RNA-binding protein QKI regulates *Glial fibrillary acidic protein* expression in human astrocytes

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Linkage, association and expression studies previously pointed to the human *QKI, KH domain containing, RNA-binding* (*QKI*) as a candidate gene for schizophrenia. Functional studies of the mouse orthologue *Qk* focused mainly on its role in oligodendrocyte development and myelination, while its function in astroglia remained unexplored. Here, we show that *QKI* is highly expressed in human primary astrocytes and that its splice forms encode proteins targeting different subcellular localizations. Uncovering the role of *QKI* in astrocytes is of interest in light of growing evidence implicating astrocyte dysfunction in the pathogenesis of several disorders of the central nervous system. We selectively silenced *QKI* splice variants in human primary astrocytes and used RNA sequencing to identify differential expression and splice variant composition at the genome-wide level. We found that an mRNA expression of *Glial fibrillary acidic protein* (*GFAP*), encoding a major component of astrocyte intermediate filaments, was down-regulated after *QKI7* splice variant silencing. Moreover, we identified a potential QKI-binding site within the 3′ untranslated region of human *GFAP*. This sequence was not conserved between mice and humans, raising the possibility that *GFAP* is a target for QKI in humans but not rodents. Haloperidol treatment of primary astrocytes resulted in coordinated increases in *QKI7* and *GFAP* expression. Taken together, our results provide the first link between *QKI* and *GFAP*, two genes with alterations previously observed independently in schizophrenic patients. Our findings for *QKI*, together with its well-known role in myelination, suggest that *QKI* is a hub regulator of glia function in humans.

INTRODUCTION

The selective RNA-binding protein quaking (*QKI*), encoded by the *Qk* gene, belongs to the signal transduction and activation of RNA (STAR) protein family and governs post-transcriptional regulation of its mRNA ligands, including pre-mRNA splicing (1), mRNA stability (2,3), transport (4) and protein translation (5). QKI binding to its downstream mRNAs is mediated by a highly conserved K homology (KH) domain that interacts directly with a short sequence termed the QKI response element (QRE), often located within the 3′ untranslated region (3′UTR) of the target mRNAs (6). In the adult mouse central nervous system (CNS), *Qk* is highly expressed in glial cells, including astrocytes and myelin-forming oligodendrocytes (7). Much attention has been given to the role of *Qk* in myelination since deletion of the *Qk* regulatory element results in severe CNS dysmyelination in quaking viable (*Qk*<sup>−/−</sup>) mutant mice (8). Multiple studies demonstrated that *Qk* plays a central role in oligodendroglia differentiation and maturation by controlling the cellular behaviour of mRNAs mediating these processes (1–4,9–11). However, the functions of *Qk* are not restricted to the control (Ctrl) of myelin gene expression. In fact, over a thousand other mRNA species have been bioinformatically predicted to be targeted by QKI proteins in mice (6). The functions of QKI in humans are less well known, but the genomic structure of the mouse *Qk* and its human orthologue *QKI*, *KH domain containing, RNA-binding* (*QKI*), are remarkably similar. Four known mRNA splice variants named *QKI5*, *QKI6*, *QKI7* and *QKI7b* are generated via alternative splicing (12). The resultant proteins differ only with regard to their short but unique C-terminal amino acid sequences.

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In humans, QKI is located in a previously identified schizophrenia (SCZ) locus in the long arm of chromosome 6 (13–17), and several independent studies showed reduced expression of QKI mRNA splice variants in distinct brain regions of SCZ patients when compared with non-affected individuals (18–20). Confirming the interest in this candidate gene, the first genome-wide RNA sequencing (RNA-seq) study of SCZ patients published up to date includes QKI among the list of genes with significant differential splicing in patients (21). These results support our previous findings showing that the expression of the splice variants QKI7, and QKI7b was preferentially down-regulated in the frontal cortex of SCZ subjects. Moreover, QKI deficiency was correlated with reduced transcription of genes critical for oligodendrocyte development (20), highlighting the link between abnormal QKI expression and oligodendrocyte dysfunction in SCZ. Whether aberrant QKI expression occurs in astrocytes and what are the possible cellular and physiological consequences remains unexplored. The involvement of astrocytes in the pathophysiology of psychiatric disorders has gained considerable interest in recent years due to the discovery of their key role in synaptic transmission and plasticity (22). Indeed, astrocyte abnormalities, such as reduced cell density and altered expression of astrocyte-related genes, have been detected in multiple brain regions of subjects with SCZ (23–27). Among these genes, reduced level of Glial fibrillary acidic protein (GFAP), encoding a major component of astrocyte intermediate filaments, was the most commonly reported (25,28–33). However, the underlying mechanism of the observed GFAP decline has not yet been described.

