The psychiatric risk gene NT5C2 regulates AMPK signalling and protein translation in human neural progenitor cells


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Abstract

The cytosolic 5’-nucleotidase II gene (NT5C2, cN-II) is associated with disorders characterised by psychiatric and psychomotor disturbances. Common psychiatric risk alleles at the NT5C2 locus reduce expression of this gene in the foetal and adult brain, but downstream biological risk mechanisms remain elusive.

Background

Distribution of the NT5C2 protein in the human DLPFC and cortical human neural progenitor cells (hNPCs) was determined using immunostaining, publicly available expression data, and reverse transcription quantitative PCR (RT-qPCR). Phosphorylation quantification of AMPK-alpha (Thr172) and ribosomal protein S6 (RPS6) (Ser235/Ser236) were performed using western blotting, to infer the degree of activation of AMPK signalling and the rate of protein translation. Knockdowns were induced in hNPCs and Drosophila melanogaster using RNA interference (RNAi). Transcriptomic profiling of hNPCs was performed using microarrays, and motility behaviour was assessed in flies using the climbing assay.

Methods

Expression of NT5C2 was higher during neurodevelopment, and was neuronally enriched in the adult human cortex. Knockdown in hNPCs affected AMPK signalling, a major nutrient sensing mechanism involved in energy homeostasis, and protein translation. Transcriptional changes implicated in protein translation were observed in knockdown hNPCs, and expression changes to genes related to AMPK signalling and protein translation were confirmed using RT-qPCR. The knockdown in Drosophila was associated with drastic climbing impairment.

Results

Conclusions

We provide an extensive neurobiological characterisation of the psychiatric risk gene NT5C2, describing its previously unknown role on the regulation of AMPK signalling and...
protein translation in neural stem cells, and its association with *Drosophila melanogaster* motility behaviour.
Introduction

The cytosolic 5'-nucleotidase II gene (NT5C2, cN-II) encodes a phosphatase associated with disorders characterised by psychiatric and psychomotor disturbances, including major psychiatric conditions (1-4), Parkinson’s disease (5), and spastic paraplegia (6). The NT5C2 enzyme cleaves purinergic monophosphate nucleotides, with particularly high affinity for adenosine monophosphate (AMP) (7). These energetic molecules are required for the extensive transcriptional reprogramming governing cell maintenance, proliferation, migration, and differentiation during neurodevelopment (8-11), and are implicated in adult brain function and psychiatric and psychomotor disturbances (12-14).

We previously showed that common psychiatric risk variants at the NT5C2 locus are associated with reduced neurological expression of this gene in population controls and in the foetus (3). As a key regulator of intracellular AMP, we hypothesise that NT5C2 modulates the AMP-activated protein kinase (AMPK), a major energy homeostasis regulator (15, 16). AMPK signalling has been previously associated with psychiatric disorders (17-20), NT5C2 function in muscle fibres (21), and also with protein translation (22-27) and motility behaviour (17, 28, 29), which are highly energy consuming processes. However, the underlying gene regulatory networks that mediate the effect of NT5C2 on AMPK signalling in the context of psychiatric disorders, cell types or developmental time point remains unclear.

Here, we have investigated NT5C2 expression, function and protein distribution in the human brain and neural progenitor cells (hNPCs); its role on the regulation of AMPK signalling and protein translation, and association with climbing behaviour in Drosophila melanogaster. First, to extend our previous work, we identified the major cell types expressing NT5C2 in the adult brain, which showed that NT5C2 protein is more expressed
in neurons relative to glial cells. Second, we provide complementary evidence that this gene is more expressed during neurodevelopment, suggesting an important role at this developmental stage. Third, we investigate the effects of NT5C2 loss-of-function on the phosphorylation of AMPK-alpha (Thr172) and ribosomal protein S6 (Ser235/Ser236) in hNPCs, suggesting a regulatory role on AMPK signalling and protein translation. Finally, based on evidence from genetic studies implicating NT5C2 in psychomotor disturbances, we tested the effect of a loss-of-function on climbing behaviour using Drosophila melanogaster (D. melanogaster) as model organism, confirming a role in motility behaviour.

The present study provides an extensive neurobiological characterisation of NT5C2, describing its hitherto unknown relationship with AMPK signalling and protein translation in neural stem cells, and a role for motility behaviour in fly. Ultimately, these data demonstrate biological mechanisms associated with NT5C2 that may explain its association with psychiatric disorders.

