MONASH University

Identification and functional validation of ADHD-associated gene variants

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Abstract

Attention deficit hyperactivity disorder (ADHD) is a neurodevelopmental disorder that affects ~5% of school aged children worldwide. Characteristic symptoms of ADHD are hyperactivity/impulsivity, and an inability to sustain attention, and as such individuals with the disorder often show impaired academic and social functioning. Research into the aetiology of ADHD has demonstrated that there is a strong genetic component, which has in turn prompted investigations into what genes are associated with the disorder. Between candidate gene and genome wide association studies (GWAS) there have been a large number of variants associated with ADHD. Despite this, there has been little work into investigating whether or not these associated variants actually functionally contribute to the development of ADHD phenotypes. Therefore, there is a need to functionally examine if the genes these variants are mapped to have roles in the development of ADHD.

In this thesis, I have utilised loss of function zebrafish models to examine three significant ADHD-associations, and provide insight into if disruptions to these genes can lead to the development of ADHD phenotypes. The first variant maps to *CHMP7*, which was functionally predicted and significantly associated with ADHD by Tong et al., (2016). The second and third variants are two of the first significant ADHD-GWAS associations, and map to *DUSP6* and *KDM4A* respectively (Demontis et al., 2019). I have generated a *chmp7* mutant line using CRISPR/Cas9 genome editing, and demonstrated that *chmp7*$^{+/-}$ fish have reduced *chmp7* mRNA levels, similar to individuals who are homozygous for the *CHMP7* ADHD risk allele. This reduction in *chmp7* mRNA leads to two ADHD phenotypes, hyperactivity, and decreased brain volume at 6 days post fertilisation. The hyperactivity phenotype can also be restored back to wildtype levels with the application of methylphenidate. Together, this evidence demonstrated that *CHMP7* is functionally important for the development of ADHD phenotypes. I have also generated a *dusp6* mutant line, and demonstrated that a loss of Dusp6 function is not sufficient to cause either a swimming activity or brain volume phenotype. There is, however, evidence to suggest a synergistic increase in activity in *dusp6*$^{-/-}$ fish treated with methylphenidate. This, in combination with the rescued activity phenotype seen in *chmp7*$^{+/-}$ fish treated with methylphenidate, provides evidence for variability in drug response in ADHD based on genotype. Finally, I have utilised *kdm4aa* and *kdm4ab* mutant models to demonstrate that loss of Kdm4aa;Kdm4ab function leads to decreased swimming activity, thus providing the first functional evidence that a significant ADHD-GWAS hit is relevant to the development of ADHD phenotypes.

Overall, I have expanded our knowledge of the genetic background of ADHD by providing functional evidence for two ADHD-associated genes. In doing so, I have demonstrated a framework for how future associations can be functionally investigated. A detailed understanding of how ADHD-

associated genes contribute to ADHD will allow us to determine what phenotypes may be exhibited, how those phenotypes may persist, and whether or not pharmacological treatments may be effective.

Publications during enrolment

Dark, C., Homman-Ludiye, J., Bryson-Richardson, R.J., 2018. The role of ADHD associated genes in neurodevelopment. Developmental Biology 438, 69–83. https://doi.org/10.1016/j.ydbio.2018.03.023

Declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes one original paper published in a peer reviewed journal and three unpublished publications. The core theme of the thesis is the functional examination of ADHD-associated gene variants using zebrafish models. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the School of Biological Sciences, Faculty of Science, under the supervision of Rob Bryson-Richardson.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of Introduction Part A, and Results Chapters 1-3, my contribution to the work involved the following:

Thesis Chapter	**Publication Title**	**Status**	**Nature and % of student contribution**	**Co-author name(s) Nature and % of Co-author's contribution**	**Co-author(s), Monash student Y/N**
Introduction: Part A	The Role of ADHD Associated Genes in Neurodevelopment	Accepted	80%. Concept, writing first and subsequent drafts	1) Jihane Homman-Ludiye, wrote section on "Glia and Microglia", designed Figure 2, 10% 2) Rob Bryson-Richardson, Input into manuscript, designed Figure 1, 10%	No No