Although the presence of QKI proteins in mouse astrocytes has been known for nearly two decades (34), their roles in this cell type remain unknown. The function of QKI in human astrocytes has not been previously investigated, except in our recent study of a human astrocytoma cell line (35). However, this tumour-derived cell line does not express the GFAP protein, thus providing a valid model only for certain types of astrocytes. To advance our understanding of QKI function in astrogala, we selectively silenced QKI splice variants in human primary astrocytes using small interfering RNAs (siRNAs) and studied the effect of siRNA-mediated QKI depletion on global gene expression using high-throughput RNA-seq. We show that silencing of QKI results in decreased expression of GFAP, linking for the first time these two deficiencies previously observed in SCZ.

RESULTS
Distinct subcellular localization of QKI proteins in human astrocytes
The expression of QKI proteins in human primary astrocytes was first examined by immunoblotting. As in rodents, the human QKI proteins are identical except for their unique C-terminal sequences, against which the isoform-specific antibodies were raised. The schematic structures of the human QKI proteins with their unique carboxy tails are shown in Figure 1A. We detected a band of ~38 kDa corresponding to QKI6, and two closely migrating bands of ~40 kDa corresponding to QKI5 and QKI7 (Fig. 1B). Antiserum specific to QKI7b is not yet available. To determine the subcellular localization of QKI isoforms in astrogala, cells were immunolabelled against QKI5, QKI6 and QKI7. All three QKI proteins were expressed in astrocytes and showed distinct intracellular distribution. QKI5 was detected exclusively in the nucleus, whereas QKI6 and QKI7 were localized in the nucleus and cytoplasm of the cell body and processes (Fig. 1C and D). Moreover, the QKI5 protein was absent from nucleoli, whereas the two other isoforms were detected throughout the nucleus. No staining was observed when primary antibodies were omitted (data not shown).

siRNA-mediated QKI knockdown in human primary astrocytes
To study the function of QKI in astrocytes, we employed a loss-of-function approach by introducing siRNAs targeting all or individual QKI splice variants in primary human astrocytes. A siRNA cocktail targeting exon 4 and exon 6, common to all QKI transcripts, was used to silence total QKI mRNA (siQKI-tot). siQKI5, targeting exon 8, was designed to suppress splice variant QKI5 and siQKI7, targeting exon 7a, was designed to silence splice variants QKI7 and QKI7b. QKI6 sequence does not contain a unique region that can be used for selective siRNA design. The schematic structure of the human QKI gene and the regions targeted by the siRNA cocktails are shown in Figure 2A. Relative expression of the QKI gene (QKI-tot) and its splice variants QKI5, QKI6 and QKI7 were measured using TaqMan-based quantitative real-time PCR (qPCR). As shown in Figure 2B, siQKI-tot treatment resulted in greater than 80% reduction in total QKI mRNA expression (QKI-tot) when compared with non-silenced Ctrl. We also detected a decline of the mRNAs encoding individual splice variants QKI5, QKI6 and QKI7, with the two last transcripts being most severely affected (~95% mRNA reduction). When the siQKI7 cocktail was used for silencing, we observed a significant reduction in the QKI7 mRNA level, but not in QKI5 or QKI6 mRNAs. Moreover, siQKI7 treatment did not significantly affect QKI-tot expression, suggesting that QKI7 accounts only for a small fraction of all QKI transcripts, as further confirmed by RNA-seq experiments (see below). Interestingly, cells treated with siQKI5 exhibited reduced expression not only of QKI5, but also of QKI6 and QKI7 transcripts to a level comparable with siQKI-tot treatment (Fig. 2B).

siRNA-induced alterations in QKI mRNA levels correlated well with changes in the abundance of endogenous QKI proteins, as shown by immunoblotting (Fig. 2C). Neither QKI mRNA expression levels (data not shown) nor the amounts of corresponding proteins were affected by the scrambled siRNA (Scram) (Fig. 2C) indicating a sequence specificity of the siQKI pools used in this study. Thus, the aforementioned siQKI5-mediated reduction in splice variants QKI6 and QKI7 is unlikely the result of siRNA off-target activity. Conversely, it may reflect QKI auto-regulatory activity, as previously proposed by others (36). No changes in cell viability were observed across the experimental groups (data not shown).
transcript profiling using RNA sequencing

