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Translational research of the quaking gene

Focusing on the conjunction between development and disease

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Abstract

Farnsworth, B. 2016. Translational research of the quaking gene. *Focusing on the conjunction between development and disease*. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology* 1381. 61 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-554-9595-4.

Quaking (*QKI*) is an RNA binding protein involved in the post-transcriptional regulation of gene expression. Originally identified as the cause of hypomyelination in a mouse mutant, it has since been consistently implicated in a wide range of neurological diseases. As a gene exclusively expressed in glial cells of the central nervous system, such associations emphasise the importance of an indirect, or non-neuronal link to aberrant neural function. A role in early neural development has also been suggested from the viable and embryonic lethal mouse mutants, yet detailed and *in vivo* study has been precluded thus far by the murine uterine gestation, and mutant lethality prior to oligodendrogenesis. This thesis examines the role of *QKI* in human neurological disease, and explores the use of the zebrafish as a model organism to allow the unimpeded study of neural development.

We first examined the expression of *QKI* in human post-mortem brain samples, in separate studies of Alzheimer's disease (AD) and schizophrenia. In AD we found that *QKI* and the splice variants *QKI5*, *QKI6*, and *QKI7* were all significantly upregulated, and were additionally implicated in the regulation of genes related to AD pathogenesis. Within schizophrenic samples, we explored the expression of *QKI6B*, a newly identified splice variant of *QKI*, alongside *GFAP*. We found that both were significantly upregulated, and a previously implicated regulation of *GFAP* by *QKI* was supported. In order to advance investigations of the potential of *QKI* to disturb neural development, we established the suitability of zebrafish for studying *qki*. This was achieved through phylogenetic and syntenic analysis, coupled with examination of the *qki* genes expression patterns. We found that *qkib* and *qki2* are orthologues of human *QKI*, and both have distinct, yet overlapping expression patterns in neural progenitors, and are not found in differentiated neurons. Following from this, we explored the effects of knockdown to *qkib* and *qki2*, finding that *qkib* exclusively led to aberrant motor neuron development, cerebellar abnormalities, and alterations to the progenitor domain. This clearly demonstrated the crucial role of *qki* in early neural development, and confirms a previously speculated, yet occluded, function prior to oligodendrogenesis.

Keywords: QKI, glia, oligodendrocyte, Alzheimer's, schizophrenia, zebrafish, statistics, morpholino

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”Science is magic that works”

Kurt Vonnegut

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I **Farnsworth, B.**, Peuckert, C., Zimmermann, B., Jazin, E., Kettunen, P. & Emilsson, L. S. (2016) Gene expression of quaking in sporadic Alzheimer's disease patients is both upregulated and related to expression levels of genes involved in amyloid plaque and neurofibrillary tangle formation. *Journal of Alzheimer's Disease*, 52(1)

II **Farnsworth, B.**, Radomska, K., Zimmermann, B., Kettunen, P., Jazin, E. & Emilsson, L. S. (2016) *QKI6B* is upregulated in schizophrenic brains and predicts *GFAP* expression. *Submitted to Schizophrenia Research*.

III Radomska, K., Sager, J., **Farnsworth, B.**, Tellgren-Roth, Å., Tuveri, G., Peuckert, C., Kettunen, P., Jazin, E. & Emilsson, L. S. (2016) Characterization and expression of the zebrafish *qki* paralogs. *PLOS ONE*, 11(1): e0146155.

IV **Farnsworth, B.**, Radomska, K., Sager, J., Jazin, E., Kettunen, P., & Emilsson, L. S. (2016). Morpholino knockdown of *qkib* leads to disturbed neural development in the larval zebrafish. *Manuscript*.

Additional Publications

The following paper was also published during the course of my doctoral studies, but is however not part of the present dissertation.

Serrien, D.J., Sovijärvi-Spapé, M.M., & **Farnsworth, B**. (2012) Bimanual control processes and the role of handedness. *Neuropsychology* 26 (6), 802.

Contents

Abbreviations

Aβ	amyloid beta
AD	Alzheimer's disease
ANCOVA	analysis of covariance
CNS	central nervous system
CRISPR	clustered regularly-interspaced short palindromic repeats
Cas9	CRISPR associated protein 9
E	embryonic day
ENU	N-ethylnitrosourea
GBM	glioblastoma multiforme
GW	gestational week
hnRNP	heterogenous ribonucleoprotein particle
hpf	hours post fertilisation
KH	K homology
LTP	long-term potentiation
MRI	magnetic resonance imaging
NHEJ	non-homologous end joining
NPC	neural progenitor cell
OLR	oligodendrocyte related
OPC	oligodendrocyte precursor cell
PC	principal component
PFC	prefrontal cortex
pMN	progenitor domain
PNS	peripheral nervous system
QRE	quaking response element
qPCR	real time PCR
siRNA	small interfering RNA
SSR	sum of squares of residuals
SST	total sum of squares
STAR	signal transduction and activation of RNA

Gene, protein, and compound symbols are not listed.

Introduction

The Brain and Neural Development

The complexity of the adult human brain is perhaps illustrated best through numbers, with 86 billion neurons, 160 thousand kilometres of axons, and 100 trillion synapses comprising what is arguably the most intricate system known to humankind [1]. The process of development that creates such an elaborate organ is also unsurprisingly complex, but worthy of interrogation, in order to further our understanding of how the brain forms, and ultimately how it functions.

The development of the first neural structure in humans begins in gestational week (GW) 3 [2]. Immediately prior to this, two layers, comprised of the upper epiblast, and lower hypoblast, gastrulate to form three layers [3]. From here, the epiblast will form all structures of the embryo, while the hypoblast will form external structures, such as the foetal placenta [2]. The epiblast will, in part, give rise to neuroectodermal stem cells (more commonly known as neural progenitor cells) that are the foundation of the central nervous system [4]. Thus, now at GW3, the first, fundamental neural structure is formed and continues to develop. The region of the embryo occupied by neural progenitor cells is known as the neural plate [2], which soon folds and fuses, giving rise to the neural tube. From the rostral end of the neural tube, the brain will develop, while the caudal end will form the spinal cord and hindbrain [5]. The neural tube is, crucially, hollow; from within this space, the ventricles will form, and neural progenitor cells will remain there throughout development and into adulthood [2]. This is one of the few sites of the mature brain in which multipotent neural stem cells are found (the other areas being the dentate gyrus of the hippocampus [6], and the olfactory bulb [7], although other regions may yet show similar features that have yet to be revealed). The neural progenitor cells of the ventricular zone will be discussed in greater detail below. The subdivision of the anterior-most area continues, with five distinct regions (the telencephalon, diencephalon, mesencephalon, metencephalon, and myelencephalon) emerging, in a rostral-caudal direction that is ultimately reflected in the topography of the adult brain. Neuron production begins around GW6, with a radial migration from the ventricular zone. Radial glial guides [8], derived from neural progenitor cells [9-11], extend and project processes from the ventricular zone, out towards the pial layer of the brain. Neurons migrate along this glial scaffolding in an “inside-out” order [12], and then begin to

differentiate [13]. Gyri and sulci of the brain appear from the foetal stage (GW9 and onwards), while neuronal migration and differentiation continue up to, and after, birth [14]. While this underlies the central components of neuronal development at prenatal stages, the process of glial development has yet to begin.

Glia Development

While the vast majority of neuroscientific literature has thus far focussed on the neurons of the brain, a vast proportion of the brain is composed of something else: glia (the exact proportional difference is not definitively known, however the most recent, and comprehensive investigation estimates the difference to be almost 1:1, with huge ranges of differences dependent on brain area [15]). Originally deriving from the Greek word for "glue", this etymology is also indicative of the prevailing view of glia throughout the development of neuroscience. It has only been within the past 40 years that modern neuroscience has appreciated and evaluated the ways in which glia underlie the functioning of the brain [16]. Originally perceived as merely support cells to neuronal function, recent research has expanded this view, additionally demonstrating the important and wide-ranging functions of glia [17].

It is only within the postnatal period that the development of glial cells begins (with the exception of the aforementioned radial glial cells), yet the differentiation and maturation of these cells continues throughout childhood [18]. Additionally, glial progenitors, particularly oligodendrocyte precursor cells (OPCs) persist throughout life, ready to differentiate in response to injury, and for ongoing myelin maintenance [2]. OPCs (also known as NG2 cells, or polydendrocytes [19]) either remain in the progenitor domain (pMN) and retain their capacity for self-renewal, or ultimately differentiate into oligodendrocytes [20]. Although the potential of OPCs to additionally differentiate into astrocytes has been reported [21, 22], this may in fact reflect a shared region of different progenitor cells, rather than the presence of bipotent precursors [23]. Evidence also exists for the ability to differentiate into neurons [24] or interneurons [25], although this has more recently been contested [26].

Three major types of differentiated glial cells exist within the brain – astrocytes, oligodendrocytes, and microglia. Each has a critical role in the formation and maintenance of the brain. Additional glial cell types exist within the peripheral nervous system (PNS), including Schwann cells, that directly form myelin ensheathments of axons, in an analogous role to oligodendrocytes [27]. Furthermore, olfactory ensheathing cells, enteric glia, satellite cells, and sensory nerve glia all play a part in PNS function [27, 28].

Oligodendrocytes

Following migration, an OPC will differentiate into a mature oligodendrocyte, and subsequently begin the process of myelination [29]. The ensheathment of axons with lipid-rich myelin begins with extensions arising from the oligodendrocyte(s) and making contact with the axon [30]. While much debate surrounds the specifics of the myelin ensheathment motion (e.g. “carpet crawler”, “serpent”, or “liquid croissant” models [31, 32]), it is widely accepted that myelination begins with the lipid membrane spiralling around the axon [33]. From this, the myelin membrane continues to grow, typically reaching an axon to myelin ratio of 0.76-0.81 [34]. Following the wrapping of the membrane, the myelin undergoes the process of compaction, in which the cells adhere closely together [35]. Ultimately, each oligodendrocyte can continue to myelinate up to 80 internodes [36]. This is a process that begins in the first postnatal year, and will continue well into early adulthood, progressing in a caudal-rostral direction, and reaching completion with the myelination of the prefrontal cortex (PFC) [37]. The basic units of myelin, and components in its development are shown in Figure 1.

The spacing intervals of myelin sheaths, called the nodes of Ranvier, have been found to emerge in a stereotypical manner even when axons are substituted with electron-spun nanofibres, or microfibres [38-40], suggesting that intranodal spacing is regulated solely by oligodendrocytes. Evidence has also shown that myelination can also be dynamically regulated by experience and environmental factors [31, 41, 42], as well as through axonal firing [43, 44]. Such changes are ultimately brought about through interaction and influence on oligodendrocyte function, further demonstrating their critical role in CNS development.