Results Chapter 1	Functional validation of *CHMP7* as an ADHD risk gene, using a CRISPR/Cas9 zebrafish model	Not Submitted	80%. Concept, collecting and analysing data, writing drafts	1) Caitlin Williams, original CRISPR injections, 2% 2) Ziarih Hawi, input into manuscript, 4% 3) Mark Bellgrove, input into manuscript, 4% 4) Rob Bryson-Richardson, input into manuscript, 10%	No No No No
Results Chapter 2	Functional investigation of an ADHD-GWAS associated gene, *DUSP6*, using a CRISPR/Cas9 zebrafish model	Not Submitted	85%. Concept, collecting and analysing data, writing drafts	1) Ziarih Hawi, input into manuscript, 2.5% 2) Mark Bellgrove, input into manuscript, 2.5% 3) Rob Bryson-Richardson, input into manuscript, 10%	No No No
Results Chapter 3	Functional validation of an ADHD-GWAS risk gene, *KDM4A*, using a zebrafish model	Not Submitted	85%. Concept, collecting and analysing data, writing drafts	1) Ziarih Hawi, input into manuscript, 2.5% 2) Mark Bellgrove, input into manuscript, 2.5% 3) Rob Bryson-Richardson, input into manuscript, 10%	No No No

I have not renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature: **Date:** 14/03/2020

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature: **Date:** 14/03/2020

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Firstly, a huge thank you to my main supervisor Assoc. Prof Rob Bryson-Richardson, who has played a vital role in helping me become the researcher I am today. You have provided me with all the guidance I could have ever needed, and I cannot thank you enough for that. Everyone says that you should try to pick a good supervisor, rather than a good project. Well I would say my project was pretty good, but having you as a supervisor was even better. Thank you again.

To my associate supervisor, Dr Ziarih Hawi, you have taught me so much about genetics and how to work in a lab, and I know I'll take the habits you drilled into me far into my career. You've also shown me how to survive in research, and now I truly feel prepared to go out on my own. I've always appreciated our talks, and I'll be happy to shout you a kebab every now and again so we can keep in touch.

To all members of the Bellgrove Lab, in particular Beth, Janette, Nicole, as well as past members Dan, Méadhbh, Tarrant, and Robyn, thank you for all your support across six years of working together. From volunteering, then to my honours, and now finishing my PhD, you have all been a wonderful group to work with. Mark you have created a truly excellent working environment, and anyone would be lucky to start their career in your lab.

To everyone in the Bryson-Richardson lab; Tamar, Lee, Clara, Rita, Emily, Coco and Dashika, thank you for putting up with me for the past four years! It's been a pleasure getting to know each and every one of you. I've learnt so much from all of you, both scientific and about life in general, and I hope to repay the favour someday. I would not have gotten this far without you all, and I know you'll all go on to do great things. Just make sure to look after Coco once I'm gone.

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Lastly, thank you to all my other friends and family, who would always ask about my "ADHD fish", and at least pretended to act interested. It's been a long time coming, but you've all helped me get to where I am today!