Methods and Materials

See Supplemental Information for further details on Methods and Materials.

Brain samples

To identify cell-type specific expression of NT5C2 in the adult cortex, we obtained samples from unaffected controls from the Medical Research Council London Neurodegenerative Disease Brain Bank, at the Institute of Psychiatry, Psychology & Neuroscience, King’s College London (UK Human Tissue Authority licence #12293).

Immunohisto- and cytochemistry
Brain sections were deparaffinised and submitted to antigen retrieval and autofluorescence removal protocols (Supplemental Information). hNPCs were fixed and processed as previously described (30). The following primary antibodies were used: NT5C2 (M02-3C1) (Abnova, Taipei, Taiwan), IBA1 (Menarini Diagnostics, Winnersh, United Kingdom), GFAP (Dako Agilent, Santa Clara, United States), MAP2, Parvalbumin and Beta-III-Tubulin (Abcam, Cambridge, United Kingdom). Fluorescently labelled secondary antibodies included Goat or Rabbit Alexa 488, 568 and 633 antibodies (Thermo Fisher Scientific, Waltham, Massachusetts, United States).

Cell lines

We used hNPCs from the CTX0E16 neural stem cell line (31) or from human induced pluripotent stem cells (hiPSCs) from an unaffected control (30), as well as human embryonic kidney cells 293T (HEK293T) to identify the subcellular distribution and function of NT5C2. The CTX0E16 neural cell line (31) was obtained from ReNeuron Ltd. under a Material Transfer Agreement. All lines were derived and maintained as described in the Supplemental Information and elsewhere (30, 31).

RNA and protein isolation and quantification

To identify gene and protein expression and phosphorylation differences in knockdown or overexpression cultures, we isolated total RNA or protein using TRI Reagent or RIPA Buffer supplemented with Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific), respectively. Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR), RNA quality control and western blotting details are available in the Supplemental Information. Primary antibodies for western blotting included: AMPK-alpha (D6) and phospho-AMPK-alpha (Thr172) (Santa Cruz Biotechnology, Dallas, Texas, United States),
total RPS6 (54d2) and phospho-RPS6 (Ser235/Ser236) (Cell Signalling, Danvers, Massachusetts, United States).

**Fly stocks and climbing assay**

We used Gal4-upstream activated sequences (UAS) (32) to knockdown CG32549 in specific tissues, by crossing a CG32549-RNAi line (KD: v30079) with UAS lines where Gal4 expression (and thus knockdown) is driven throughout the body (ACT5C: BL4414), in neurons and neural progenitor cells (ELAV: BL8765), or in gut (GUT: DGRC113094). Crosses were submitted to the negative geotaxis assay (33), a cost-effective test that has been previously used to investigate the association between genes and motility (34-36). Climbing success was calculated as % flies per tube which climbed over an arbitrary mark, and survival was determined as % flies alive 17-20 days post emergence, out of starting flasks containing 20 flies (n = 4+ flasks per condition).

**Statistical analysis**

To infer statistical differences between more than two independent groups, we used one-way ANOVAs followed by Tukey post hoc tests (for normally distributed values), or Kruskal-Wallis tests followed by Dunn’s tests (for non-normally distributed values). To infer differences between two groups, we performed two-way independent parametric t-tests. Multiple testing correction was applied as described in Results. Microarray expression data were analysed using linear regressions (see Supplemental Methods). Gene overlap significance was calculated in R (package ‘GeneOverlap’) using the Fisher’s exact test. Statistical analyses were performed in R and SPSS.

**Results**
Expression of NT5C2 is enriched in neurons in the adult brain

To extend our previous work and investigate the relationship between NT5C2 expression and psychiatric disorders, we investigated which cell types express this gene in the adult brain. We examined single-cell RNA-sequencing data from the mouse cortex (37), which revealed cell-type specific profiles of NT5C2 expression (Kruskal-Wallis test, \( H(4) = 52.44, P < 0.001 \)). Post hoc analyses confirmed that NT5C2 was more frequently observed in pyramidal neurons (95% percentile = 3 (counts)) than astrocytes (95% percentile = 1, Dunn’s post hoc test, corrected \( P < 0.001 \)), and in interneurons (95% percentile = 3) rather than microglia (95% percentile = 2.05, corrected \( P < 0.01 \)) or astrocytes (95% percentile = 1, corrected \( P < 0.001 \); Supplemental Figure 1).