We first used RNA-seq for a comprehensive analysis of the QKI transcriptional profile across the experimental groups. Sequence reads mapping to the reference genome revealed the presence of all four previously described QKI splice variants, QKI5 (ENST00000361752), QKI6 (ENST00000453779), QKI7 (ENST00000275262) and QKI7b (ENST00000392127) in the examined material (Fig. 3A). Expression levels across all QKI exons were significantly reduced in cells treated with siQKI-tot, and siQKI5 when compared with non-silenced Ctrl (P-value = 2.85E-06 and P-value = 9.46E-08, respectively), confirming the qPCR results. Also in line with the qPCR data, most exons did not show obvious alterations in expression upon siQKI7 silencing, indicating that QKI7 and QKI7b make up only a small proportion of all QKI transcripts. Specific silencing of these two splice variants may be visualized by the decreased read coverage in the region marked in green in Figure 3B. By comparing number of reads mapping to exonic regions exclusive to QKI6 and QKI7 and QKI7b (green) and the regions shared by QKI6, QKI7 and QKI7b (red), we estimated that the expression of QKI6 transcript is 1.8 times higher than the combined expression of QKI7 and QKI7b splice forms. Interestingly, in addition to the four well-known protein-coding QKI splice variants, we detected high expression of another transcript (ENST00000361758) that has not been previously characterized in humans. We termed this transcript QKI6b, since its coding region, and thus a predicted protein product, are identical to the one of the QKI6 messenger. QKI6b harbours an alternatively spliced 3′ UTR region that overlaps partially with the 3′ UTR exon of the QKI5 variant (Fig. 3A and B, yellow). The relative abundance of QKI6b mRNA compared with the more restricted expression of QKI6 can be visualized by comparing the yellow region with the blue region, exclusively present in QKI5 (Fig. 3B). We estimated that QKI6b transcript was seven times more abundant than QKI5. Together, these observations indicate that QKI6b and QKI6 are the major transcripts expressed by human astrocytes. Of note, these two mRNA species were not distinguishable in our qPCR study, and both could be detected by the QKI6 probe. Moreover, the siQKI5 cocktail designed to silence QKI5 could also target the overlapping 3′ UTR of the QKI6b mRNA, offering a plausible explanation for the observed siQKI5-mediated reduction in the QKI6 probe signal intensity (Fig. 2B). However, this overlap cannot explain QKI7 decline after siQKI5 treatment. The possibility remains that the nuclear QKI5 protein regulates levels of QKI7 mRNA.

Effect of QKI silencing on global gene expression

We next used RNA-seq to study the effect of siRNA-mediated QKI depletion on global gene expression. Since siQKI5 treatment failed to induce splice variant-specific QKI mRNA...
silencing, we noted reduced expression of GFAP ($P$-value $= 0.002$, Supplementary Material, Table S2 and Fig. 3C). Among all differentially expressed genes, GFAP is the only one with known functions in astrocytes (as described in the ‘Discussion’ section) and therefore, it has been selected for the follow-up analysis and validation studies.

Since QKI is known to modify alternative splicing of some target mRNAs, we wished to compare the splicing pattern of GFAP in siQKI7-treated cells and non-silenced Ctrl. Three major GFAP transcripts are present in the human CNS-GFAPα, GFAPβ and GFAPγ, with the first being most highly expressed (37). GFAPα was also the most dominant isoform in our primary astrocytes, followed by GFAPβ. The expression of GFAPγ was negligible. As shown in Figure 3C, the differential GFAP expression observed in this study is mostly due to a decrease in splice variant GFAPα. Whether or not differential splicing was affected could not be clearly determined.

Glial fibrillary acidic protein is a novel putative target for QKI

To validate the RNA-seq results, we examined relative mRNA expression levels of GFAPα in siQKI-treated versus Ctrl cells using quantitative real-time PCR. We detected $>$50% reduction in the expression of GFAP, whereas other well-characterized astrocyte markers, such as Vimentin (VIM) or Glutamine-ammonia ligase (GLUL), were unaffected upon silencing with both siQKI-tot and siQKI7 (Fig. 4A). Since QKI7, but not QKI5 or QKI6, was down-regulated in both experimental groups, we believe that reduction in QKI7 splice variant (and/or QKI7b) accounts for the observed GFAP transcriptional changes.

To evaluate whether GFAP mRNA may directly bind to QKI protein, we searched for the presence of potential QRE motifs in the GFAP sequence. A majority of the previously identified mouse QKI targets contain a bipartite QRE consisting of a core sequence NCACAAAY (where N is any base and Y is a pyrimidine) and a neighbouring half-site YAAY (with 2–21 nucleotide spacing) (6). Interestingly, a single-core sequence is localized within the 3′UTR region of the human GFAP messenger (splice variant GFAPα) as shown in Figure 4B. We also identified a potential half-site located upstream. Alignments of the 3′UTR of GFAPα in human and mice revealed that while the potential core site is conserved in both species, the potential half-site is not, raising the possibility that GFAP is a target for QKI in humans but not in mice.