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List of Abbreviations

5-CCPT: five choice continuous performance test

5-CSRTT: five choice serial reaction time task

ADHD: attention deficit hyperactivity disorder

ASD: autism spectrum disorder

bp: base pair

CADD: Combined Annotation Dependent Depletion

CHMP7: charged multivesicular body protein 7

CNS: central nervous system

CNV: copy number variation

CP: cortical plate

CPT: continuous performance test

DLPFC: dorsolateral prefrontal cortex

DPF: day post fertilisation

DUSP6: dual specificity phosphatase 6

DZ: dizygotic

ESC: embryonic stem cell

ESCRT: endosomal sorting complex required for transport

GFP: green fluorescent protein

GWA: genome wide association

GWAS: genome wide association study

GWAVA: Genome Wide Annotation of Variants

HPF: hour post fertilisation

IFG: inferior frontal gyrus

IZ: intermediate zone

JTT: Jones-Taylor-Thornton

KDM4A: lysine demethylase 4a

KIM: kinase interaction motif

KO: knock-out

LD: linkage disequilibrium

LGE: lateral ganglionic eminence

LTD: long term depression

LTP: long term potentiation

LV: lateral ventricle

MDCK: Madin-Darby canine kidney

MEGA: Molecular Evolutionary Genetics Analysis

MGE: medial ganglionic eminence

MpH: methylphenidate

MRI: magnetic resonance imaging

MZ: marginal zone

MZ: monozygotic

NPC: neural progenitor cell

NSC: neural stem cell

OFC: orbitofrontal cortex

OPC: oligodendrocyte precursor cell

PAGE: polyacrylamide gel electrophoresis

qRT-PCR: quantitative reverse transcription polymerase chain reaction

RT-PCR: reverse transcription polymerase chain reaction

S: somite

SEM: standard error of the mean

SHR: spontaneously hypertensive rat

SLC6A3: dopamine transporter

SNARE: soluble N-ethylmaleimide-sensitive factor attachment protein receptor

SNP: single nucleotide polymorphism

ST: striatum

TU: *Tübingen*

VLPFC: ventrolateral prefrontal cortex

vmPFC: ventral medial prefrontal cortex

VNTR: variable number tandem repeat

VORT: virtual object recognition test

VZ/SVZ: ventricular/subventricular zones

Introduction: Part A

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The role of ADHD associated genes in neurodevelopment

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ABSTRACT

Attention deficit hyperactivity disorder (ADHD) is a highly heritable neurodevelopmental disorder of childhood. It is primarily characterised by high levels of activity, inattention, and impulsivity, and has strong negative impacts on academic functioning. Children with ADHD show a reduction in volume, and hypoactivity, in a range of brain regions. The underlying mechanisms behind these phenotypes are unknown, however, variants in several genes with known roles in neurodevelopment are associated with ADHD. In this review we discuss how these ADHD associated genes contribute to neurodevelopment, and how variants in these genes could give rise to the neurological phenotypes seen in ADHD.

1. Introduction

Attention deficit hyperactivity disorder (ADHD) is a common neuropsychiatric disorder of childhood, affecting 5% of school-aged children worldwide (Polanczyk et al., 2007), and persisting into adulthood in 30–50% of cases (Faraone and Biederman, 2005; Polanczyk et al., 2007). The disorder, characterised by high levels of inattention, uncontrollable hyperactivity, and impulsivity, is classified into three clinical subtypes: predominantly inattentive, predominantly hyperactive, and combined (American Psychiatric Association, 2013). ADHD is reported more often in males than females, with population and clinical studies showing male: female ratios of 4:1 and 9:1 respectively (Biederman et al., 2002; Cuffe et al., 2005). The disorder has been shown to have negative impacts on family relations and academic functioning (Mannuzza et al., 1993), and is associated with a greater likelihood of risk taking behaviours and drug use (Konstenius et al., 2015).

The aetiology of ADHD remains poorly understood, although both environmental and genetic factors are known to contribute to the onset of the disorder. Environmental factors such as prenatal exposure to alcohol, cigarettes, and illicit drugs have all been associated with an increased risk of ADHD (Banerjee et al., 2007; Langley et al., 2005; Sagiv et al., 2013). Low birth weight and adverse life experiences have also demonstrated associations (Banerjee et al., 2007; Heinonen et al., 2010). Despite this, only a small portion of the aetiology of ADHD can be explained by environmental factors. Family and twin studies provide estimates of heritability at around 76% (Faraone et al., 2005). Furthermore, concordance rates in monozygotic (MZ) twins are consistently higher than those in dizygotic (DZ) twins (~80% and ~40%, respectively; Levy et al., 1997). There is, therefore, a significant genetic contribution to ADHD risk.