To investigate correlates with the human cortex, we performed a series of immunocolocalisation experiments using post-mortem brains. Initially, we investigated the specificity of an antibody for NT5C2 by probing HEK293T cells and CTX0E16 hNPCs overexpressing myc-NT5C2 (Supplemental Figures 2 and 4), and a loss-of-function in hNPCs (Figure 3C). While immunolabelling of endogenous expression and heterologous systems may produce different results due to existence of tissue-specific isoforms, these findings corroborate the suitability of this antibody for our aims. This antibody was used to investigate the distribution of NT5C2 in the prefrontal cortex using standard immunoperoxidase staining with DAB as chromogen (Supplemental Figure 3). A qualitative analysis of NT5C2-positive immunostaining with Nissl counter-stain to reveal cell morphology suggested that NT5C2 was present in neurons, glia and the surrounding neuropil. However, we noted that not all putative glial cells expressed NT5C2 (red arrows; Supplemental Figure 3), corroborating a previous observation by the Human Protein Atlas that expression is lower in these cells (38). To confirm this, we quantified the cell type-
specific expression profile of NT5C2 in the cortex by quantifying immuno-co-localisation of this protein with markers of mature neurons (microtubule-associated protein 2, MAP2), a sub-class of gamma-amino butyric acid (GABA) interneurons (parvalbumin, PARVALB), astrocytes (glial fibrillary acidic protein, GFAP), and microglia (ionized calcium-binding adapter molecule 1, IBA1) (Figures 1A-E). These cells were selected based on the wealth of evidence implicating them in the pathophysiology of psychiatric disorders (39-42). Co-localisation quantification was performed using a semi-automated ImageJ macro (43, 44) (details in Supplemental Methods), which revealed cell type specific profiles of NT5C2 expression, based on the co-localisation with these markers (One-way ANOVA, F (3,44) = 39.12, P < 0.001, n = 4 unaffected controls, 4 technical replicates each, 20 fields of view per technical replicate). Co-localisation occurred more frequently with neuron and interneuron than non-neuronal markers (Tukey post hoc tests: MAP2 (7.5% (cluster colocalization relative to all clusters in image) ± 2.0 (standard deviation)), PARVALB (6.9% ± 2.1), GFAP (3.13% ± 1.1), IBA1 (1.4% ± 0.93); P < 0.001 for all comparisons, except MAP2 vs. PARVALB, and GFAP vs. IBA1 (P > 0.05); Figure 1E). NT5C2 expression at the transcript and protein levels appear to be highly-expressed in neurons within the adult brain, consistent with recent observations that risk variants implicated in schizophrenia are enriched for neuronal genes (40).

**NT5C2 is highly expressed and ubiquitously distributed in hNPCs**

The role of NT5C2 in psychiatric disorders has been previously hypothesised to commence during neurodevelopment, a period underscored by complex processes implicated in major psychiatric disorders (45), with this risk mechanism persisting in the adult brain (3). The contribution of NT5C2 to neurobiology, however, should be greater at the developmental stage when this gene is more expressed. Thus, we investigated NT5C2 expression in the Human Brain Transcriptome atlas (46), which revealed that expression peaks during
neurodevelopment (Figure 2A). Considering that established cell lines are cost-effective and easy-to-use tools to study neurodevelopment, we tested whether the CTX0E16 hNPC line (30, 31) would constitute an appropriate model. We measured expression of the NT5C2 main RefSeq transcripts (NM_012229 and NM_001134373) in these cells, specifically in hNPCs and immature neuronal cultures terminally differentiated for 28 days (DD28) which we previously showed to comprise of neurons (~80%) and glial cells (~10%) (30, 31). The expression of NT5C2 RefSeq transcripts NM_012229 and NM_001134373 in hNPCs (Day 0; NM_012229: 94.22 ± 5.85; NM_001134373: 85.67 ± 9.77) was 30% higher in hNPCs compared to DD28 cultures on average (NM_012229: 45.09 ± 5.59; NM_001134373: 70.45 ± 2.93; t-tests: NM_012229, t(6) = 12.14, P < 0.001, Bonferroni corrected P < 0.001; NM_001134373, t(6) = 2.99, P < .0245, Bonferroni corrected P = 0.049). These findings are consistent with higher expression at an earlier developmental stage with persistent expression after differentiation (Figure 2B).