Oligodendrocytes and their constituent myelin sheaths are involved in various processes that aid the functioning of the CNS. First and foremost, the myelin improves signal velocity of axonal signalling (action potentials), by establishing saltatory conductance. The electrical current travels fastest along the myelin insulation, and establishes a new action potential at the nodes of Ranvier, allowing rapid conduction without a loss of signal fidelity. Further to this, oligodendrocytes ensure the maintenance of myelin in both healthy [45, 46], and diseased brains [47]. Oligodendrocytes and the myelin sheath itself have been shown to be critical for axonal survival, providing both lactate (a metabolite used in the production of ATP within the axon [48-50]), and exosomes (cell-derived vesicles), in a neuroprotective manner [51]. Furthermore, McKenzie et al [52] used transgenic mice incapable of producing new oligodendrocytes from OPCs, and trained them with a motor learning task. The mice were unable to master the task, as compared to wild-type mice, demonstrating that oligodendrocytes have a role in learning and behaviour.

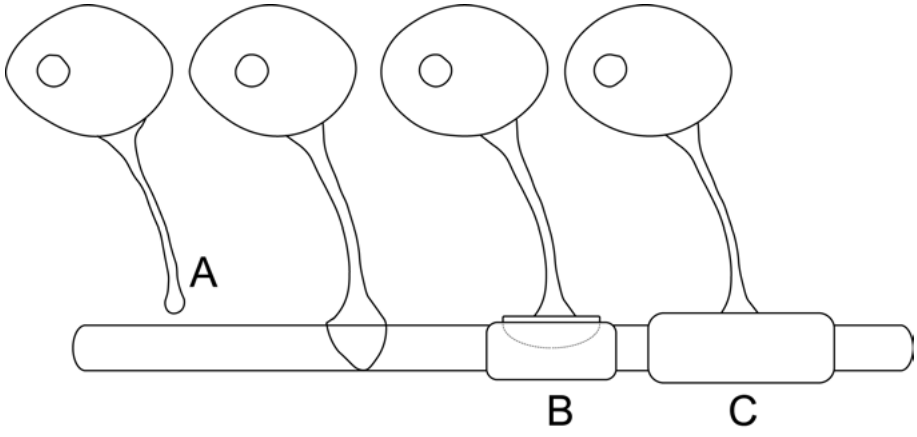

Figure 1. Development of myelin sheaths from oligodendrocytes. A – Oligodendrocyte extends process that reaches an axon. B – myelin sheath is produced, curling around axon and underneath the prior layer. C – Myelin sheath internodes are formed, with nodes of Ranvier between. In the developing CNS, this process could be carried out by a single oligodendrocyte with multiple extensions.

Astrocytes

While it is largely assumed that astrocytes follow a similar developmental progression as oligodendrocytes, this has not been conclusively shown (largely due to a lack of markers specific for astrocyte precursors [53]). It has however been demonstrated that astrocytes form from the ventricular zone, akin to oligodendrocytes [54], before specifying to an astrocytic cell-fate and migrating [53]. Evidence has implicated a regulated patterning of astrocytes, dependent on their point of origin within the ventricular zone [55], with further specification dependent on cell subtype (i.e. fibrous or protoplasmic [56]). Upon maturation, astrocytes typically make contact with blood vessels, and with either synapses (in the case of protoplasmic astrocytes), or nodes of Ranvier (in the case of fibrous astrocytes), or with other astrocytes [57]. Astrocytes ultimately propagate throughout the entirety of the CNS, and can make contact with up to two million synapses [58].

Astrocytes have a wide variety of functions within the CNS, ranging from energy metabolism [59], and neurotransmitter recycling [60], to controlling synapse formation [61, 62] and elimination [63]. Even complex synaptic functions such as long-term potentiation (LTP) have been found, at least in some cases, to be under the regulation of astrocytes [64]. Furthermore, astrocytes contain receptors, ion channels, and cell surface molecules [65], permitting an involvement in brain signalling, and blurring the line between glia and neuron [66]. This is particularly evident in the context of calcium signalling, a feature discovered in the early 1990's, *in vitro* [67, 68]. Subsequent *in vivo* studies have shown that synaptic neurotransmitter "spillover" (i.e. neurotransmitters not absorbed at the synapse) can initiate oscillating Ca^{2}+ signals, released

from astrocytes [69, 70]. As a result of increased Ca^2+, gliotransmitters are released, which can affect neurons [71], signal to glia [72], can regulate cortical blood flow [73], and can even be traced to behavioural outcomes (albeit, thus far only in sleep [74]). It therefore appears that the involvement of astrocytes in the CNS is difficult to underestimate, and the increasing accuracy of imaging techniques points to even greater levels of sophistication [75].

Microglia

Microglia arise through haematopoiesis at around GW4.5 [76] and enter the brain primordium through the developing meninges, choroid plexus, and ventricular zone [77, 78], areas of aforementioned neural proliferation. From here they play an active role in regulating the development of the CNS and can initiate apoptosis [79], promote migration, and stimulate axonal growth [80]. Ultimately they will constitute 10-15% of the adult brain [81]. Initially amoeboid in appearance, as microglia mature they attain a ramified (or resting) morphology [82]. As the blood-brain-barrier becomes functionally effective (the timing of which is strongly debated, [83]) microglia remain within the brain parenchyma [84] and remain ramified except in the case of pathology [85]. Ultimately they play a principal role as resident immune cells of the brain, with an involvement in tissue repair, and in activating further immune responses [86].

Genetic Regulation of Glia

There are a number of genes that tightly regulate the development of the aforementioned cell types in the development of the CNS. As has been shown, each glial cell type has far reaching roles in the development, maintenance, and proper functioning of the CNS. Glia therefore represent critical, and often underexplored targets for investigation in both normal and aberrant neural outcomes. Furthermore, the various genes that are involved in the regulation of gliogenesis are of particular value when investigating neurodevelopmental disorders. Particular attention has surrounded the quaking (*QKI*) gene, due to a wide range of evidence implicating an involvement in various neurological diseases [87, 88].

Quaking

The *Qk* mutant mouse was first identified in 1961, and was characterised in 1964 as responsible for an autosomal recessive mutation within the same organism [89]. The mutant mouse subsequently developed tremors of the hindquarters (hence the term “quaking”) at around 10-12 days after birth, which

increased in intensity to a peak at around 3 weeks. This phenotype can ultimately result in tonic-clonic seizures in adult mice [90]. Upon investigation, the mouse was found to be severely deficient in myelin. No evidence of destruction was found, suggesting a developmental origin [89]. The gene was subsequently cloned by Ebersole et al in 1996 [91], and was found to be strongly expressed at the peak of myelination. This was further evidence of a critical role in myelinogenesis, and by proxy, in oligodendrocyte function.

Later investigations of *QKI* (and *Qk* in the mouse) showed it to be an RNA-binding protein exclusively expressed in glial cells in the brain [92] with a role in mRNA translation [93], turnover [94], stabilisation [95], and splicing [96, 97]. *QKI* has several splice variants in humans, termed *QKI5*, *QKI6*, *QKI6B*, *QKI7*, and *QKI7B* [98, 99]. While QKI was found to have fairly widespread expression, it is predominantly expressed within glia cells of the CNS in adult human tissue [100]. The splice variants show widespread conservation across their sequences, only differing in their UTR (untranslated region), and by around 30 amino acids in their Carboxyl (C)-termini [101]. The C-terminal region of QKI5 has been found to contain a noncanonical nuclear localisation signal [102], which is reflected in the detected nuclear expression [92]. Qk6 and Qk7 are found to be predominantly cytoplasmic [92], although Qk6 can be found in lower quantities (and Qk7 to an even lesser degree) within the nucleus, suggested to be a result of dimerization with Qk5 [103]. While there is a large degree of conservation between each splice variant, studies of cellular expression, downstream targets, and differences in expression in disease all point towards potentially distinct functions. For example, *QKI7* has been specifically suggested to regulate the expression of glial fibrillary acid protein (*GFAP*; [98]), while *QKI7* and *QKI7B* are found to be differentially expressed in schizophrenia, distinct to other splice variants [104]. These studies are discussed in further detail below.

All of the *QKI* splice variant sequences contain a hnRNP (heterogenous ribonucleoprotein) K homology (KH) domain (and as such, belong to the eponymously named KH motif protein family [105]). This is the principal region through which QKI binds to RNA [106]. Upon RNA binding, QKI can impact the aforementioned processes, such as translation and turnover. The sequences to which QKI binds to are defined by their harbouring of a Quaking Response Element (QRE, [107]). The minimal QRE nucleotide sequence that will be bound by QKI is defined as UACU(C/A)A [108], while Galarneau and Richard [107] define a bipartite consensus sequence as ACUAAY-N$_{(1\text{-}20)}$-UAAY (in which Y refers to cytosine or thymine, and N to any nucleotide, with the numbers in subscript referring to the length of the nucleotide chain). There are nuanced tolerances within the binding affinity of the sequence, and the half-site (UAAY) does not appear to be essential [107], however sequences found to be containing this QRE offer the best glimpse of QKI targets. Recent work has identified the formation of complexes, through X ray crystallography, with further differing nucleotide sequences and the KH domain

[109]. *QKI* isoforms also harbour two subdomains that flank the KH domain: Qua1, and Qua2. This total region (i.e. Qua1-KH-Qua2) is termed either the STAR (signal transduction and activation of RNA) domain, or the GSG (GRP33, Sam68, GLD-1) domain [110, 111]. Qua1 is required for homodimerization, and point mutations within this area prevent self-association and initiate apoptosis [112]. The Qua2 sequence is found to be essential for sequence-specific RNA binding, as an extension of the KH domain [113]. In addition to these regions, a tyrosine rich area is found proximal to the C-terminus, suggesting that *QKI* itself may be subject to mediation through tyrosine phosphorylation [110, 114, 115]. A schematic of the structure and domains of the QKI gene is shown in Figure 2.

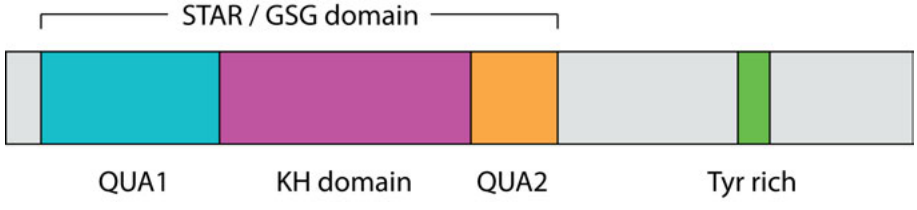

Figure 2. Domains of *QKI*. Text above and below the figure indicates the name of the region. Sizes of the components are reflective of the basepair length of the region. Grey regions denote areas without identified domains. The figure shows *QKI5*, although other splice variants only differ in their C-termini. The figure is adapted from Volk et al., 2008 [114].

Homologues of *QKI* are found within several species additionally to human and mice, including *Drosophila melanogaster* (fruit fly, [116]), *Gallus gallus* (chicken, [117]), *Xenopus laevis* (African clawed frog, [118]), and *Danio rerio* (zebrafish, [119], which is discussed in detail below), amongst others. The sequence is remarkably conserved throughout all species and all splice variants, with the KH binding domain also consistently present, suggesting the domain is subject to strong selective pressure [120].