Coordinated increase of QKI7 and GFAP mRNA after haloperidol treatment

We have previously shown that the antipsychotic drug haloperidol specifically increases the QKI7 mRNA level in the human astrocytoma cell line U343 (35). We, therefore, tested whether haloperidol induces a similar response in primary astrocytes, and if so, whether this would reverse the effect of siQKI7 silencing. Cells were treated with 0.2 μM haloperidol, resembling the clinical plasma concentration observed in patients (38). qPCR results were indicative of a substantial (2.8-fold change) increase in the QKI7 mRNA level after 6 h exposure to haloperidol (Fig. 4C). These

Figure 2. siRNA-mediated silencing of QKI splice variants in primary human astrocytes. (A) Exon–intron structure of the human QKI gene and the regions targeted by small interfering RNAs (siRNAs). Boxes represent exons, and black boxes indicate coding regions. The conserved KH domain is encoded by exons 2, 3 and 4. Coding regions including QUA1 and QUA2 motifs are also indicated. Regions targeted by three siRNA cocktails are shown as grey boxes. siQKI-tot was designed to silence all QKI splice variants (QKI-tot), siQKI5 targets QKI5, and siQKI7 silences both, QKI7 and QKI7b. (B) Relative quantification of QKI splice variants using quantitative real-time PCR. Percentage of remaining mRNA expression level for QKI-tot, QKI5, QKI6 and QKI7 are shown after silencing with siQKI-tot, siQKI5 and siQKI7. Asterisks indicate significant deviation in the mRNA levels compared with non-silenced Ctrl. (C) Western blot analysis of QKI protein isoforms in the lysates derived from the control cells (Ctrl), cells treated with scrambled siRNA (Scram) and cells silenced with siQKI-tot, siQKI5 or siQKI7. Actin, beta (ACTB) was used as a loading control.

ablation, we focused our analysis on siQKI-tot and siQKI7 silenced cells. Significantly up- or down-regulated genes after silencing with siQKI-tot and siQKI7 are listed in the Supplementary Material, Tables S1 and S2, respectively. The most significant change after siQKI-tot treatment was the reduction in the expression level of the QKI gene itself. Very few additional genes were significantly modified after correction for multiple testing (Supplementary Material, Table S1). Among significantly differentially expressed transcripts upon siQKI7
Figure 3. (A) RNA sequencing reads of control and siQKI-silenced cells mapping to the QKI gene region according to the UCSC reference genome (hg19). Y-axis indicates the number of reads covering each position along the transcript. Reads aligned within eight annotated QKI exons (Ensembl, version 65). The schematic representations of the Ensembl transcripts for QKI are shown at the bottom of the figure. QK15 = ENST00000361752, QK17b = ENST00000392127, QK17 = ENST00000275262, QK16 = ENST00000453779 and QK16b = ENST00000361758. (B) Detailed view on the QKI alternatively spliced 3’UTR exons. Reads mapping to chromosomal regions included in the transcribed sequence of each splice variant are colour coded. Green = sequence specific for QK17 and QK17b, red = sequence shared by QK16, QK17, and QK17b, yellow = sequence specific for QK15 and QK16b, blue = sequence exclusive for QK15. (C) RNA sequencing reads of control and siQKI17 silenced cells mapping to the GFAP gene region according to the UCSC reference genome (hg19). The schematic representations of the Reference Sequence (RefSeq) transcripts for GFAP are shown at the bottom of the figure. NM_001131019 = GFAPa, NM_002055 = GFAPb and NM_001242376 = GFAPc. Decreased read counts across nine annotated exons, corresponding to a significant reduction in GFAP transcript abundance (P-value = 0.002), are shown after siQKI17 treatment. GFAP mRNA decline is mainly due to a decrease in the level of splice variant GFAPa as shown by reduced read coverage mapping to the α-specific exon 9.