As sub-cellular protein distribution can inform gene function, we immunolabelled hNPCs from the CTX0E16 and human induced pluripotent stem cell lines (hiPSC) to study NT5C2 localisation. We ectopically expressed a myc-tagged NT5C2 construct in hiPSC-NPCs, and labelled these cells using myc or NT5C2 antibodies, which revealed that myc-NT5C2 was abundantly expressed in punctate structures in the cell soma and along neurites (Figure 2C; Supplemental Figure 4). Similarly, endogenous NT5C2 was distributed in punctate structures through the cell and neurites (Figures 2D and E), suggesting that this protein is ubiquitously distributed in hNPCs, consistent with the expected distribution of a cytosolic protein.

NT5C2 regulates the phosphorylation of AMPK and ribosomal protein S6 (RPS6)
The knockdown of NT5C2 activates AMPK signalling in muscle fibres (21), and considering the relevance of AMPK to psychiatry (17-20), we tested whether this also occurred in hNPCs. The knockdown in CTX0E16 hNPCs was performed using two independent small interfering RNA (siRNA) sequences, A and B. Initially, the transfection protocol efficacy was determined by assessing the uptake of fluorescently labelled oligonucleotides (BLOCK-iT), which revealed a transfection rate of 90% ± 0.02 (n = 4 biological replicates per condition, 4 technical replicates each; Figure 3A). We obtained knockdown cultures and confirmed reduced NT5C2 expression using RT-qPCR (linear regression to identify the effect of siRNAs on NT5C2 expression controlling for biological replicate and qPCR batch: F(2, 5) = 13.9, P = 0.009, R² = 0.92; Tukey post hoc tests against scramble (3.29 (dCt) ± 0.23 (standard deviation)): siRNA A (3.79 ± 0.09), fold-change = 0.71, P = 0.005; siRNA B (3.29 ± 0.23), fold-change = 0.81, P = 0.028; Figure 3B). The ability of these siRNAs to knockdown NT5C2 protein was further assessed in independent cultures (Figures 3C and D), which revealed a significant decrease in protein abundance in knockdown conditions (One-way ANOVA, F (2, 41) = 12.23, P < 0.001; Tukey post hoc tests against scramble (100.0 ± 14.7): siRNA A (58.8 ± 34.7), P < 0.001; siRNA B (62.4 ± 21.5), P < 0.001).

To test the effect of the knockdown on AMPK signalling, we measured total abundance and relative phosphorylation of AMPK-alpha, a subunit of AMPK. We observed a significant effect of the knockdown on AMPK-alpha abundance, which was associated with a mean 132% increase in total protein (Kruskal-Wallis test, H(3) = 12.2, P < 0.001; Dunn’s post hoc tests against scramble (Mdn = 100.0): siRNA A (236.1), P = 0.002; siRNA B (182.8), P = 0.017; Figure 3E). Additionally, we observed a significant effect of the knockdown on the relative phosphorylation of AMPK-alpha (Thr172), with the knockdown associated with a mean 55% increase in phosphorylated AMPK, suggesting activation of this cascade
Kruskal-Wallis test, $H(3) = 7.65$, $P < 0.013$; Dunn’s post hoc tests against scramble (Mdn = 100.0): siRNA A (141.2), $P = 0.033$; siRNA B (160.7), $P = 0.033$; Figure 3E).

Considering that protein translation is partly regulated by AMPK (23) and is one of the most energy consuming cellular processes (47), we hypothesised that NT5C2 function could affect the rate of protein synthesis. To test this, we assessed the effects of the knockdown on the phosphorylation of ribosomal protein S6 (RPS6) (Ser235/Ser236), which is routinely used as a proxy to estimate the rate of protein translation in neurons, as it correlates with mammalian target of rapamycin complex 1 activation (mTORC1) (48). No difference was observed in total RPS6 abundance (Kruskal-Wallis test, $P > 0.05$; Figure 3F), but we observed that the knockdown with siRNA A was associated with a mean 23% increase in phosphorylated RPS6 (Kruskal-Wallis test, $H(3) = 8.22$, $P = 0.002$; Dunn’s post hoc test against scramble (Mdn = 100.0): siRNA A = 115.9, $P = 0.012$; Figure 3F). The knockdown with siRNA B elicited a mean 10% increase in RPS6 phosphorylation, but this did not survive correction (siRNA B = 110.20, $P = 0.09$), probably as a consequence of the less efficient knockdown achieved with this siRNA (Figure 3B).