With regards to the role of *QKI* in the regulation of glia, much attention has been paid to an involvement with oligodendrocytes, due to the aberrant hypomyelination within mouse mutants. Insufficient myelination from Schwann cells also occurs in the PNS, although not as drastically as in the CNS [121, 122]. Hardy et al [92] showed that the lack of myelination in quaking viable mutant mice (the first identified *Qk* mutant, Qk^{v}) is associated with specific splice variant expression. Both *Qk6* and *Qk7* were found to be absent from any myelin-forming cells (i.e. oligodendrocytes and Schwann cells), while *Qk5* was only absent in the most severely affected regions [92]. It is therefore apparent that the function of *Qk* within oligodendrocytes can have a dramatic impact on the formation of myelin. However, further investigation suggested

that the resulting dysmyelination phenotype in *Qk* mutants may actually be produced by the role of *Qk* at an earlier timepoint in development, rather than at the point of myelination itself. For example, delayed oligodendrocyte maturation has been found upon *QKI* knockdown induced via short interfering RNAs (siRNA) [123], while overexpression of *QKI* increased differentiation. Furthermore, rescue of this phenotype could be partially achieved through siRNA resistant splice variants, *QKI5* and *QKI6*, (but not *QKI7*). This was found to be unrelated to the level of cell proliferation, or cell cycle progression, suggesting a critical role of *QKI5* and *QKI6* in oligodendrocyte development, and therefore prior to myelin formation [123]. Further to this, both *Qk6* and *Qk7* have been shown to, through an upstream process, bind to and stabilise *p27Kip1* [94] which is involved in regulating the cell cycle of OPCs [124]. Zearfoss et al [125] also show that Qk regulates *Hnrnpa1* expression in OPCs. Downstream of this, both *Mag* (myelin associated gene) and oligodendrocyte-specific *Plp1* (proteolipid protein 1) were under the control of Hnrnpa1, possibly in addition to other genes. This suggests that Qk can regulate the oligodendrocyte / myelin related genes, *Mag*, and *Plp1*, via regulation of *Hnrnpa1*. Recently, QKI was also found to bind and promote the translation of VE-cadherin and β-catenin mRNA [126]. VE-cadherin is a component of endothelial junctions [127], while β-catenin is involved in cell-cell adhesion and gene transcription, specifically as a co-activator of the Wnt signalling pathway [128, 129]. The Wnt signalling pathway is involved in multiple processes, including embryonic development through direction of cell proliferation, cell polarity, and cell fate determination [129]. Furthermore, Dai et al [130] show that *Wnt*/β-catenin signalling disruption can delay oligodendrocyte maturation in the developing mouse brain. Further investigation is required to confirm or refute the involvement of this process in the aberrant myelination / oligodendrocyte phenotype encountered in *Qk* mutant mice, although there are intriguing similarities and links in both processes.

Quaking and Disease

In addition to the dysmyelination phenotype seen in mouse mutants, *QKI* has also been found to be associated with a wide range of neurological diseases, and subsequent abnormalities in the CNS [87, 88]. Baumann, in 1982 [131], was one of the first researchers to speculate on how the resulting hypomyelination in *Qk* mouse mutants could be related to human diseases. Further to this, Rondot et al. in 1986 [132], were the first to point towards a potential association with Parkinson's disease, which drew further interest after it was revealed that the Qk^{v} mutant led to a spontaneous deletion of *PARKIN* and *PACRG* [133]. It was however revealed that the mutation failed to replicate the complete neuropathology associated with Parkinson's disease [134], and Itier [135] showed that *PARKIN* mutants do not display the characteristic dysmyelination, tremors, or seizures found in *Qk* mutants [87]. These studies have

therefore shown that the dysmyelination phenotype is under the control of the *Qk* gene, and are not representative of Parkinson's disease pathology. Further research has established an involvement of *QKI* in a wide variety of neurological diseases, which will be discussed in the following sections. Due to the glial expression of *QKI*, and the putative role in glial regulation, evidence indicating a glial involvement in disease will also be discussed where relevant.

Quaking and ataxia

Ataxia is a neurological disorder resulting in imbalance and deficient, or absent, motor co-ordination [136]. Severe ataxia is found in Qk^{v}, and Qk^{e5} mouse mutants [89, 137], and axonal swelling of Purkinje cells was one of the first uncovered neuropathological features, in addition to the evident hypomyelination [137], and is similar to the neuropathology of ataxia in humans [138]. In a yeast two-hybrid screen of ataxia related proteins, Lim et al. [139] also identified *QKI* as a potential hub of interaction within the "ataxia-ome" [87]. Associations have also been made with other KH domain containing proteins and ataxia-like syndromes [140, 141], suggesting an involvement of this domain in the disease [87]. Additionally, Bergmann glia both abundantly express QKI [142], and are implicated in the pathogenesis of several forms of spinocerebellar ataxia [143-145], although this, and other associations remain only suggestive until further research can confirm or refute the association of *QKI* and ataxia.

Quaking and glioma

Gliomas are the most common form of malignant brain tumour, and arise from glial cells in the CNS [146]. Reduced expression of *QKI* was found in human glioma samples [99], although not in schwannomas or meningiomas, in accordance with the glial cell specificity of *QKI*. Deletions of *QKI* have also been found with specific gliomas such as anaplastic astrocytoma [147], and glioblastoma multiforme (GBM), which is the most common tumour of the CNS, and invariably fatal [148]. *QKI* has been found to be a GBM tumour suppressor, through stabilising miRNA, which mediates downstream cancer-related genes [149]. GBM cell lines also show deletions of *p53* [148], the perturbation of which is required for the development of most cancers [150], and is an inducer of *QKI* activity [149]. This suggests that the development of glioblastoma multiforme could lead to (or result from) a deletion of *p53*, which subsequently leads to a reduction of *QKI*. Further to this, loss of *p53* within Qk^{v} mice increases the mortality rate, although the exact cause of death is not known [151]. This again suggests that *QKI* expression may be altered as a consequence of *p53* alterations, increasing phenotypic severity, although this has yet to be definitively determined. A recent study of angiocentric (pertaining to blood vessels) gliomas found that the fusion protein of MYB (myeloblastosis transcription factor) and QKI was a specific and single candidate

driver for angiocentric glioma development [152]. This fusion protein produces a hemizygous deletion at the 5' end of *QKI*, which reduces the expression of *QKI*, thereby also diminishing the role it plays as a tumour suppressor gene, and increasing tumorigenicity [149, 152, 153].

Quaking and schizophrenia

Schizophrenia is a heterogeneously exhibited neurodevelopmental disorder consisting of "positive" (hallucinations, delusions) and "negative" symptoms (apathy, social withdrawal) [154, 155]. The first direct genetic association of *QKI* to schizophrenia was made in 2006, with the finding that two splice variants, *QKI7* and *QKI7B*, were downregulated in the prefrontal cortex of schizophrenic brains, as compared to controls [104]. This finding was soon repeated, and a downregulation was also found in several cortical regions via microarrays, and in the cingulate cortex via real-time PCR (qPCR) [156]. Further research utilising *in situ* hybridization also determined a reduction of *QKI* within the anterior cingulate cortex in post-mortem schizophrenic brains [157]. However, Huang et al [158] failed to find any evidence of a difference in *QKI* expression within a Han Chinese population. Nevertheless, the research represents a relatively robust consensus of gene expression differences. Additionally, other genes under the regulation of *QKI* have also been found to be altered in schizophrenic brains. Åberg et al [159] revealed that several oligodendrocyte-related (OLR) genes appear to be both differentially expressed in schizophrenic brains, and under the regulation of *QKI*. This suggests that an oligodendrocyte link, that is also present in the Qk^{v} mouse, may have some relevance within schizophrenia. Further to this, in a siRNA experiment, *QKI7* appeared to regulate the expression of several interferon-related genes [160], in line with evidence showing a link between immune-related genes and schizophrenia [161, 162]. Additionally, *QKI7* appears to regulate the expression of glial fibrillary acid protein (*GFAP*) in astrocytes [98], which has consistently been found to be differentially expressed within schizophrenic brains [163-165]. While further experiments will be required to definitively establish the regulation by *QKI* of multiple genes implicated in schizophrenia, the evidence does appear to suggest that *QKI* could play an important role in schizophrenia development. Rosenbluth and Bobrowski-Khoury [166] explored the original Qk^{v} mutant mouse neuropathology, and how it may relate to neural abnormalities in schizophrenic brains. While they discovered numerous contrasting aspects of CNS pathology, several similarities also emerged. For example, abnormal neural oscillations, as seen in schizophrenia [167] may arise as a result of dysmyelination and subsequent velocity decreases, while defects in the dorsal visual stream are speculated as a result of dysmyelination, and found within schizophrenic brains [166, 168].

In addition to the apparent genetic link between schizophrenia and *QKI*, several neuropathological lines of evidence indicate commonalities between

the disorder and *QKI* function. For example, reduced myelin has been repeatedly found in post-mortem brains of schizophrenic patients [169, 170], and *in vivo*, through the use of MRI (magnetic resonance imaging) [171], reminiscent of the *Qk* mutant mouse dysmyelination. Furthermore, reduced oligodendrocyte density has been found in several brain regions of schizophrenics [172, 173], as seen in *Qk* mutant mice [174]. Similarities in neuronal disturbances are also found, with increased dopamine metabolism in *Qk* mutant mice [175], which is also implicated in schizophrenic brains [176]. A shortening of both apical and basal dendrites of pyramidal neurons in layer II-III of the anterior cingulate cortex was also found in *Qk* mutant mice [174], which is replicated for basal, but not apical dendrites in the PFC of schizophrenic brains [177, 178]. It is clear that a complex and heterogeneous human disorder such as schizophrenia is not (and is unlikely to be) recapitulated by a single mouse mutant, yet there are intriguing parallels that merit further study.

Quaking and depression

Depression encompasses a range of symptoms that must be present daily for at least two weeks for a diagnosis, including decreased interest or pleasure, depressed mood or irritability, decreased activity, and fatigue, amongst others [179]. *QKI* was found to be downregulated within multiple brain regions of individuals with major depressive disorder, who died by suicide [180]. This was assessed via microarray, followed by qPCR, and immunoblotting to assess protein levels. However, no differences were found in the variation of the promotor, or in the methylation status of *QKI*, suggesting a different mechanism is responsible for the expression differences. A reduced glial cell density has been repeatedly found in post-mortem brains of depressed individuals [181-183], suggesting a glial involvement. Glia has also been implicated in another study of post-mortem brains of depressed individuals, through transcriptional profiling and subsequent discovery of an overrepresentation of expression differences of glia-related genes [184].

Quaking and anxiety

Anxiety has historically been difficult to define [185], but a relative consensus surrounds the term as "a future-oriented mood state associated with preparation for possible, upcoming negative events" [186]. There are numerous anxiety disorders [179], but all converge within this definition. In a screening of brain tissue and blood from mice treated with anxiogenic and anti-anxiety drugs, *Qk* was found to be a candidate gene for anxiety disorders [187]. This approach was cross-referenced within the same study with previous data of transgenic model behaviour, in which the Qk^{v} mouse showed abnormal responses to a novel object, symptomatic of "anxious" behaviour in mice [188]. A study within guinea pigs exposed to prenatal stress [189], showed that offspring displayed more anxious behaviours, and had decreased protein expression of myelin basic protein (MBP) and GFAP (both of which contain a QRE

and are speculated to be targeted by QKI; [98, 107, 115]). This suggests a neurodevelopmental role of glia in the formation of anxious behaviours, but has yet to be confirmed in humans. Additionally, analysis of the human genome of chronically stressed individuals showed alterations to *QKI* [187, 190]. As anxiety has a high diagnostic co-morbidity with schizophrenia [191] and depression [192], it is perhaps not entirely unsurprising that differences in *QKI* expression could be found in light of previous research concerning these disorders (as previously speculated by Klempan et al. [180]).