DISCUSSION

This study shows that QKI proteins are expressed in a subcellular-specific manner in human primary astrocytes, and that silencing of QKI produces a significant reduction in the mRNA level of GFAP, a widely recognized astrocyte differentiation marker constituting the major intermediate filament protein in mature astrocytes. Our interest in QKI function in astrocytes was fuelled by previous work by Larocque et al. related to the role of Qk in neural progenitor cell fate determination (2). In that study, the retrovirus-mediated ectopic expression of QKI6 and QKI7 in mouse telencephalon neural progenitors induced oligodendrocyte differentiation (2). Although astrocyte fate was not the focus of their study, the authors observed that ~30% of QKI-infected progenitors differentiated into GFAP-positive astrocytes (fig. 5 in 2), prompting us to hypothesize that Qk may be equally important for astrocyte specification and/or maturation. Despite this interesting observation, functional studies on the Qk gene in the brain have focused entirely on its involvement in oligodendrocyte development, neglecting the potential role in astroglia. In humans, QKI deficiency is associated with SCZ, a disease in which the abnormal expression of several key astrocyte structural and functional molecules has been reported (24–27). However, QKI expression and function in human astrocytes has not been previously described.

Here, we demonstrate that all three major QKI protein isoforms, QKI5, QKI6 and QKI7, are expressed in primary human astrocytes and occupy distinct cellular compartments. QKI5 immunoreactivity was found exclusively in the nucleus, consistent with the presence of a nuclear localization signal in its C-terminus. The other isoforms, QKI6 and QKI7, were uniformly distributed throughout the nucleus, cell body and processes. QKI differential intracellular localization implies that its function in astrocytes may, similarly to oligodendroglia, be spatially regulated. Our results are in line with a previous report on QKI expression in murine astrocytes (34), except that QKI5 was detected in astrocyte nuclei and proximal processes and not, as in our study, restricted to the nucleus. Although the QKI amino acid sequence is identical in the human and the mouse, potential differences in post-translational regulation may explain distinct subcellular localizations in the two species. In fact, post-translational modifications are often involved in directing the nascent proteins to distinct cellular compartments. For instance, tyrosine

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phosphorylation and arginine methylation of RNA-binding protein SAM68 (belonging together with QKI to the STAR protein family) have both been shown to promote its nuclear entry (38). However, SAM68 is the major embryonic isoform and its expression dramatically declines after post-natal day 14, during the peak of myelination (34). Human QKI5 mRNA, although highly expressed in the foetal brain, does not show significant reduction during post-natal development (41). These observations suggest that details of the molecular mechanism of QKI5-mediated post-transcriptional gene Ctrl, including interactions with other QKI splice variants, may not be identical in both species at all times during development.

We used RNA-seq to interrogate at a genome-wide level changes produced after silencing of QKI splice variants in human primary astrocytes. Differential gene expression in Ctrl versus siQKI-treated cells was limited to a small subset of genes suggesting narrow QKI target specificity in astrocytes. Among the list of QKI-regulated genes, AF339817, a transcript of unknown function, deserves special mention because of its location in a region previously linked to SCZ and major depression (42,43). We focused our attention to the siQKI7-mediated decrease in the GFAP mRNA level because besides its well-known structural role (cytoskeleton assembly, maintenance of cell shape, mechanical stability), GFAP has been implicated in mediating a broad spectrum of processes including brain development, synaptic plasticity, brain–blood barrier integrity, regeneration, ageing and CNS diseases pathogenesis (see (37) for a recent review). Abnormally elevated GFAP expression is a hallmark of a wide variety of CNS pathologies (37) among which Alexander disease is the only known example caused by a mutation in the GFAP gene itself (44). Less frequently reported a GFAP decline (as observed in our study) has been associated with growth of gliomas (45), mood disorders, SCZ (25,28–31,33,46) and in response to chronic viral infections (47–49). Although the role of abnormal GFAP post-transcriptional processing has been postulated, the underlying molecular mechanisms are not completely understood. We propose here that GFAP mRNA fate may be regulated via binding to QKI proteins. Notably, GFAP mRNA reduction observed in our study appears to be mediated by silencing of QKI7, the same splice variant previously found by our group to be preferentially down-regulated in SCZ patients (13). Our results link for the first time these two deficiencies observed in SCZ and point towards QKI as a potential regulator of astrocytic GFAP expression in vivo. In support of this hypothesis, we identified a putative QRE sequence located within the 3’UTR of the major GFAP transcript (GFAPα). To our surprise, Gfap was not listed among thousands of predicted putative QKI targets in mouse (6). However, inspection of a GFAP sequence revealed that while the core sequence is conserved in both species, the half-site is unique to humans, raising the possibility that GFAP mRNA is a ligand for QKI in humans but not in rodents. Also supporting the idea of a functional interaction between QKI and GFAP in humans, the expression levels of these two genes can be modulated in a coordinated way in astrocytes: QKI7 silencing produced GFAP decline, while haloperidol-mediated induction of QKI7 resulted in augmented GFAP expression. Interestingly, no changes in the GFAP protein expression has been detected in the rat brain following chronically administered haloperidol (25,50,51), suggesting interspecies differences in molecular pathways involved in GFAP processing.