Research into the genetic basis of ADHD initially focussed on candidate genes identified from animal models or knowledge of drug targets. In particular, genes involved in catecholamine (dopamine, noradrenaline) and serotonin transmission have been thought to be important to the aetiology of ADHD, and several of these have demonstrated replicable evidence of association (Faraone and Biederman, 2002; Gizer et al., 2009). More recently, hypothesis free genome wide association studies (GWAS) have been used to identify single nucleotide polymorphisms (SNPs), and copy number variations (CNVs) associated with the disorder. These approaches scan the genomes of cases and control individuals for thousands of SNPs to determine if any SNPs or CNVs (as identified by consecutive sets of SNPs) are associated with the disorder. For the detection of associated SNPs, this approach has, until recently, mostly been unsuccessful (Akutagava-Martins et al., 2016), with only one quantitative trait loci GWAS, examining six traits derived from ADHD clinical and symptom measures, identifying two significant associations (Lasky-Su et al., 2008). However, in what is the biggest ADHD GWAS to date, (Demontis et al., 2017) utilised 20,183 ADHD cases and 35,191 controls to identify 12 hits significant at the GWAS level ($p \leq 5 \times 10^{-8}$). With regards to CNVs, there has been success in identifying significant associations between ADHD and several genes mapped to these CNVs (Hawi et al., 2015). There are several limitations with this however, noting in particular low penetrance of variants, minimal overlap with previously reported ADHD common variants, and an inconsistency of individual variants being carried by different

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ADHD patients (Hawi et al., 2015). Despite this, the evidence from candidate gene, GWA-SNP and GWA-CNV studies has suggested many genetic associations with ADHD. A database of ADHD genetic associations and the study which identified them is available at (Zhang et al., 2012).

ADHD often co-exists alongside other psychiatric disorders such as oppositional defiant disorder, conduct disorder, anxiety disorder, depression, tic disorder, bipolar disorder, Tourette's syndrome, and substance use disorder (in adult cases) (Jensen and Steinhausen, 2015; Kessler et al., 2006; Steinhausen et al., 2006), suggesting a common aetiology. In addition, ADHD has been shown to share a significant genetic component with other neurodevelopmental cognitive disorders including schizophrenia, autism spectrum disorder (ASD), and X-linked intellectual disability (Cristino et al., 2013). Therefore, genes associated with these conditions may also play a role in ADHD.

ADHD is associated with macroanatomical changes in multiple brain regions, resulting from disrupted neurodevelopmental mechanisms. In the largest imaging meta-analysis to date, Hoogman et al. (2017) demonstrated significantly smaller volumes in ADHD cases for the accumbens, caudate, putamen, amygdala, hippocampus, as well as reduced intracranial volume as a whole, adding to previously identified changes. While these studies identify regions affected in ADHD (Table 1, Fig. 1), how these changes manifest has not yet been elucidated. In addition to changes in volume, cortical thickening in the prefrontal areas is delayed in ADHD, taking around 2.5–5 years longer than matched controls to achieve normal cortical thickness (Almeida et al., 2010; Montes et al., 2013; Shaw et al., 2007). Alongside the morphological changes in these structures, functions associated with these regions are disrupted. Studies have demonstrated hypoactivation during response inhibition tasks in frontal and parietal regions, as well as the thalamus, basal ganglia, and cingulate cortex (Dickstein et al., 2006; Hart et al., 2013). Furthermore, in attention demanding tasks, decreased activity in frontal regions, as well as the basal ganglia, thalamus (pulvinar), and the parietal and temporal lobes was identified (Dickstein et al., 2006; Hart et al., 2013). In addition to decreased activity in attention demanding tasks and response inhibition, both directly related to the ADHD phenotype, an array of other functions is disrupted in ADHD. These include reduced activity in the striatum in reward anticipation tasks (Scheres et al., 2007), and in the cerebellum in cognitive tasks, motor timing, and in the resting state (Suskauer et al., 2007; Tian et al., 2006; Vloet et al., 2010). Overall, consistently decreased brain volumes and hypoactivation of regions known for their roles in inhibition and attention are consistent with the behavioural