Quaking and Alzheimer's disease

Alzheimer's disease (AD) is an incurable neurodegenerative disease, accounting for around 50-70% of all cases of dementia [193]. It is chiefly characterised by memory impairment and executive dysfunction [194], but may also present with co-morbidity for depression, anxiety, and other neuropsychiatric disorders [195]. Despite being identified over a hundred years ago [196], advances in understanding and treatment has yielded little success [193].

QKI was recently identified in a microarray screening of AD patient samples to be upregulated, in accordance with disease severity, compared to non-AD samples [197]. Additionally, several other genes related to neurogenesis were found to be differentially regulated, suggesting a neurodevelopmental component of disease pathology. Various aspects of glial cell pathology are also implicated in AD. Activation of astrocytes has been repeatedly documented [198-200], which forms an immune response [201] that can ultimately increase disease progression [202, 203]. OLR myelin defects have been found [204], in addition to a general reduction of myelin in post-mortem brains [205-208]. As with depression, two speculated targets of QKI, *MBP* and *GFAP*, have been linked to AD progression. MBP co-localises with Aβ (a protein integral to AD pathology; [209]), and is found to be increased within post-mortem AD brains [210]. *GFAP* is similarly upregulated [211, 212], and is a central component of the aforementioned astrocytic activation [213].

A note on microglia

As *QKI* has not been shown to be expressed within microglia, such associations of these glial cells will not be explored in detail. While there is also an abundance of research linking microglia alterations and various neurological diseases, the main scope of this thesis is to explore the potential of *QKI* as a hub for disease. However, many reviews already exist elaborating on the involvement (speculated or confirmed) of microglia in schizophrenia [214], bipolar disorder [215], depression [216], autism [217, 218], AD [219, 220], Parkinson's disease [221], and amyotrophic lateral sclerosis [222], amongst others. While this glut of associations to neurological disease is certainly noteworthy, the involvement may not be entirely surprising considering the role

of microglia as an immune response, and the way in which so many neurological diseases activate factors related to the brain's immune response (e.g. oxidation, inflammation, and other pathologies).

Mouse Models of QKI

In addition to the initial investigations of the original Qk^{v} mutant, a great amount of research has subsequently explored the developmental trajectory of both the Qk^{v} mutant, and other mutants (including the *Qk* null mouse, $Qk^{-/-}$). Several N-ethylnitrosourea (ENU)-induced mutant mice have been generated, all of which are found to be embryonic lethal, or have severely increased mortality. The E48G mutant (also known as $Qk^{kt3/4}$), is the result of a point mutation (an adenine to guanine transition) changing glutamic acid 48 to glycine within the STAR domain. The mice show defects in *Qk* dimerization, but interestingly, not in RNA binding [91, 112, 223]. This mutant survives only until embryonic day (E) 9.5, displaying cranial and heart defects. A further mutant is found to be deficient in binding RNA as a result of a thymine to adenine transversion (V157E mutant; [224], or Qk^{k2} [223]), and shows no defects with dimerization, yet is ultimately embryonic lethal at E10-E12.5. This mutant shows abnormal somite development, and similarly to E48G, displays cranial and heart defects [225]. Another mutant, Qk^{kt1}, is also found to survive only until E9.5, and shows the same defects at death as both $Qk^{kt3/4}$ and Qk^{k2} [223]. The exact location of the mutation for Qk^{kt1} has not yet been delineated, but screening by Cox et al [226] showed that the mutation is not present in either the coding region, or in the C-terminus, which is in marked contrast to the other lethal mutations, and raises further questions about the structural function of *Qk*. Another ENU-induced mutant, Qk^{l-1} [226, 227], fails to produce the *Qk5* isoform as a result of an adenine to guanine transition that abolishes the start site required for *Qk5* transcription [225]. This mutant also shows the critical role of *Qk5* for embryonic development, as embryonic lethality occurs at E8.5-11.5, due to vascular remodelling defects [225]. Furthermore, the isoform *Qk5* is found to be deficient in function or expression for all of the embryonic lethal ENU-induced mutants [228].

Noveroske et al [137] were the first to produce an ENU-induced viable *Qk* mutant mouse since Qk^{v}, although only around 35% survive until 5 months. Named Qk^{e5}, the mice show ataxia, and dysmyelination in a manner similar to, but much more severe than Qk^{v}. Additionally, Purkinje cell axonal swellings are exhibited, a pathology not seen in the original Qk^{v} mutant, and symptomatic of neurodegeneration. As with the Qk^{kt1} mutant, the precise location of the mutation is not currently known, however the Qua2 region (required for sequence-specific RNA binding [113]) appeared unchanged, as probes were able to bind to this region [137]. Of further interest for *Qk5*, the Qk^{e5} mutant shows a reduction of this isoform, whereas the Qk^{v} mutant does not; this is suggested to be a potential reason for the increased phenotypic severity [228].

The *Qk* null mutation ($Qk^{-/-}$) is predictably embryonic lethal; mice show a range of defects in neural tube development, pericardial infusion, and embryonic turning, with mice surviving only until E9.5-10.5 [229].

The clear and critical requirement of *Qk* for normal survival and development is resolutely indicative of essential functionality throughout gestation. While the criticality of *Qk* in development, and the links to human disease are apparent, it is also evident that aberrations in human disease are subtler than the aforementioned mouse mutations. Furthermore, the early embryonic lethality of the mouse mutants precludes investigations of later prenatal, and postnatal developmental mechanisms that *Qk* is suggested to be involved in.

Furthering research of *QKI* and disease

From the abundance of direct and indirect links to neurological disease, it is apparent that the function of *QKI* merits further investigation. Despite over fifty years of research, much remains to be elucidated with regards to how *QKI* can impact neural development, as the majority of *Qk* mouse mutants are embryonic lethal [88]. The non-viable *Qk* mouse mutants die at around E8.5-12.5 (depending on the type of mutation), which is prior to the earliest stages of oligodendrogenesis (around E12.5-13; [230, 231]), preventing further study of this process. As the formation of oligodendrocytes ultimately gives rise to myelination, the occlusion of this process is a barrier in the study of *Qk*, as dysmyelination is the central phenotype. Other model systems offer the prospect of at least partially comparable physiology, and a viable developmental system in which to observe and investigate *QKI* (or rather the homologues, of *QKI*) function. While many model organisms exist, in choosing one, a difficult balance must be taken between biological relevance (particularly with regards to human comparisons), and practical tractability (which could encompass everything from fecundity and generation time, to genome sequences, standardised protocols and tools). In terms of biological relevance, it would appear that genetic distance from humans would determine the relative utility for comparisons of the organism being studied, although the actual experimental feasibility precludes many "close relatives" [232]. The ideal model organism would therefore have both a recent common ancestor, and an established experimental record. As mammals offer little advantage in comparison to mice in terms of experimental feasibility (i.e. ease of use and access within a laboratory setting), the utility of other organisms beyond this must be explored. One such organism that offers such feasibility, and a relatively close evolutionary relationship, is the zebrafish, or *Danio rerio* [233].

The Zebrafish

The use of zebrafish as a model organism dates back to the late 1960's, although it was only from around 1990 that their utilisation in research gained

traction [234]. Zebrafish were the first vertebrate to be cloned [235], and innumerable subsequent studies have exemplified their significance in genetics research [233]. Zebrafish are a member of the Cyprinidae family, and the genus *Danio*; the commonly used species for research is *Danio rerio*. As a teleost, they underwent an additional teleost-specific whole-genome duplication that occurred in a common ancestor, around 345 million years ago [236, 237].

Adult zebrafish are both relatively easy to breed and maintain, and can lay up to 300 eggs a week [238]. This fecundity and inexpensive maintenance costs provide a system ideal for high-throughput research. Coupled with a transparent and rapid *ex utero* development, organogenesis can be readily studied, in a marked contrast to murine development. Studies pertaining to the development of the central nervous system are therefore well-suited to the zebrafish [239].

It therefore follows that investigations in the zebrafish of *QKI*, or the zebrafish homologue *qki*, are also well-suited. The external and rapid development of the zebrafish embryo provides an ideal system in which to study glial development. This is demonstrated with OPC development arising as early as 48 hours post-fertilisation (hpf) [239, 240], and myelination beginning at 3 days post-fertilisation (dpf) [241]. However, as with mammals, myelination continues into adulthood [37, 242]. Nevertheless, the rapid and visible development of myelination offers a suitable opportunity in which to investigate early myelinogenesis [240], the point at which *QKI* function is hypothesised to have the greatest impact [123]. Additionally, multiple genetic methods exist for the zebrafish, including morpholinos and the CRISPR/Cas9 system, which are discussed in the section *Future Perspectives*.

The zebrafish genome has around 26,000 genes [243], with which roughly 70% of human genes have an orthologue (69% in the opposite direction), and 47% have a one-to-one relationship [237]. The quantities of shared proteins across the human, mouse, and zebrafish are shown in Figure 3.

While there are differences in genome evolution, genetic conservation has allowed for direct comparisons to humans. For example, a central gene in myelin formation in humans is *MBP* which is found as two ohnologues (genes retained from a whole genome duplication [244, 245]), *mbpa*, and *mbpb*. Both of these genes, while having slightly different amino acid sequences to human *MBP*, appear to have largely conserved cellular functions [246]. The two genes also exhibit partially distinct expression patterns, which suggests a degree of neofunctionalisation has occurred.

Similar to human / mammalian CNS development, OPCs emerge from the pMN domain in zebrafish [247]. However, in contrast to mammalian development, OPCs are able to differentiate into motor neurons, under the influence of a sonic hedgehog (shh) gradient [248]. Although, similar to mammals, the gene signalling of oligodendrocyte transcription factor 2 (*olig2*) does appear to determine oligodendrocyte development. Numerous other transcription factors have also been reported to function in the same manner in both zebrafish

and mammals (e.g. *sox10*, *olig1* with regards to glia development), further establishing the applicability of zebrafish as a model organism in which to study CNS development [240].

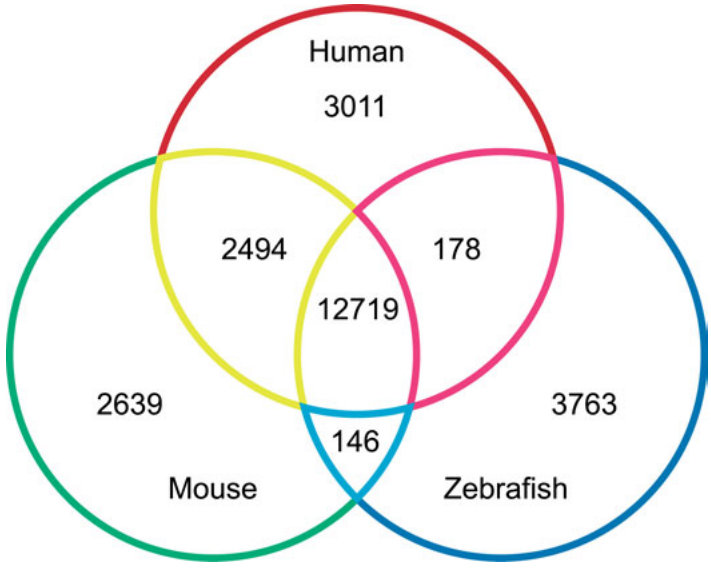

Figure 3. Orthologue genes shared across human, mouse, and zebrafish, shown by numbers within coloured regions. The figure is adapted from Howe et al., 2012 [237].