In rodents, Gfap loss leads to decreased astrocyte process branching, abnormal synaptic functioning, late-onset myelin
defects and aberrant behaviour (52–55). In humans, the physiological consequences of GFAP decline are poorly understood, but reduced GFAP-immunoreactivity in the prefrontal cortex of SCZ patients was proposed to account for the observed astrocyte processes atrophy (30). Moreover, increased packing density of GFAP-positive somata has been detected suggesting that the stability or transport of GFAP molecules along the astrocyte processes may be affected in SCZ. QKI has been previously shown to regulate stability and export of some target mRNAs in oligodendrocytes (2–4). Whether similar mechanisms are involved in QKI-dependent post-transcriptional mRNA Ctrl in astrocytes needs further investigation. A current hypothesis is that GFAP-mediated structural abnormalities of fine distal astrocytic processes, which are normally intimately associated with synapses, may impair synaptic functioning and thus affect behaviour.

Since purified astrocytes, as used in our study, are devoid of neuronal contact, they do not develop extensive branching typical for astrocytes in vivo. Instead, only few proximal processes were formed, making the potential morphological effects of QKI-mediated GFAP decline difficult to assess. Neuron-astrocyte co-culture systems may be in the future a valuable tool to study the effects of QKI ablation in the context of neuro–glia interactions. Mouse models are commonly used to overcome the limitations of in vitro systems. However, the available viable Qk knock-out mice are devoid of Qk specifically from the myelin-forming cells, while its expression in astrocytes remains intact (34). Therefore, these mice cannot be used for studies of Qk role in astrocytes. Future conditional strategies may address this issue. Another limitation of our study refers to the use of RNA-seq technology. Lack of replicates makes the statistical modelling less robust in terms of finding minor deviations in expression levels between treatments. However, all reported findings are statistically significant and the QKI and GFAP decline were confirmed using an independent validation method (qPCR).

In conclusion, we propose GFAP as a novel putative target for QKI binding in human astrocytes. Considering that GFAP expression is associated with astrocyte differentiation, QKI-mediated post-transcriptional gene Ctrl may not only be critical for astrocyte structure and function in the adult brain, but also play a role in astroglia specification and/or maturation. We believe that future developmental studies addressing the role of QKI in astroglia in health and disease will be as rewarding as studies of QKI-dependent oligodendrocyte determination have been in the past.

**MATERIALS AND METHODS**

**Cell culture conditions**

The human primary astrocytes were obtained from 3H Biomedical (Uppsala, Sweden). These primary cells are derived from the human brain cerebral cortex, and stain positive for GFAP consistent with their astrocytic origin, as characterized in detail previously (56). Cells were cultured in poly-L-lysine-coated T75 tissue culture flasks (Sarstedt) in the manufacturer-supplied Astrocyte Medium supplemented with 2% foetal bovine serum, 1% astrocyte growth supplement

| Table 1. Sequences of siRNA duplexes designed to silence all QKI splice variants (siQKI-tot), splice variant QKI5 (siQK15) or QKI7 (siQK17) |
|---|---|
| siRNA cocktail | 5’- siRNA sequences-3’ |
| siQKI-tot | gaaagacagacagacagacagag | gacagacagacagacagacagag |
| siQK15 | gacagacagacagacagacagag | gacagacagacagacagacagag |
| siQK17 | gacagacagacagacagacagag | gacagacagacagacagacagag |

and 1% penicillin–streptomycin solution (3H Biomedical). Cultures were maintained at 37°C and 5% CO2. All experiments were performed within the first two passages of the defrosted cells, following manufacturer’s recommendations.

**RNA silencing**

A pool of four siRNA duplexes was designed to silence all QKI splice variants (QKI-tot), while two other siRNA pools were designed to specifically silence splice variants QKI5 or QKI7 (Dharmacon, Custom SMARTpool® siRNA Design). All siRNA sequences are shown in Table 1, and the position of the sequences with respect to the human QKI gene structure is shown in Figure 2A. Astrocytes were split to six-well tissue culture plates (Sigma) 48 h before transfection. No antibiotics were used in the culture medium. Transfection was performed using TransIT-TKO Transfection Reagent (Mirus) according to the manufacturer’s instructions. 25 nM of each siRNA pool, or scrambled (Scram) siRNA (siGENOME Non-Targeting siRNA #3, Dharmacon), harbouring no homology to any mammalian mRNA was delivered to each well. Mock (no siRNA) transfection Ctrl, comprising of cells treated with the transfection mix only, was used in the parallel transfections. Biological triplicates were prepared for each treatment. After 48 h cells were harvested in Trizol Reagent (Life Technologies) and stored at −80°C until RNA extraction.