We obtained complementary evidence supporting the association between NT5C2 and AMPK and RPS6 regulation using HEK293T cells. Overexpression of pNT5C2-myc in these cells resulted in a mean 64% decrease in phosphorylated AMPK-alpha (t-test, control (no vector): 223.00 (normalised expression) ± 76.99 (standard deviation), overexpression: 81.05 ± 30.14, $t(15) = 4.88$, $P < 0.001$, $P$ Bonferroni-adjusted $P$ (4 tests) < 0.001), whilst no difference in total AMPK levels were observed ($P > 0.05$; Figure 3G). These results are consistent with our previous data and indicate that NT5C2 is a negative regulator of AMPK signalling. Subsequently, we observed a mean 28% decrease in total RPS6 abundance (t-test, control: 159.10 ± 48.52, overexpression: 108.8 ± 48.52, $t(16) = 2.88$, $P = 0.011$,
corrected $P = 0.044$), and a highly significant 300% increase in RPS6 phosphorylation (t-test, control: $31.03 \pm 10.66$, overexpression: $124.10 \pm 8.20$, $t(16) = 20.76$, $P < 0.001$, corrected $P < 0.001$; Figure 3G). The effect of exogenous NT5C2 on RPS6 here was opposite to what we observed in hNPCs, highlighting the complex and time-dependent nature of the intracellular cascades governing protein translation, which are examined in the discussion.

**NT5C2 is associated with transcriptional changes implicated in protein translation**

In order to determine the regulatory gene networks governing the effect of NT5C2 on AMPK signalling in hNPCs, we profiled the transcriptome of these cultures using microarrays (Figures 4A and B). We aimed to characterise expression differences that were shared between both siRNA treatments, to reduce off-target effects associated with individual siRNAs (49). The concordant transcriptomic changes consisted of an overlap of 69 genes (Figure 4C), which is statistically unlikely to occur by chance (genes in microarray = 21,196; affected by siRNA A: 881 genes; siRNA B: 741 genes; Fisher’s exact test, $P < 0.001$, Jaccard index < 0.001, odds ratio = 2.6; gene list in Supplemental Table 1). We observed that there was a high correlation between samples within the same siRNA groups, despite the modest number of overlapping, differentially expressed genes (Pearson’s $r > 0.99$, $P < 0.001$ for all correlations; siRNA A, $n = 3$ biological replicates; siRNAs B and scramble, $n = 4$). The list of overlapping gene expression changes was subdivided by directionality of effect, and the up- and downregulated gene network topologies were calculated using GeneMania (50) (Supplemental Figure 5). This analysis revealed multiple connections between genes due to co-expression and co-localisation, corroborating their functional association. The up- and downregulated gene lists were analysed for enrichment of gene ontology (GO) terms (Figure 4D, Supplemental Table 2), which revealed downregulated genes ($q < 0.05$) pertaining to the regulation of protein translation, and of the cytoskeleton.
(which is highly dependent on the transcriptional machinery (51)). Furthermore, ribosomal
genes including *RPL15, RPL22, RPL5* and *RPL6*, were downregulated, consistent with
activation of AMPK signalling and inhibition of protein translation. The top upregulated GO
term suggested the involvement of *NT5C2* in cell adhesion, but this was not significant after
correction (*q* > 0.05).

We used RT-qPCR to validate a panel of gene expression changes observed in the
microarray analysis (*Supplemental Figure 6*), which were related to protein translation and
AMPK signalling, including the heterogeneous nuclear ribonucleoprotein A1 (*HNRNPA1*),
the proteasome 26S subunit, ATPase 4 (*PSMC4*), and the autophagy-related cysteine
peptidase gene (*ATG4B*). *HNRNPA1* is involved in protein translation (52), while *ATG4B*
regulates AMPK signalling and energy homeostasis (53), and *PSMC4* physically interacts
with AMPK (54) and is involved in Parkinson’s disease (55). Considering the effects of
*NT5C2* in AMPK and RPS6 regulation, the transcriptional changes observed here
corroborate a role for *NT5C2* on protein translation, providing evidence of the gene networks
involved.