Statistics

In addition to the use of appropriate animal models in investigations of genes, other methodologies can be implemented or improved to gain a better understanding of current data. Statistics offers an accessible route into such a deeper exploration. This is particularly relevant in the case of gene expression data, that superficially may appear to offer little more than essentially binary measurements (i.e. high / low), yet can actually be utilised in numerous ways, akin to any large dataset.

One such statistical method is analysis of covariance (ANCOVA), a statistical technique used to assess significant differences across variables, after accounting for confounding variables [249]. This approach increases the sensitivity of the *F*-test (any statistical test using a continuous probability distribution), making the error term smaller, and therefore less likely to present a type II error [250]. Within the test, the group means are adjusted, dependent on the magnitude of the effect that the covariate has on the outcome. This allows the

model to account for some (although crucially, not all [251]) of the error variance introduced by other factors that have an impact on the data [249].

In addition to the requirements inherent in experimental design that are essential for correctly using an ANCOVA (generally, that the data is of the correct type, and that independence of observations is maintained), further assumptions about data distributions and relationships must be largely adhered to. For any linear model, it is required that the data has a normal distribution (normality), that the variance of the predictor variables is equal (homoscedasticity), and that the variance of the sample populations are equal (homogeneity of variance) [252]. Furthermore, two additional assumptions must be met specifically for an ANCOVA model, that of linearity of regression, and homogeneity of regression slopes. The former requirement concerns whether the independent and dependent variables are independent from one another; that they exhibit a linear relationship. The latter assumption states that the regression lines for each interaction term (i.e. a covariate) must be parallel. The homogeneity of regression slopes is a particularly pertinent factor for ANCOVA models, and ensures that the variance accounted for by each covariate does not overlap, and therefore remove, the variance of the experimental condition, or vice versa [252]. Once these assumptions are met, the simplest form of the analysis is shown by the following equation.

$$y_{ij} = \mu + \alpha_{ij} + \beta\omega_{ij} + \epsilon_{ij}$$

Where y_{ij} is the response variable, for the *i*th group, with the *j*th observation, μ is the grand mean of the values, α_{ij} is the treatment factor(s) for the *i*th group, with the *j*th observation, β is the regression coefficient for ω_{ij}, which is the covariate(s) (for the *i*th group, with the *j*th observation), and ϵ_{ij} is the term for the unobserved error.

As an ANCOVA uses an underlying *F*-test, it cannot determine the direction of any significant differences, just that the explained variance of the data is greater than the unexplained variance of the data [251]. Post-hoc testing is therefore essential for determining directionality. Pairwise comparisons using a Bonferroni correction is one of the most common methods for deducing the specifics of group differences [253], while also correcting for the inevitable biases that arise from multiple testing [254]. There are various other post-hoc tests, including the Šidák correction [255], and Tukey's range test [256], both of which are less conservative (but therefore offer more power).

In addition to ANCOVA models, there are various other statistical procedures that permit comprehensive interrogations of data, beyond simple comparisons. An example is a multiple linear regression, which essentially forms the basis for an ANCOVA model [257]. This method gives a prediction of a dependent variable from independent variable values, or can provide a test of the strength of a relationship between two or more variables. The equation for a simple linear regression is shown below.

$$\hat{y} = b_o + b_1 x_1$$

In which $\hat{y}$ is the predicted value, b_o is the intercept, and $b_1 x_1$ is the estimate of the slope of values, multiplied by the independent variable value. This is essentially the same as a slope-intercept equation. The equation can also be expanded for multiple independent variables, and an error term can be added, as shown below.

$$\hat{y} = b_o + b_1 x_1 + \cdots + b_n x_n + \epsilon$$

Wherein the same terms apply as above, but $b_n x_n$ refers to the *n*th independent variable, and ϵ is $y - \hat{y}$ (the actual values minus the predicted values, giving the error).

An adaption of this technique is known as hierarchical multiple regression, in which each independent variable is entered into the equation as a separate entity (in contrast to a standard linear regression, in which all the variables are entered simultaneously). This allows the variance of a dataset to be predicted in a predefined order, and the predictive capacity of each independent variable to be viewed separately [258]. In this way, independent variables can act in a similar manner to covariates, when entered before an independent variable of interest. This is performed through iterative steps repeating the above equation for each independent variable, and the remaining variance (the amount not accounted for, or predicted by, each independent variable) is left to the next independent variable, in a sequential manner. To calculate how effective each independent variable is at predicting the dependent variable, the coefficient of determination, or R^2 is used. This allows a quantification of the percent of data that each independent variable accounts for, before or after applying other independent variables [259]. In a general form, the R^2 equation requires the sum of residuals (SSR; the difference between the observed data, and the predicted data, squared, and then summed), and the total sum of squares (SST; the difference of the observed data from the overall mean, squared, and then summed). This can be shown by the following equations.

$$SSR = \sum (\hat{y}_n - \bar{y}_n)^2$$

$$SST = \sum (y_n - \bar{y}_n)^2$$

Where $\hat{y}_n$ refers to the *n*th predicted value and y_n refers to the *n*th observed value, as above, while $\bar{y}_n$ refers to the mean of the data. This can therefore be generally shown as the following equation, for R^2.

$$R^2 = 1 - \frac{SSR}{SST} = 1 - \frac{\sum(\hat{y}_n - \bar{y}_n)^2}{\sum(y_n - \bar{y}_n)^2}$$

However, adjustments must be made to R^2, as the amount of variance predicted from each additional independent variable always increases. Furthermore, overfitting with too many independent variables can produce a high prediction value, where one may not exist [260]. Therefore, adjusted R^2 ($\bar{R}^2$) is used. This accounts for the degrees of freedom in the model, and is shown by the equation below.

$$\bar{R}^2 = 1 - (1 - R^2)\frac{n - 1}{n - m - 1}$$

In which *n* refers to the sample size, and *m* refers to the number of independent variables. This way, the variance predicted by each independent variable is controlled for and can be assessed in the context of other variables.

The applications of both ANCOVA and multiple regression models are numerous. The ANCOVA approach is particularly well suited to judging group differences across large datasets, where the ability to control for cofounders is important. This could be the case for data regarding gene expression, or behavioural testing. Multiple regressions can help delineate the magnitude of the effect that an independent variable has on the data, such as factors that could influence gene expression, or to determine what sources of data account for the observations. In this way, statistical methods offer insight into data that is otherwise unobservable or quantifiable, and greater understanding can be gained from and about the sources of information.

Research Aims

While *QKI* has been extensively linked to human disease and aberrations in CNS development, the function, downstream targets, and the potentially different roles of each *QKI* isoform remain largely unexplained. This is principally due to the embryonic lethality of *Qk* null mouse mutants, which motivates and also obfuscates further investigation of this gene. The embryonic lethality is itself evidence of a role prior to gliogenesis, as this stage in development is not yet reached at the time of death. The use of the zebrafish as a model organism in which to study the effects of *QKI* (or rather the zebrafish homologues, *qkia*, *qkib*, and *qki2*) on CNS development is therefore summarily incentivized.

The overarching aim of this thesis was tripartite: to further investigate the breadth of human disease that *QKI* and *QKI* isoforms could be involved in, to ascertain the suitability of the zebrafish as a model organism, and finally, to investigate how *qki* can impact CNS development in the zebrafish, with implications for human disease.

Specifically, the aim of each paper was as follows:

Paper I – Explore the expression of *QKI* and *QKI* splice variants within human Alzheimer's disease brains, alongside canonical Alzheimer's disease related genes.

Paper II – Investigate the expression of *QKI6B*, a recently identified and scarcely studied splice variant of *QKI*, within human schizophrenic brain samples.

Paper III – To determine the suitability of the zebrafish as a model organism in which to study the development of *QKI*, and to ascertain the comparability of the zebrafish *qki* genes.

Paper IV – To study the impact of *QKI* perturbation on zebrafish development, and to establish the suitability for future research utilising gene targeting.

Results and Discussion

Paper I

Gene expression of Quaking in sporadic Alzheimer's disease patients is both upregulated and related to expression levels of genes involved in amyloid plaque and neurofibrillary tangle formation.

As *QKI* has been previously implicated in a variety of diseases showing glial aberrations (as discussed above), we sought to clarify a previously suggested involvement in Alzheimer's disease (AD). Gomez Ravetti [197] previously linked *QKI* to AD in a microarray screen. While this data is highly indicative, it is by no means definitive, as microarrays can be prone to false positives [261, 262]. We therefore utilised qPCR as a more accurate mRNA quantification technique (often termed a "gold standard" method; [263]), in order to investigate these findings further. We also sought to examine the expression levels of genes related to the pathogenesis of AD, due to the potentially far-reaching regulatory capabilities of *QKI* [107], and the breadth of glial cell involvement with both AD and AD-related gene function [198, 199], suggesting the possibility of a shared link. The AD-related genes that we chose to examine were amyloid precursor protein (*APP*), presenilin-1 (*PSEN1*), presenilin-2 (*PSEN2*), and microtubule associated protein tau (*MAPT*). Within the disease context, overproduction of neurotoxic amyloid-β_{42} (Aβ) results from the aberrant cleavage of APP, by γ-secretase [264]. The γ-secretase complex consists of four proteins, one of which can be either PSEN1 [265], or PSEN2 [266]. Aberrant γ-secretase function (that can be initiated by mutations in either *PSEN1* or *PSEN2*) can therefore cause an accumulation of Aβ plaques [267]. Additionally, abnormally hyperphosphorylated tau proteins (from *MAPT*) form neurofibrillary tangles. Whether or not the hyperphosphorylation of tau proteins is due to the presence of Aβ, or triggers Aβ accumulation itself, is still contended [268-270].

These proteins eventually cause cell death, likely by a combination of excitotoxicity [271, 272], oxidative stress [273, 274], and mitochondrial damage [275], amongst other factors [276]. This is a paraphrasing of the amyloid hypothesis, and despite robust criticism [269], it remains the most widely ac-

cepted explanation of AD pathogenesis [277]. While there are other genes implicated in the disease process, these remain central components of the prevailing hypothesis of AD emergence [267].

