.1.0 (18) pre-ranked analysis. If the gene set being queried was generated from a study using the Affymetrix Rat230 2 array, the gene set and ranked list were directly analysed in GSEA using probesets. When using a gene set or gene set database with human gene symbols as identifiers, the probeset with the highest absolute value of fold change (FC) was reported for that gene, and all rat gene symbols were converted to human gene symbols before conducting GSEA preranked analysis. Pathway enrichment of the ranked list was tested against mSigDB, and custom gene sets generated from the literature. When broadly testing pathways corrections for multiple hypothesis testing were performed as described in Subramanian (18).

For the candidate gene set approach, the raw *P*-value was used, as only one hypothesis was tested. A standard hypergeometric test was employed to calculate enrichment between two sets of genes. The test was run utilizing parameters of universe size, number of genes in the gene set, number of genes observed to change in the relevant direction, and number of genes both present in the gene set and modulated in the relevant direction.

## Gene expression RNAseq analysis of HD versus WT mouse model

Publicly available RNAseq count level data of the human huntingtin gene knockin mouse model with varying CAG repeats were downloaded from http://chdifoundation.org/datasets/ and normalized using the voom transform from limma v3.18.13 in R v3.1.3. Changes in expression levels between mouse striatum samples homozygous for the 20 CAG repeat allele (Q20), representing wild-type, and those heterozygous for the 175 CAG repeat allele (Q175), exhibiting HD pathology, were queried using lmFit from the limma package. All fold changes (FC) reported from this analysis are log fold change.

## Dose-range confirmatory study and qPCR analysis

SD male rats were divided into 6 groups ($n = 10$ per group) and treated daily by oral gavage with pridopidine at doses of 0.3, 3, 15, 30, 60 mg/kg or vehicle for five days. On day 5, 90 min following the last drug administration, brains were removed and processed as described earlier. The sample mix, cDNA 125 ng and respective primers were loaded onto a primed Fluidigm Dynamic platform and qPCR was performed per manufacturer's instructions. Fluidigm Real-Time PCR Analysis software calculated cycle threshold (ct) values with the following settings: Quality Threshold 0.65, Baseline Correction Linear, Ct Threshold Method Auto. Two negative controls including a no enzyme control (NEC) and a no template control (NTC) were included in the real-time reaction. All samples were processed in duplicate. Delta-delta Ct values for each assay per sample were calculated using an average of three housekeeping genes including RPS15a, HK1, and HPRT. The statistical significance of differential expression of each dose versus vehicle was assessed using a two-sample t test on the delta Ct values. Differentially expressed genes were selected for testing based on statistical significance observed in genome-wide microarray analyses and biological relevance. Probes were selected to represent the significant array probeset as closely as possible. 16 Taqman probes were used for qPCR analysis: HOMER1A Rn01489773_m1; EGR2 Rn00586224_m1; KLF5 Rn00821442_g1; CEBPB Rn00824635_s1; NR4A1 Rn01533237_m1, SGK1 Rn01537468_g1; NFKBIA Rn01473657_g1; DUSP1 Rn00587176_g1; PDK4 Rn00585577_m1; ARC Rn00571208_g1; EGR1 Rn00561138_m1; CDKN1A Rn01427989_s1; DDIT4 Rn01433735_g1; HK1 Rn00562436_m1, RPS15A Rn00821570_g1, HPRT1 Rn01527840_m1.

## BDNF secretion analysis in neuroblastoma rat cells via in-situ ELISA

B104 cells secrete higher BDNF levels than other cell lines and hence were selected as the model system for induction of BDNF

secretion post-treatment. B104 rat neuroblastoma cells were maintained in 1:1 mixture of Eagle's MEM and F12 containing 10% FBS, PS (1%) at 37 °C with 5% $CO_2$ as described (21). Cells were plated in 96-well Nunc MaxiSorp surface polystyrene flat-bottom immunoplate (Ref Dutscher: 055133) precoated with an anti-BDNF monoclonal antibody at a density of 60,000 cells/well. The procedure was carried out as previously described. Briefly, after 1.5 h of incubation to ensure cell attachment to the plate, cells were incubated for additional 2 h with fresh culture medium to remove any residue of the ELISA washing solution. Cells were grown for 5 days in serum-free DMEM containing pridopidine (100 nM or 1 μM), control medium or cutamesine 1 μM (SA4503;Tocris) with or without the S1R antagonist NE100 (Sigma, batch 023M4623V 1 μM). SA4503 (cutamesine), a reference S1R agonist was used to induce BDNF secretion. After 5 days, cells were carefully washed with TBST (20 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.05% (vol/vol) Tween 20) and were then incubated with the polyclonal anti-human BDNF antibody overnight. The amount of specifically bound polyclonal antibody was detected by the use of the anti-IgY-horseradish peroxidase tertiary antibody, which when exposed to a chromogenic substrate (TMB reagent; Promega), changes colour in proportion to the amount of BDNF protein present in the sample. Colour intensity was quantified by measuring the absorbance at 450 nm. BDNF standards, ranging in concentration from 0 to 500 pg/ml were added in parallel wells in the same medium used for B104 cells. Data are expressed as mean ± SEM (6 per condition). A global analysis of the data was performed using an unpaired *t*-test with an alpha level of 0.05. In situ ELISA assay was performed at NeuronExperts SAS, France.

## Supplementary Material

Supplementary Material is available at *HMG* online.

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