**RNA extraction and real-time RT–PCR**

Total RNA was isolated from the cells using the PureLink RNA MiniKit (Life Technologies) according to the manufacturer’s instructions. RNA concentration and quality was monitored using Agilent 2100 Bioanalyser RNA Pico 6000 chip. Samples were stored at −80°C until further use. From each biological replicate, 400 ng of RNA was reverse transcribed into cDNA using TaqMan Reverse Transcription Reagents (Applied Biosystems). The final concentrations of reagents were: 1× RT buffer, 5.5 mM MgCl2, 2 mM dNTPs mixture, 2.5 μM random hexamers, 0.4 U/μl RNase inhibitor and 1.25 U/μl reverse transcriptase in RNAase-free water to a final volume of 10 μl. The mix was incubated at 25°C for 10 min (primer annealing), 48°C for 1 h (synthesis) and 95°C for 10 min (enzyme inactivation). cDNA samples were diluted with RNAse-free water to a final concentration of 5 ng/μl and stored at −20°C.

All real-time PCR experiments were performed in ABI Prism 7000 Sequence Detector System (Applied Biosystems)
using 96-well optical plates (ABI). The reaction mix included 4 μl of cDNA sample, 9.2 μl of MilliQ water, 0.66 μl (10 μM) of forward primer, 0.66 μl (10 μM) of reverse primer, 0.66 μl TaqMan probe and 9.8 μl of TaqMan Universal PCR Master Mix (ABI). Sequences of primers and probes are listed in Table 2. Expression of VIM and GLUL was quantified using SYBR Green as previously described (35). The thermal cycle was as follows: 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. A dissociation step was added for SYBR Green runs. Gene expression was quantified using the standard curve method and normalized against two housekeeping genes, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Actin, beta (ACTB). The two-tailed t-test was used to evaluate the statistical significance of differential gene expression.

### Western blotting

Transfected and Ctrl cells were lysed in the lysis buffer [50 mM Tris, 150 mM NaCl, 1% Triton X, protease inhibitor cocktail (Roche)] on ice for 20 min. The lysates were centrifuged (15 min, 4°C) and the supernatant was collected. Protein concentration was determined using the protein assay DC (Bio-Rad) according to the manufacturer’s instructions. Equal amounts of protein (150 μg) were electrophoresed on Mini-Protean TGX gels (4–15%, Bio-Rad) in transfer buffer (0.025M Tris base, 0.192M glycine, 8% 0.01 M Tris, 0.05% Tween 20, pH 8.0) containing DAPI for nuclear stain. The membranes were then incubated for 1 min in blocking buffer (0.025 M Tris base, 0.192 M glycine, 0.1% SDS). PageRouser prestained protein ladder (Fermentas) was used for molecular weight determination. Proteins were transferred to Immobilon-P PVDF transfer membranes (Millipore) in transfer buffer (0.025 M Tris base, 0.192 M glycine, 20% methanol) at 4°C. After blocking for 1 h in blocking buffer [5% non-fat milk powder (Bio-Rad), 0.15 M NaCl, 0.01 M Tris, 0.05% Tween 20, pH 8.0] the membranes were incubated with a primary rabbit antibody (anti-QKI5, -QKI6 or -QKI7, Chemicon, 1:500 in blocking buffer) overnight at 4°C. After rinsing, a secondary antibody (horseradish-conjugated goat anti-rabbit, 1:10000, Life Technologies) was applied for 1 h at room temperature. The membranes were then incubated for 5 min in 1:1 mixture of luminol/enhancer : peroxidase buffer solution (Bio-Rad) and developed on high performance chemiluminescence film (GE Healthcare). To ensure equal protein loading, membranes were stripped and probed for ACTB (mouse anti-β-actin, 1:5000, Life Technologies/horseradish-conjugated goat anti-mouse, 1:10000, Life Technologies).

### Immunofluorescence

The expression of QKI protein isoforms in primary human astrocyte cultures was analysed by immunocytochemistry. Cells were fixed with 4% paraformaldehyde (Sigma) for 15 min and blocked for 1 h in 0.25% bovine serum albumin in phosphate-buffered saline (PBS) with 0.025% Triton X-100. Each of the following primary antibodies: anti-QKI5, -QKI6, -QKI7 (rabbit, 1:1000, kindly provided by Prof. K. Artzt) was incubated together with anti-GFAP (chicken, 1:1000, AbCam) overnight at 4°C. After washing with PBS, the corresponding secondary antibodies were applied for 4 h at room temperature: Alexa 594 goat anti-rabbit and Alexa 488 goat anti-chicken (1:400, Life Technologies). Immunlabelled cells were mounted with DTG mounting media [2.5% DABCO (Sigma), 50 mM Tris–HCl pH 8.0, 90% glycerol] containing DAPI for nuclear stain. Cell imaging was performed using a confocal microscope (Zeiss LSM 510 META, Göttingen, Germany). The images were assembled in Photoshop CS4 (Adobe).