Using 123 post-mortem brain samples from the PFC of individuals with AD or without (62 and 61 samples, respectively), we quantified the mRNA expression of *QKI* (non-specific), *QKI5*, *QKI6*, and *QKI7*, alongside the AD-related genes *APP*, *PSEN1*, *PSEN2*, and *MAPT*. From these measurements, and using an ANCOVA model, we detected a significant upregulation of *QKI* and all *QKI* splice variants, but did not detect any differences for the AD-related genes. The latter finding was not entirely unexpected, as reported differences in AD-related gene expression have been persistently inconsistent. While the genes have been identified for their functional role in AD progression and emergence, it appears that expression quantity is not a robust, significant determinant of AD at the group level. We next sought to explore the possibility of a relationship between *QKI*, and *QKI* splice variants, with AD-related genes; despite AD-related gene quantities not appearing to drive AD pathogenesis, an association would at least merit further research. It remains a possibility that AD-related gene expression quantities could be relevant at an individual level, or that regulatory / deregulatory processes constitute an aspect of disease pathogenesis.

Using a multiple linear regression, we explored the predictive capacity of expression values of *QKI* and each measured *QKI* splice variant with AD-related gene expression. The regression used the geometric mean (used to increase stability of combined measurements [278]) of reference gene expression (in this case, actin, beta (*ACTB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)) as the first variable in the model, to account for general gene expression quantities (which explained roughly 20% of the variance). We furthermore found that *QKI* accounted for between 23% and 36% of AD-related gene variance. Each isoform then accounted for between 1% and 6% of the variance after the prior two variables had been input. This was echoed when a principal component (PC) was formed from the expression values of *APP*, *PSEN1*, and *PSEN2*, which are directly implicated in a shared AD pathway. While the PC is not a direct reflection of disease genetics, the encapsulation is at least representative of the hallmarks of the canonical pathway gene expression. From these findings, it was apparent that *QKI* and *QKI* isoforms had the potential to be involved in the regulation of AD-related genes, although this cannot be definitively known from the statistical findings. To further explore the potential link between *QKI* and AD-related genes, we carried out a bioinformatic exploration of the sequences of *APP*, *PSEN1*, *PSEN2*, and *MAPT*, finding the presence of putative QRE sequences in all but *PSEN2*. The potential interaction of QKI with the putative QRE sites has not been confirmed, but is suggestive of a link. Additionally, we split the samples by status type (i.e. AD or control) to explore the potential regulation within groups. As widespread genetic dysregulation has been found upon AD onset [279], it was

speculated that the relationship between *QKI* and AD-related genes within AD samples would also widen. This was found to be the case, with a generally higher predictive capacity of *QKI* in control samples, and lower in AD samples. This suggests that any potential regulation by *QKI* is diminished within AD brains.

While further evidence is undoubtedly required to uncover any direct mechanisms of interaction between *QKI*, *QKI* isoforms and the aforementioned AD-related genes, the general association of *QKI* with AD is clear. In terms of disease directionality (i.e. *QKI* being associated with the causes or effects of disease onset), little has been determined. However, as it should be assumed that any pathology involving changes in *QKI* expression would arise through changes in oligodendrocytes or astrocytes, it appears likely that the disease onset would initially trigger changes in *QKI*. This is due to the location of Aβ and tau deposition, which is likely centred around the postsynaptic cleft [280], and therefore not directly pertaining to glia. Subsequently, astrocytes have been shown to harbour Aβ and become activated [281], while Aβ has also been found to be toxic to oligodendrocytes [282], both of which could have downstream consequences for *QKI* activity. The response of *QKI* to pathology could therefore potentially act in tandem, both as a result (potentially compensatory and / or protective), and as an element of further neurological damage (through dysregulation of genes involved in oligodendrocyte / astrocyte function). Future research examining the actions that QKI may have on genes involved in AD would clarify the underlying processes and involvement of QKI in AD pathogenesis.

Paper II

QKI6B is upregulated in schizophrenic brains and predicts GFAP expression.

Schizophrenia was the first human disease in which *QKI* was shown to be differentially expressed in the sample of interest (i.e. not in cells [104]). While each previously identified splice variant was examined within that investigation (*QKI5*, *QKI6*, *QKI7*, and *QKI7B*), a later study carried out by Radomska et al [98], discovered a novel splice variant, *QKI6B*, within astrocytic cells. This study also found that *GFAP*, an astrocytic marker [57], was likely under the regulation of *QKI7*. As *GFAP* has been previously shown to be associated with schizophrenia, this implicates the possibility that *QKI* could be the upstream element, determining such differential expression in a disease state. We therefore set out to complete the picture of *QKI* splice variant expression within schizophrenic post-mortem brain samples, through measurement of the

mRNA, compared to non-schizophrenic (i.e. control post-mortem brain samples). Furthermore, we measured expression of *GFAP*, and explored the possibility of regulation between these two genes using a statistical approach.

The tissue for the experiment was obtained from three brain banks, and totalled 110 samples, split evenly for schizophrenic and controls. The first step in this process required isolating the mRNA from the tissue. This could then be converted to more robust cDNA and measured using qPCR. Quantification was then carried out, and with the use of stably expressed reference genes (*GAPDH* and *ACTB*), we were able to make comparisons across the different samples. We found that both *QKI6B* and *GFAP* were differentially expressed in schizophrenic tissue, showing an upregulation relative to controls. This result was obtained using an ANCOVA model, allowing other factors related to gene expression outcome to be incorporated and accounted for. The equation for this model is summarised below:

$$y_{ij} = \mu + \alpha_{ij} + \beta cov_{ij} + \epsilon_{ij}$$

Where μ is the overall mean value, α encompasses the mean value of the categorical factors of interest, denoted by *i* (including whether or not the individual had been diagnosed with schizophrenia or not, the sex of the individual sample, the brain bank that the sample was obtained from, and the plate effects), while *j* denotes the individual values (or observations) of the factors. Additionally, β is a regression coefficient for the covariates, denoted by *cov* (which include age of the individual at time of death, the time post-mortem before the sample was obtained, and the geometric mean of the reference genes, *ACTB*, and *GAPDH*), again for the categorical factors *i*, with the values of those factors, *j*. The error term ϵij refers to the uncontrolled random variation that occurs for each brain sample, dependent on factors such as individual and experimental variation (assumed to be constant). The amount of variance contributed by the covariates (of which some are known, or speculated to affect mRNA quantity [283]) could therefore be constrained, to an extent, providing more validity to the analysis. Additionally, with such a relatively large number of samples coupled with the high resolution quantification of qPCR, a degree of precision in measurements was afforded that is not obtainable with other methodologies, such as within a microarray study.

As both genes were found to be upregulated, and due to the prior hypothesis of *QKI* regulating *GFAP*, we sought to explore how this could be investigated through the current findings. Using a multiple linear regression model, we were able to evaluate the amount of variance that could be predicted of *GFAP*, by *QKI6B*. The regression model first included the geometric mean of the reference genes, to account for the variation that could be explained by general gene quantity. Secondly, measurements of total *QKI* (taken from a probe that covers all splice variants, and thus measures the total *QKI* quantity) were then

used, to account for general amounts of *QKI*. Lastly, the measurements of *QKI6B* were used. The regression therefore tested whether or not *QKI6B* quantity was a significant predictor of the quantity of *GFAP*, above and beyond the predictive ability of general gene expression, and general *QKI* expression. This was found to be the case, with *QKI6B* individually accounting for 17% of the variance of *GFAP* quantity, significant at $p < 0.0001$. It should be noted that this is in addition to the 23% of variance that both the reference genes and total measurements of *QKI* accounted for.

As *GFAP* contains a QRE [98], any regulation is likely to occur through an interaction of one, or several *QKI* isoforms with this sequence. Interestingly, previous research examining *QKI* in schizophrenic tissue found *QKI7* and *QKI7B* to be significantly downregulated (the opposite direction to what is currently seen with *QKI6B*), and it has also been suggested that *QKI7* and *QKI7B* can regulate *GFAP* expression, as *QKI7* silencing also leads to diminished *GFAP* expression in astrocytes [98]. We therefore also used previous measurements of *QKI7* and *QKI7B* in the same regression equation as above, to determine the proportion of *GFAP* quantity that could be accounted for by these two splice variants. Both splice variants were found to be significant predictors, but with notably limited potential, accounting for 2% of the variance. This ostensive disparity in regulatory potential could be due to the apparent abundance of *QKI6B* expression within astrocytes [98], which would more readily impact the expression of *GFAP* (as an astrocyte marker) than *QKI7* and *QKI7B*, which are expressed at a much lower level in astrocytes. This could also suggest a partial degree of cellular specificity, with *QKI6B* having a primary role in astrocytes, and *QKI7* and *QKI7B* having a primary role in oligodendrocytes. While this has yet to be determined, Lauriat et al [284] found developmental and regional differences in expression values of *QKI* splice variants, suggesting that a certain degree of specificity occurs. Further to this, we set out to examine whether previous research suggesting a regulation of OLR genes by *QKI7* and *QKI7B* could be shown by *QKI6B*. Using previous measurements of OLR genes, we used a multiple linear regression as before, with each gene, and a principal component (PC) formed from the combined values of OLR gene expression. We found that *QKI6B* was not able to significantly predict the expression of any OLR gene (or of the PC). While this isn't direct support for cellular specificity, it is at least in line with such speculation, and does suggest a different role of *QKI6B*, at least in relation to OLR genes. It remains to future research to examine the potential of expression differences across alternate glial cell types, and whether or not this has the potential to regulate different networks of genes.

Paper III

Characterization and expression of the zebrafish qki paralogs.

While the previous two papers certainly implicate the central importance of *QKI* in disease pathology, the embryonic lethality of *QKI* null mouse mutants precludes mechanistic study. To understand the developmental functions of *QKI*, we chose to explore the applicability of the zebrafish due to their rapid *ex utero* development and embryonic transparency.

To clarify the suitability of the *qki* genes (the zebrafish has three: *qkia*, *qkib*, and *qki2*), we first sought to evaluate their evolutionary relationship and conservation. Protein sequence analysis reveals a remarkable degree of conservation, with *qkib* and *qki2* containing an amino acid (aa) sequence that is 89% to 96% identical with human *QKI* splice variants, while *qkia* shows at least 77% identity. Sequence differences are almost entirely contained within the alternate 3' exons of splice variants, suggesting that selective pressure preserved the central domains, including the KH domain, responsible for RNA binding.

In order to clarify the relationship of each zebrafish (the following text will state the zebrafish as *Danio rerio* for consistency with other species' names) gene to the human counterpart, we carried out a Bayesian-based phylogenetic analysis, uncovering that *qki2* and *qkib* are orthologues of human *QKI* (evolving from a common ancestor), whereas *qkia* is a paralogue (arising from duplication). This is supported by syntenic analysis, an approach that examines the position of genes, or the loci of genes, to determine their positional conservation across the genomes of different species. Two *qki* genes were found in both *Lepisosteus oculatus* (spotted gar), and *Latimeria chalumnae* (coelacanth). The genomes of these species are currently the closest proximates of the last common ancestor of fish and tetrapods, suggesting that the *qki* gene was present at that point. Further evidence for *qki2* and *qkib* as ohnologues is found through the syntenic conservation of *qki2* in *Tetraodon lineatus* (Fahaka pufferfish) and *Danio rerio*, but not *Lepisosteus oculatus*. Both *Tetraodon lineatus* and *Danio rerio* emerged from a common ancestor in which a whole genome duplication event occurred, whereas *Lepisosteus oculatus* diverged prior to this. The presence of *qkib*, and the lack of *qki2*, in *Lepisosteus oculatus* and *Danio rerio*, and the presence of *qki2* in in *Tetraodon lineatus* therefore suggests that *qki2* is the result of the teleost-specific whole genome duplication. As both *qki2* and *qkib* can therefore be considered orthologues to human *QKI*, we set out to explore their expression in *Danio rerio* further.