**Knockdown of CG32549 in Drosophila melanogaster is associated with impaired
climbing**

Considering the genetic link between *NT5C2* and disorders associated with psychiatric and
psychomotor disturbances, and the importance of AMPK in energy consuming tasks such
as motility (19, 56), we investigated a potential role of Drosophila’s *NT5C2* homologue in
climbing. The human NT5C2 protein shares 60.5% sequence identity and 80.2% sequence
similarity with CG32549 (*Supplemental Figure 7*), suggesting that these proteins exert the
same or similar function. To investigate the role of CG32549 in motility whilst controlling for
potential confounding effects in muscles, we generated flies using the Gal4-UAS system to
obtain crosses with reduced expression of this gene ubiquitously (driven by \textit{ACT5C} promoter), in neurons and neural progenitor cells (\textit{ELAV}), or in gut, as a control (\textit{GUT}; \textbf{Figure 5A}). The ubiquitous knockdown was associated with reduced \textit{CG32549} expression in the brain (UAS line (control, no RNAi cassette) = 0.042 (dCt) ± 0.027; UAS-KD (knockdown) flies = 0.007 ± 0.004; fold-change = 0.88; t-test: \(t(6) = 2.6, P = 0.043\), \(n = 4\); \textbf{Figure 5B}). No difference in survival was observed across genotypes (UAS versus UAS-KD lines, t-tests, \(P > 0.05\), \(n = 5\) flasks on average; \textbf{Figure 5C}). We observed a significant impairment in climbing success associated with the knockdown using the \textit{ELAV} promoter (UAS = 96.9\% ± 2.2, UAS-KD = 85.2\% ± 8.5; \(t(11) = 3.53, P = 0.005\), adjusted \(P = 0.014\), \(n = 7\) per condition on average; \textbf{Figure 5D}). There was also a nominally significant reduction in climbing success upon knockdown of \textit{CG32549} using the \textit{ACT5C} promoter (UAS = 90.6\% ± 9.7, UAS-KD = 77.7\% ± 13.4, t-test: \(t(17) = 2.4, P = 0.029\), \(n = 10\) per condition on average, Bonferroni \(P\) (adjusted for 3 comparisons) > 0.05). This impairment was not observed in flies with the knockdown in gut (UAS = 98.5\% ± 2.3, UAS-KD = 97.4\% ± 2.3, t-test, \(P > 0.05\), \(n = 8\) per condition on average). These findings suggest there is an effect of neuronal \textit{CG32549} in \textit{D. melanogaster} motility, and provide an insight at the function of \textit{NT5C2} at a systems level.

\textbf{Discussion}

The \textit{NT5C2} gene is implicated in risk for psychiatric and neurological conditions (1-3, 5, 6) and it has been recently classified as a high confidence schizophrenia risk gene by PsychENCODE (4), but the biological mechanisms responsible for these associations remain elusive. We previously showed that psychiatric risk alleles at the \textit{NT5C2} locus are associated with reduced expression of this gene in the adult and developing brain (3). Here, we observe that reduced \textit{NT5C2} expression is associated with differential regulation of
AMPK signalling and RPS6 in hNPCs, suggesting a regulatory role on energy homeostasis and protein translation. We also observe that neurological expression of NT5C2 peaks during neurodevelopment but persists at later developmental stages, corroborating our previous hypothesis that the NT5C2 risk mechanism is an ongoing process that starts from embryonic development (3). The cell-type specific NT5C2 expression profile observed in the adult brain further revealed an enrichment for neuronal expression, suggesting that reduced NT5C2 expression in the mature cortex could be particularly detrimental to neurons. These findings are consistent with recent studies showing that schizophrenia risk variants are enriched for genes implicated in neurodevelopment and neuronal function (40, 57).

The manipulation of NT5C2 expression in hNPCs and HEK293T cells corroborate the existence of a complex regulatory network governing protein translation, with observations suggesting, at first glance, opposing effects of AMPK on the rate of protein synthesis. However, on closer inspection, we observed that our findings with HEK cells corroborate that AMPK activation inhibits protein translation by repressing mTORC1 and eEF2 (23, 24, 58, 59). Our findings with the hNPC model, in turn, corroborate AMPK activation leading to increased rates of protein synthesis over time, despite an initial period in which translation is halted, likely due to a negative feedback loop (60). As a result, we observed increased abundance of AMPK-alpha in these cultures, while no differences in expression of AMPK transcripts were detected.

We also observed that a knockdown of the NT5C2 homologue CG32549 in D. melanogaster was associated with abnormal climbing behaviour, more specifically when driven by a neuronal promoter, supporting a role for NT5C2 in motility. It is unrealistic to correlate fly motility with complex psychomotor disturbances experienced by patients, but our results
corroborate previous genetic associations between NT5C2 and diseases associated with motor symptoms (1-6). The effect of CG32549 could be mediated by regulation of AMPK signalling and RPS6 activation, which are implicated in Drosophila motility (61, 62). A study showed that CG32549 is downregulated in a Drosophila model of seizure (28), and work by another group demonstrated that distance moved during seizure-like activity can be reduced (rescued) upon AMPK activation using the drug metformin (29).