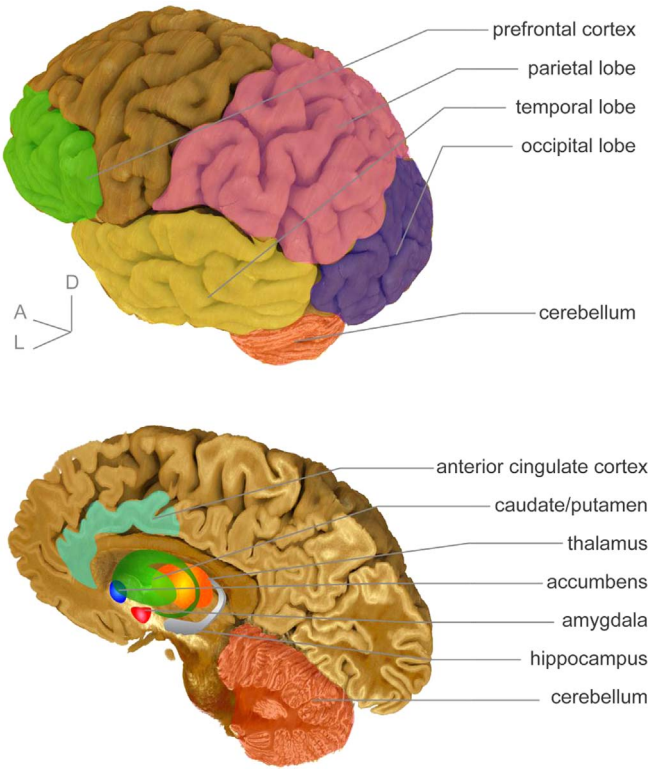

Fig. 1. Brain regions affected in ADHD. 3D rendering of an adult male brain obtained from the Big Brain project (Amunts et al., 2013) and rendered using Drishti (Limaye, 2012). **A** anterior, **D** dorsal, **L** lateral.

ADHD phenotype. Given the neurodevelopmental phenotype, we might expect a developmental role for ADHD associated genes, and genes known to be involved in neurodevelopment may provide candidates for ADHD.

The changes observed in the brains of ADHD cases result from impaired development during pregnancy and/or early postnatal life. The formation of a functioning brain occurs in a conserved sequence. Initially, pools of neural progenitors distributed across multiple neurogenic zones proliferate and give rise to the different classes of neurons. The newly formed neurons migrate across the developing brain and, upon reaching their final destination, establish a network of

Table 1
Changes in brain volumes seen in ADHD.

Region	Function	Volume change	References
Accumbens	Reward processing	Reduced	(Hoogman et al., 2017)
Amygdala	Memory, emotional regulation	Reduced	(Hoogman et al., 2017)
Anterior cingulate cortex	Executive functioning	Reduced	(Pliszka et al., 2006)
Caudate	Learning and motor control	Reduced	(Hoogman et al., 2017)
Cerebellum	Motor coordination, inhibition, executive functioning	Reduced	(Valera et al., 2007)
Cortex	Sensory processing and cognition	Reduced Thickness	(Narr et al., 2009)
Hippocampus	Short to long term memory transfer, emotion regulation	Reduced	(Hoogman et al., 2017)
Occipital lobe	Visual processing	Reduced	(Durston et al., 2004)
Parietal lobe	Visuo-spatial, selective attention	Conflicting evidence: both reduced and increased volumes reported	(Castellanos et al., 2002; Sowell et al., 2003)
Prefrontal cortex	DLPFC: attention, working memory VLPFC: inhibition OFC: social behaviour, balance of inhibition and disinhibition, emotional regulation	Reduced	(Mostofsky et al., 2002;Sowell et al., 2003)
Putamen	Learning	Reduced	(Hoogman et al., 2017)
Temporal lobe	Visual and auditory association, memory, emotional regulation	Conflicting evidence: both reduced and increased volumes reported	(Castellanos et al., 2002; Sowell et al., 2003)
Thalamus (pulvinar)	Attention	Reduced	(Ivanov et al., 2010)