Using qPCR, we evaluated the developmental expression of each *qki* gene, finding that *qki2* and *qkib* share a similar quantitative trajectory, distinct from *qkia*. The RNA of both *qki2* and *qkib* shows a gradual increase from 7hpf, to a peak at around 3dpf, which then declines and appears stable at 21dpf. In

contrast, *qkia* is most highly expressed at 7-14hpf and then declines. As *qkia* has previously been shown to be involved in muscle development [285], it is of note that the RNA is most abundant at the timepoints associated with early muscle development [286]. Incidentally, the developmental trajectory of both *qki2* and *qkib* shows a resemblance to the points of maximal oligodendrocyte and astrocyte development, and myelination [241, 287].

Using *in situ* hybridization, we were able to add a spatial resolution to the temporal dynamics of developmental expression. Expression (non-maternal) of *qkia* and *qki2* was detected from the 5 somite stage (approximately 11hpf) in adaxial cells. Also at the 5 somite stage, *qkib* expression was found in regions of midbrain and hindbrain. This pattern persisted with expression detected in the somites of *qkia* and *qki2*, and within brain regions for *qkib*, at 15 somites (approximately 15hpf). From 24hpf, *qki2* showed increased expression in the brain region, more akin to *qkib*, while *qkia* continued to show expression within the myotome (dorsal region of somites). From 48hpf, *qki2* and *qkib* showed similar expression within the CNS (specifically the dorsal hindbrain), whereas *qkia* was found around the craniofacial musculature. This is also reflected at 72hpf, with *qkia* detected in a similar location, while *qki2* and *qkib* both appear to reside in the lateral line, a mechanosensory network containing Schwann cells [288]. The findings therefore show shared and distinct expression patterns, in accordance with speculated functional components (i.e. *qkia* expressed primarily around sites of muscular development, and both *qki2* and *qkib* expressed primarily in the CNS).

As both the aforementioned bioinformatics investigation, and the spatiotemporal expression evidence implicate the relevance of studying *qki2* and *qkib* with regards to human *QKI*, we sought to explore their development in greater detail, utilising a combined fluorescent *in situ* and immunohistochemical approach with confocal microscopy. By using a sox2 antibody (a marker of neural progenitors [289]), we were able to show co-labelling at 72hpf within the midline ventricular zone of the brain and spinal cord, where neural progenitors reside [290]. By using the HuC/D antibody, we were also able to show that this expression was not overlapping with differentiated neurons (which HuC/D labels [291]), within the same regions. This suggests a role in early neural development. Both *qki2* and *qkib* showed similar, yet distinct expression within these regions, suggesting shared and unique roles in neural development. This offers the possibility of selectively perturbing these genes to obtain region-specific effects. For example, *qkib* appears to be more prevalent within the cerebellar plate than *qki2*. Additionally, *qki2* appears to be expressed within a region proximal to or consisting of the floor plate, whereas *qkib* is absent from this region. Selective perturbations to either of these genes may result in specific neurodevelopmental aberrations, allowing dissections of regional developmental differences in the CNS. However, these speculations remain tentative without definitive co-localisation with specific markers for these regions.

Ultimately, the findings indicate the relevance of studying both *qki2* and *qkib*, as they appear to be the orthologues of human *QKI*, have a spatiotemporal expression similar to what has been documented in mice [92], and are indicative of a role in neural development. We therefore concluded that the zebrafish is an appropriate model organism in which to further dissect the roles of *qki*.

Paper IV

Morpholino knockdown of qkib leads to disturbed neural development in the larval zebrafish.

From our previous work in paper III, showing the suitability of zebrafish to study *qki*, we sought to develop our understanding of the function of *qki* further, using gene knockdown experiments. This was performed with morpholino oligonucleotides, which are short sequences that bind to RNA and disrupt either the correct translation, or splicing of the sequence, which diminishes subsequent protein production. Binding of morpholinos occurs via complementary base-pairing, wherein the alternative structure of the morpholino prevents correct translation or splicing [292, 293]. Injection of splice-blocking morpholinos specific for *qki2* and *qkib* was carried out at the 1-cell stage, and produced an additional lower molecular weight amplicon, suggesting a decrease in normal splicing events. Morphant embryos injected with the *qki2*-morpholino appeared physiologically normal, even at relatively high doses. However, embryos injected with the *qkib*-morpholino developed aberrantly, including a shorter, thinner body and head, and an apparent impairment of motility. These results are from comparisons to non-injected zebrafish, and zebrafish injected with control morpholinos, which are similar in sequence, but have 5 base pairs mismatched. The mismatch should prevent binding, and otherwise consists of the same molecular properties; it therefore functions as a relative proxy for the impact of non-specific binding. Mismatch control morpholinos were used throughout, alongside non-injected zebrafish as controls; subsequent statements regarding observed differences are therefore in relation to these zebrafish. While off-target effects aren't uncommon with morpholino administration [294], these features may also reflect true disturbances in physiological development. This will require further investigation to confirm or refute, with the use of rescue-mRNA, or alternative gene targeting. Notwithstanding the potential non-specificity of these morpholino effects, further investigation of the developing embryos yielded consistent, and definitive developmental abnormalities. This was carried out with transgenic embryos labelled with several fluorescent reporter proteins. Specifically, transgenic lines with labelling of olig2 (a marker of primary motor neurons, oligodendrocytes,

and eurydendroid cells; [247, 295]), and sox10 (a marker of oligodendrocytes in the spinal cord, amongst other regions; [296]) were used to follow CNS development, using *in vivo* confocal imaging of agarose-embedded embryos at 28hpf, 48hpf, and 72hpf.

We first sought to visualise the development of oligodendrocytes, as this has repeatedly been reported as one of the more striking features of aberrant CNS development in mice mutants [142]. Visualising the progenitor domain at 72hpf in the Tg(*olig2*:*DsRed2*) zebrafish injected with the morpholino for *qkib*, we found a reduction of migratory and differentiated OPCs. This time point is particularly crucial for oligodendrocyte development, enhancing the importance of studying OPCs at this time. This finding was confirmed with the Tg(-4.*9sox10*:*eGFP*) line at 48hpf; this timing is prior to myelinogenesis [241], wherein the transgenic line specifically labelling OPCs in the spinal cord [296].

Within the qkib-morphant, we also found irregular branching and arborisation of motor neuron projections from the spinal cord, in the Tg(*olig2*:*DsRed2*) at 72hpf. As the same transgenic line has been found to label cells within the cerebellum (specifically, oligodendrocytes and glutamatergic eurydendroid cells, equivalent to deep cerebellar nuclei within mammals [297]), and due to the presence of *qkib* within what is likely the developing cerebellar plate, we sought to examine the impact of *qkib* perturbation within this area. We discovered a striking absence of DsRed2-positive cells, and due to the proportion of eurydendroid cells compared to oligodendrocytes, it can be assumed that this was largely due to a deficit of the former cell type.

From these experiments, and prior research on mouse development, it is apparent that the formation of the CNS is critically dependent on *qki* / *Qk* function. The produced aberrations appear to result from changes in neural progenitor regions. The reduction of mature / differentiated oligodendrocytes, diffuse motor neuron arborisation, and absent eurydendroid cells indicates that a wide range of neural development is perturbed, at an early stage. We therefore additionally examined the progenitor domain of the spinal cord at 28hpf, using the *Tg*(*olig2*:*DsRed2*) embryos injected with the *qkib*-morpholino. This stage is prior to OPC differentiation, yet a reduced number of cells was still apparent, suggesting that the proliferative capacity of neural progenitor cells was impacted. Furthermore, we quantified expression of *gfap* RNA, as this gene specifically labels neural progenitors within the ventricular zone [298], and provides an estimate of neural progenitor cell quantity. *gfap* was found to be significantly reduced upon *qkib*-morpholino injection at 28hpf.

Interestingly, none of the observed differences were replicated for the *qki2*-morpholino injected embryos, despite ultimately receiving a considerably higher morpholino dose. This may be due to compensatory effects from *qkib* (or possibly other genes), or other unknown causes [294]. It is also possible that despite similarities in expression and sequence, *qki2* may not play an important role in neural development. Further dissection and detailed screening

of *qkib* and *qki2* mutants / transgenics will aid in a greater understanding of the developmental effects of these genes.

Ultimately, the results seen within embryos injected with the *qkib*-morpholino are encouraging for a greater understanding of *qki* function, with regards to both the developmental effects, and the relevance to human disease. Reduced grey matter has been found within schizophrenic brains [174], at least partially similar to the perturbation seen with eurydendroid cells. A reduction of oligodendrocytes has also been documented [172], akin to the current findings. While there are obvious and inherent differences in human and zebrafish development, the similarity of the resulting neural changes combined with the prior genetic associations merits further investigation.

Conclusions

This thesis represents my work on the *QKI* / *qki* gene with regards to human disease, how it can be studied, and the impact it has on neural development. The work has utilised both human brain samples, and zebrafish as a model organism to further delineate the functions and processes in which this gene is involved. *QKI* has been found to have a robust and widespread involvement in human disease, which can now be readily and reliably studied in the zebrafish, and therefore without the use of mouse models. Furthermore, the morpholino-derived impacts on neural development suggest that future developmental studies within the zebrafish will yield greater insights with relevance to human neurological disease pathogenesis.

Within paper I, samples taken from AD patient brains were analysed for expression differences of *QKI*, *QKI* splice variants, and other genes previously related to the pathogenesis of the disease. It was found that total measurements of *QKI*, and the variants *QKI5*, *QKI6*, and *QKI7* were all upregulated in AD patient brains, as compared to controls. The other genes, *APP*, *PSEN1*, *PSEN2*, and *MAPT*, were not found to be significantly different. However, their expression levels were significantly predicted by *QKI* and *QKI* isoforms using a multiple linear regression model. Furthermore, putative binding sites within *APP*, *PSEN1*, and *MAPT*, were suggestive of a possible route of regulation. The relationship between *QKI*, *QKI* isoforms, and the AD-related genes was also found to be less tightly conferred in patient samples, possibly due to widespread genetic dysregulation that occurs upon disease emergence.

Within paper II, we explored the association of a newly identified splice variant of *QKI*, *QKI6B*, within schizophrenic brain samples. We find this splice variant to be upregulated alongside *GFAP*, a marker for astrocytes. Previous research also indicated that *GFAP* may be under the regulation of *QKI6B*, and we find the expression values of these two genes to be closely related through a multiple linear regression approach. This relationship appears stronger than the previously predicted link between *QKI7*, and *QKI7B*, with *GFAP*. However, we also uncover that *QKI6B* is not related to oligodendrocyte-related gene expression. This could suggest that *QKI6B* is specifically expressed, or specifically functional, in astrocytes. Future research on this specific isoform could explore this further, and additionally determine whether any potential cellular specificity exists for other *QKI* isoforms.