There are limitations to our study which should be acknowledged. First, we obtained a modest knockdown of NT5C2 in hNPCs, which we hypothesise this to be due to the proliferative nature of these cells. To support the link between NT5C2, AMPK signalling and RPS6 activation, we overexpressed NT5C2 in HEK293T cells and confirmed the differential regulation of AMPK and RPS6. Second, we observed a modest overlap of differentially expressed genes between siRNA treatments, which we hypothesise to be due to the low specificity associated with the siRNAs. This could be overcome by using a more effective gene silencing method, such as CRISPR interference (63). Third, we quantified the rate of protein translation using relative abundance of phosphorylated RPS6, but we did not investigate overall protein synthesis using methods such as the surface sensing of translation (SUnSET) (64). We have, however, provided support for the role of NT5C2 on protein translation at the transcriptional level using our microarray and RT-qPCR data.

By exploring the role of NT5C2 in neurobiology, we observe that the study of individual risk factors for complex disorders has the potential to advance our understanding of common biological pathways contributing to disease. Functional studies using model organisms or cell culture may not entirely capture the heterogeneity and complexity of psychiatric
disorders, but may have potential to accelerate the identification of novel drug targets and biomarkers for psychiatric disorders.

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Disclosures
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**Authorship contribution**

Conceived and designed experiments: RRRD, DPS, NJB. Performed the experiments: RRRD, NDB, GAH, MCC. Analysed the data: RRRD, TRP. Contributed reagents, biological material, expertise: SHL, SS, IAW, CT, GDB, ACV, IE, DFN, RMM, NJB, TRP. Wrote the paper: RRRD, TRP, DPS.

**References**


Figure 1. Distribution of the psychiatric risk protein NT5C2 in DLPFC sections of post-mortem unaffected patients. (A) Co-localisation of NT5C2 staining with MAP2 (neuronal marker), (B) PARVALB (interneuron marker), (C) GFAP (glial marker), (D) and IBA1 (microglia marker). Scale bars are 50 μM. (E) Quantification of the co-localisation of NT5C2 with markers from A to D revealed a cell-type specific expression of NT5C2 in the mature cortex (one-way ANOVA, Tukey pairwise comparisons: ***P < 0.001 for all comparisons).

Figure 2. Neurodevelopmental expression of NT5C2, and protein distribution in human neural progenitor cells. (A) Neurological expression of NT5C2 across human development, according to the Human Brain Transcriptome Atlas (46), showing that expression peaks during foetal development. (B) The expression of NT5C2 RefSeq transcripts NM_012229 and NM_001134373 in hNPCs (Day 0) and cultures differentiated for 28 days (DD28). Expression is significantly higher at the neural progenitor state (t-tests: ***P < 0.001, *P < 0.05). (C) Distribution of ectopic NT5C2 was assessed by transfecting hNPCs with pNT5C2-myc and peGFP plasmids, followed by immunolabelling using antibodies raised against myc or GFP (GFP was used as morphological marker). (D) Subcellular localisation of endogenous NT5C2 in hNPCs derived from a hiPSC line, and from (E) the CTX0E16 cell line. NT5C2 was ubiquitously distributed in hNPCs from both models. Scale bars are 20 μM.

Figure 3. Knockdown of NT5C2 in hNPCs is associated with differential phosphorylation of AMPK and RPS6. (A) The efficacy of the siRNA transfection was
determined by uptake of BLOCK-iT, a fluorescently labelled oligonucleotide. (B) \textit{NT5C2} expression was significantly reduced in knockdown cultures (linear regression, Tukey post hoc tests, \( **P < 0.01, *P < 0.05 \)). (C, D) The ability of the siRNA treatments to significantly reduce \textit{NT5C2} expression in independent hNPC cultures at the protein level (one-way ANOVA, Tukey post hoc tests, \( ***P < 0.001 \)). (E) The \textit{NT5C2} knockdown was associated with increased total AMPK-alpha, and in phosphorylated AMPK-alpha (Thr172) in hNPCs (Kruskal-Wallis test, Dunn’s post hoc tests, \( **P < 0.01, *P < 0.05 \)). (F) The knockdown did not alter total RPS6 levels, but was associated with increased phosphorylated RPS6 (Ser235/Ser236). siRNA A was associated with a mean 23% increase in phosphorylation (Kruskal-Wallis test, Dunn’s test, *P < 0.05), and siRNA B a modest 10% mean increase, which was not significant after correction (\( P = 0.09 \)). Full blots for E and F are available in Supplemental Figure 8. (G) The overexpression of \textit{NT5C2} in HEK293T cells causes a significant decrease in phosphorylated AMPK-alpha levels and in total RPS6, and a significant increase in phosphorylated RPS6 (t-test, \( ***P < 0.001, *P < 0.05 \)). Full blots are available in Supplemental Figure 9.