### Library preparation and RNA sequencing

RNA obtained from three biological replicates for each treatment were pooled and used for cDNA library preparation at the SciLife facilities at the Uppsala Genome Centre. Briefly, an rRNA depletion step was performed with 56 mg as input amount for all samples, using the RiboMinus Eukaryote kit (Life Technologies). Whole transcriptome libraries were then constructed using the SOLiD total RNA-Seq kit (rev B, July 2011, Life Technologies). Emulsion PCR was performed using the SOLiD EZ Bead System (Life Technologies) and the libraries were then sequenced on three lanes with the SOLiD 5500xl System (Life Technologies).

### Sequence alignment and data analysis

All sequencing reads were aligned to the hg19 build of the human genome using the LifeScope software (version 2.0, Life Technologies). Reads mapping to exonic regions were counted using the HTSeq software (http://www-huber.embl.de/users/anders/HTSeq/doc/overview.html date last accessed, January 11, 2013) and it was based on the known genes list from the University of California, Santa Cruz (UCSC) Genome Browser (57). Reads counting for calculations of the relative expression of distinct QKI splice variants was performed using BEDTools (58). The differential gene expression between Ctrl and siQKI-silenced cells was calculated using the

### Table 2. Primer and TaqMan probe sequences used for quantitative real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTB</td>
<td>gacctcagagetgcctgacg</td>
<td>gtagtttcttgtagtcacacaggact</td>
<td>ctcacacctaacatggagagggctcc</td>
</tr>
<tr>
<td>GAPDH</td>
<td>gggacgcctactgtgcaagggc</td>
<td>tagacggcaggtccgcagggc</td>
<td>ccaccaactgtctcgactggtagtcaggg</td>
</tr>
<tr>
<td>QKI-tot</td>
<td>gcaactacagagttgccacattata</td>
<td>cttggcttggtggcaagaga</td>
<td>aaataacagcttctctctcttcctglttc</td>
</tr>
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<td>QKI5</td>
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<td>cattgctttgaggaagegttca</td>
<td>acctaggtgcgtttagaagttgaggttcc</td>
</tr>
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<td>GLUL</td>
<td>cactcagacagtttacactg</td>
<td>gtagtttcttgtagtcacacaggact</td>
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</tr>
</tbody>
</table>

---

**Note:** The table lists the primer and TaqMan probe sequences used for quantitative real-time PCR. The sequences include forward primers, reverse primers, and the probe sequences, all necessary for the TaqMan assay. The sequences are given with standard abbreviations for primer designations (e.g., ACTB, GAPDH) and the specific nucleotide sequences for each primer and probe.

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**Reference:** Table 2 includes primer and TaqMan probe sequences used for quantitative real-time PCR, which are essential for gene expression analysis in real-time PCR experiments. These sequences are used to amplify specific gene fragments, enabling the quantification of gene expression levels. The table lists the primer and probe sequences for genes such as ACTB, GAPDH, QKI-tot, QKI5, QKI6, QKI7, GFAP, VIM, and GLUL, detailing the forward primers, reverse primers, and probe sequences. This information is crucial for researchers performing gene expression studies, allowing for accurate and specific detection of gene expression levels.
DESeq software (version 1.8.3) (59). DESeq approximates the null distribution using a negative binomial model under the assumption that the two conditions have the same read abundance, in order to calculate the probability of genes having a differential expression between two samples. Normalization of the raw read count was performed using the DESeq estimateSizeFactors function. The normalized values (shown in Supplementary Material, Tables S1 and S2) will be referred to as base mean normalized units. Genes showing a high probability of differential expression were manually inspected, and those containing PCR artefacts suspected to inflate significance values were removed from the list. Correction for multiple testing was done by DESeq using the procedure of Benjamini and Hochberg (59).

Haloperidol treatment

Before drug treatment, astrocytes grown in T75 flasks were split equally into six-well tissue culture plates (Sigma) and cultivated to ∼50% confluence. Biological triplicates were treated with 0.2 μM haloperidol delivered in antibiotic-free astrocyte culture medium, or with medium alone. Cells were harvested after 6 h using Trizol Reagent (Life Technologies) and total RNA was extracted as described above.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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