Paper III set out to establish the suitability of the zebrafish as a model organism in which to study *qki*. This was done as a means to bypass the technical

challenges resulting from the embryonic lethality of *Qk* mouse mutants. We first characterised the relationship of each of the three zebrafish *qki* genes; *qkia*, *qki2*, and *qkib*. Using syntenic and phylogenetic analysis it appeared that *qkia* is an ancestral paralogue, while both *qki2* and *qkib* are orthologues of human *QKI*. A high degree of conservation was also found, strengthening the comparability of zebrafish to other organisms with regards to genetic evidence. Expression analysis using *in situ* hybridization and immunohistochemistry, revealed distinct, yet overlapping regions of expression for each *qki* gene. The expression patterns on *qki2* and *qkib* showed a particularly high level of overlap, and were largely present in the ventricular zone and other areas of neural proliferation. This was confirmed through co-labelling with markers specific for neural progenitors, and a lack of co-labelling with differentiated neurons. Ultimately, this further suggested the involvement of *qki* at early stages of neural development, and established the suitability of studying *qki* within the zebrafish.

In paper IV, we sought to determine the effects of *qkib* and *qki2* perturbation through morpholino knockdown. We found that administering the *qkib*-morpholino appeared to have effects on the gross morphological development, while the *qki2*-morpholino did not produce any observable effects. Subsequent confocal imaging permitted a visualisation of the developing CNS. No differences were observed for any of the embryos injected with the *qki2*-morpholino, however, multiple aberrations were seen upon *qkib*-morpholino injection. Irregularities were found in the number of proliferating or differentiated oligodendrocytes, while motor neuron arborisation from the spinal cord appeared diffuse and disordered, and the eurydendroid cells of the cerebellum appeared wholly absent. Additionally, a reduction of cells in the progenitor domain of the spinal cord was discovered. All of which suggests that *qkib* has a role in regulating neural development from an early stage. This finding is also intriguingly similar to the perturbation seen within mouse mutants, further establishing the suitability of zebrafish for studying *qki*. Further research could therefore derive further the mechanisms of neural development that *qki* is involved in.

Future Perspectives

The above work presents the basis, and beginnings, of understanding how *qki* may affect neural development, how this may be relevant to human *QKI*, and the numerous associated neurological diseases and disorders. However, much of course remains to be done. Examples of future directions are discussed below, although it is evident that new research approaches and methodologies may advance the knowledge of *qki* / *QKI* in unexpected, or unpredictable ways. The approaches discussed are therefore tentatively prescient.

One of the most exciting breakthroughs within biological research to occur in recent times has been the discovery and application of targeted gene editing using CRISPR/Cas9 (Clustered regularly-interspaced short palindromic repeats / CRISPR associated protein 9) [299-302]. While the proteins in their natural setting operate as part of an adaptive microbial immune response, usually to invading viruses [303], researchers have co-opted this function to edit genes of interest in a variety of organisms [304], including in human embryos (although with limited success; [305]). The gene editing system requires two central components, the Cas9 protein, and single guide RNA (sgRNA) that act in tandem to cut the target DNA sequence. The sgRNA is a synthetic combination of the originally separate CRISPR RNA (crRNA), and trans-activating crRNA (tracrRNA) [306], that guides the Cas9 protein to the DNA target within a cell. The Cas9 protein will then create a double-stranded break at the DNA target site [304]. The endonuclease activity of the Cas9 protein is entirely contingent upon the presence of a protospacer-associated motif (NGG, where N is any nucleotide, and G is guanine) at the 3' end of the DNA target [307, 308]. This double-stranded break will ultimately lead to activation of error-prone non-homologous end joining (NHEJ) [309], and subsequent insertions and deletions that disrupt the targeted sequence [304].

Intriguingly, different forms of the Cas9 protein have been produced through mutations. For example, Cas9D10A only cleaves one strand of DNA, and therefore does not induce NHEJ [310]. This can increase the stringency of the targeting if two sgRNA sequences are used, as an increased number of base pairs must bind to permit Cas9 activity. Additionally, dCas9 has been created and used to target and bind sequences, but not to initiate any cleavage. The dCas9 system can therefore be used in gene silencing [311], activation [312], visualisation [313], amongst other uses [314].

It is difficult to overestimate the potential utility of CRISPR/Cas9, and biological research will undoubtedly explore and advance such myriad uses. This use has been extended into research involving zebrafish, and has been found to operate successfully [315]. There are several avenues of approach that in particular would benefit research into *qki*. Directed gene knockout of *qkib* and *qki2* in particular would allow dissection of individual gene function throughout development in the zebrafish. As previous experiments utilising morpholinos operate at the level of knockdown, but not knockout, the entire ablation of gene function would offer a new level of insight into the gene function, and the potential compensation offered by other genes. Null mutants have even been produced within the founder, potentially allowing high-throughput investigations of gene function [316]. While the use of CRISPR/Cas9 will provide reliable and quick gene knockouts, the consequences may not be entirely equivalent to gene knockdown, such as those seen following morpholino injections. It was recently shown in the zebrafish, that a CRISPR/Cas9 induced gene knockout, but not morpholino induced knockdown, produced a seemingly compensatory upregulation of genes that can rescue the original gene function [317]. This suggests that any resulting alteration, or lack thereof, to zebrafish development following gene knockout may require particular scrutiny. However, the use of CRISPR/Cas9 to interrogate the *qki* genes will undoubtedly yield further insight, and may provide a better understanding of their role in development.

Summary in Swedish / Svensk sammanfattning

Denna avhandling representerar det arbete jag har utfört avseende genen quaking (*QKI* / *qki*), dess påverkan på mänsklig sjukdom, hur den kan studeras, samt slutligen dess roll under neural utveckling. I mitt arbete har jag analyserat prov från mänskliga hjärnor samt använt zebrafisken som en modellorganism för att vidare klargöra de funktioner och processer som denna gen är involverad i. *QKI* har tidigare visat sig ha en robust och omfattande involvering i mänsklig sjukdom, och kan nu på ett tillförlitligt sätt kan studeras i zebrafisken. Detta innebär att genen kan studeras utan de svårigheter som musmodeller innebär. Vidare påvisar den inverkan som morfolinadministrering haft på neutral utveckling att fortsatta utvecklingsbiologiska studier med zebrafisken som modellorganism kan bidra med ytterligare förståelse kring utveckling och sjukdomsförlopp hos mänsklig neurologisk sjukdom.

I den första artikeln analyserades hjärnprov tagna från patienter med Alzheimers sjukdom för skillnader i genuttryck av *QKI*, alternativt splitsade genvariationer av *QKI*, samt gener som tidigare har relaterats till sjukdomens utveckling. Resultaten visade att de totala måtten av *QKI* samt varianterna *QKI5*, *QKI6* och *QKI7* var uppreglerade hos patienter med Alzheimers jämfört med kontrollgrupp. Övriga gener – *APP*, *PSEN1*, *PSEN2* och *MAPT* – uppvisade ingen signifikant skillnad; däremot predicerades deras uttrycksnivåer av *QKI* och *QKI*-isoformer i en multipel linjär regressionsmodell. Möjliga bindningsytor inom *APP*, *PSEN1* och *MAPT* indikerade även möjliga regulationsvägar. Förhållandet mellan *QKI*, *QKI*-isoformer och Alzheimers-relaterade gener var i lägre utsträckning korrelerat i patientprov, möjligen till följd av den omfattande genetiska dysreglering som sker när man drabbas av sjukdomen.

I den andra artikeln utforskade vi associationer till den nyligen identifierade genvariationen, *QKI6B*, i hjärnprov tagna från patienter som drabbats av schizofreni. Vi fann att *QKI6B* var uppreglerat tillsammans med *GFAP* – en känd markör för astrocyter. Tidigare forskning har även visat att *GFAP* kan regleras av *QKI6B*. Genom användandet av en multipel linjär regressionsmodell fann vi ett tydligt samband mellan dessa två geners uttrycksnivåer. Detta förhållande tycks starkare än det tidigare förutsedda sambandet mellan *QKI7*, *QKI7B* och *GFAP*. I denna studie påvisar vi även att *QKI6B* inte är relaterat till genuttryck av oligodendrocyt-relaterad gener. Detta skulle kunna antyda

att *QKI6B* är specifikt uttryckt eller specifikt funktionellt i astrocyter. Implikationer för forskning gällande denna specifika isoform rör således att vidare undersöka detta, samt att avgöra huruvida det föreligger någon potentiell cellulär specificitet för andra *QKI*-isoformer.

Den tredje artikeln menade att etablera zebrafiskens lämplighet som modellorganism för att studera *qki*. Syftet med detta var att kringgå de tekniska svårigheter som resulterar av embryonal död hos möss med muterat *Qk*. I första hand karaktäriserades förhållandet mellan zebrafiskens tre *qki*-gener; *qkia*, *qki2* och *qkib*. Via syntonisk och fyliogenetisk analys framkom att *qkia* är en nedärvd paralog, medan både *qki2* och *qkib* är ortologer av mänskligt *QKI*. Resultaten påvisade även en hög grad av konservation, vilket stärker zebrafiskens lämplighet för jämförelse med andra organismer avseende genetisk evidens. Uttrycksanalys utförd med hjälp av in situ-hybridisering, självständigt likväl som kombinerat med immunhistokemi, påvisade distinkta men överlappande uttrycksregioner för varje *qki*-gen. Uttrycksmönster för *qki2* och *qkib* visade på ett påfallande stort överlapp, och fanns i hög utsträckning i den ventrikulära zonen samt andra regioner som karaktäriseras av närvaron av neutrala progenitorceller. Detta konfirmerades genom sammarkering med markörer specifika för neutrala progenitorceller samt för differentierade neuron. Detta påvisade ytterligare inblandningen av *qki* i tidiga faser av neural utveckling, och fastställde zebrafisken som en lämplig modell för att studera *qki*.

I den fjärde artikeln eftersträvade vi att avgöra effekterna av *qkib*- och *qki2*-nedreglering genom användning av morfolin. Administreringen av *qkib*-morfolinet påvisades ha effekter på grov morfologisk utveckling, medan administrering av *qki2*-morfolinet inte tycktes leda till några observerbara effekter. Påföljande avbildning i konfokalmikroskop möjliggjorde en visualisering av det centrala nervsystemet under utveckling. Inga skillnader kunde observeras för de embryon som injicerats med *qki2*-morfolin – ett flertal defekter kunde dock observeras efter *qkib*-injektion. Avvikelser kunde ses i antalet ökande eller differentierade oligodendrocyter, i att motorneuronens förgrening från ryggmärgen tycktes diffus och desorienterad, samt i att eurydendroidceller i cerebellum syntes helt frånvarande. Slutligen upptäcktes även en minskning av celler i progenitordomänen i ryggmärgen. Sammantaget antyder dessa resultat att *qkib* har en roll i regleringen av neural utveckling från ett tidigt stadie. Dessa fynd har även påfallande likheter med de störningar som setts i musmutanter, vilket ytterligare stärker användandet av zebrafisken i studiet av *qki*. Framtida forskning skulle därför vidare kunna härleda de mekanismer av neural utveckling som involverar *qki*.

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