Figure 4. Transcriptional changes associated with the knockdown corroborate a role for \textit{NT5C2} on protein translation. (A) Volcano plots indicating nominally significant transcriptomic changes elicited by siRNA A, and (B) siRNA B. (C) Venn diagrams indicating the number of genes differentially regulated by siRNA A and B, and the overlapping gene set, which is unlikely to occur by chance, according to a Fisher’s exact test (\( P < 0.001 \)). (D) Gene ontology terms enriched within genes concordantly, differentially expressed in both knockdown conditions. The line indicates the significance threshold (\(-\log_{10} q < 0.05\)).
Figure 5. The knockdown of CG32549 (NT5C2 homologue) in D. melanogaster using the UAS-Gal4 system. CG32549-RNAi was induced ubiquitously (ACT5C promoter), in gut (GUT), or in neural progenitor cells and neurons (ELAV). (A) Experimental design of the knockdown. (B) CG32549 was less expressed in the brain of knockdown flies (t-test, *P < 0.05). (C) There was no difference in survival percentage between UAS lines vs UAS-knockdown lines 17-20 days post eclosure (t-tests, P > 0.05). (D) Climbing success observed in UAS lines vs UAS-knockdown lines highlights the effect of ubiquitous, neuron- or gut-specific knockdowns on motility (t-tests, *P < 0.05, **P < 0.01), but not in gut (P > 0.05).
Duarte et al. Figure 1
A. **NT5C2 expression**

![Graph showing NT5C2 expression over age (days)].

B. **NT5C2 expression**

![Bar graph showing normalized expression (dCt) for NM_012229 and NM_001134373].

C. **GFP + myc + DAPI**

- hiPSC-NPCs

D. **DAPI + NT5C2**

- hiPSC-NPCs

E. **DAPI + NT5C2**

- CTX0E16-NPC

**Duarte et al. Figure 2**
A. BLOCK-IT

B. NT5C2 (qPCR)

C. NT5C2 (ICC)

D. TUBB3 (Tuj1) NT5C2

E. Scramble siRNA A siRNA B

F. p-AMPK Total AMPK β-Actin

G. p-rpS6 (Ser235/6) Total rpS6 GAPDH

H. pNT5C2-myc overexpression (NT5C2++) HEK293T cells

i. Normalized expression

j. Total AMPK pho-AMPK total rpS6 pho-rpS6

k. N.S. P < 0.001 P = 0.011 P < 0.001

Duarte et al. Figure 3
Duarte et al. Figure 4

A. **NT5C2 KD - siRNA A**

B. **NT5C2 KD - siRNA B**

C. Enriched GO terms NT5C2 KD

D. Enriched GO terms NT5C2 KD
**P-AMPK (Thr172)**

**Total AMPK**

**β-Actin**

**p-rpS6 (Ser235/6)**

**Total rpS6**

**GAPDH**
**Total AMPK**

**p-AMPK (Thr172)**

**Total rpS6**

**p-rpS6 (Ser235/6)**

**myc**

**mAb**

**kDa:**

- 100
- 75
- 50

**Ladder**

**Control**

**NT5C2++**
**Specific GAL4 promoter:**
- Ubiquitous (*ACT5C*)
- Neural progenitor/neuronal (*ELAV*)
- Gut (*GUT*; control)

**A.**
- GAL4
- CG32549-RNAi

**GAL4-driven promoter for gene of interest:**
- CG32549-RNAi

**B.**
CG32549 expression (qPCR)

- Knockdown in gut (*GUT*)
- Knockdown in neurons (*ELAV*)
- Ubiquitous knockdown (*ACT5C*)

- Fold-change ($2^{\Delta\Delta Ct}$)

- * $P = 0.043$

**C.**
Survival

- Survival of flies after 17-20 days
- n.s.

**D.**
Climbing success

- % Flies climbed over mark
- n.s.
- * $P = 0.029$
- ** $P = 0.005$

**Duarte et al. Figure 5**