Abbreviations: DLPFC, dorsolateral prefrontal cortex; VLPFC, ventrolateral prefrontal cortex; OFC, orbitofrontal cortex.

Table 2
ADHD associated genes that play a role in neurodevelopment.

Gene	Study type	References	Neurodevelopmental process
BDNF	Candidate Gene	(Hawi et al., 2017; Kent et al., 2005)	Synaptogenesis, Selective cell death, Glia and Microglia
CDH13	GWAS-SNP	(Lasky-Su et al., 2008)	Neurogenesis, Connectivity, Synaptogenesis
CHRNA7	GWAS-CNV	(Williams et al., 2012)	Synaptogenesis, Glia and Microglia
DRD5	Candidate Gene	(Daly et al., 1999; Gizer et al., 2009)	Glia and Microglia
FOXP2	GWAS-SNP	(Demontis et al., 2017)	Neurogenesis, Migration, Synaptogenesis
GIT1	Candidate Gene	(Won et al., 2011)	Glia and Microglia
GRM1	GWAS-CNV	(Elia et al., 2012)	Neurogenesis, Synaptic Plasticity, Selective cell death
GRM5	GWAS-CNV	(Elia et al., 2012)	Neurogenesis, Synaptogenesis, Selective cell death
GRM7	GWAS-CNV	(Elia et al., 2012)	Neurogenesis, Synaptic Plasticity, Selective cell death
5-HT1B	Candidate Gene	(Gizer et al., 2009; Hawi et al., 2002)	Synaptic Plasticity
LPHN3/ADRGL3	Candidate Gene	(Arcos-Burgos et al., 2010; Ribases et al., 2011)	Connectivity, Synaptogenesis
MEF2C	GWAS-SNP	(Demontis et al., 2017)	Neurogenesis, Synaptogenesis
NOS1	Candidate Gene	(Reif et al., 2009)	Neurogenesis, Synaptic Plasticity, Selective cell death, Glia and Microglia
PARK2	GWAS-CNV	(Jarick et al., 2014)	Neurogenesis, Selective cell death
PCDH7	GWAS-SNP	(Demontis et al., 2017)	Connectivity
PTPRF	GWAS-SNP	(Demontis et al., 2017)	Synaptogenesis, Selective Cell Death
SEMA6D	GWAS-SNP	(Demontis et al., 2017)	Connectivity
SLC6A2	GWAS-SNP	(Lasky-Su et al., 2008)	Glia and Microglia
SLC6A3	Candidate Gene	(Cook et al., 1995; Gizer et al., 2009)	Synaptic Activity, Synaptic Plasticity
SLC6A4	Candidate Gene	(Gizer et al., 2009; Manor et al., 2001)	Neurogenesis, Migration, Synaptic Plasticity, Selective Cell Death
SLC9A9	Candidate Gene, GWAS-SNP	(de Silva et al., 2003; Lasky-Su et al., 2008)	Synaptic Activity
SNAP25	Candidate Gene	(Brophy et al., 2002; Gizer et al., 2009)	Synaptic Activity, Selective Cell Death
SORCS3	GWAS-SNP	(Demontis et al., 2017)	Synaptic Plasticity
ST3GAL3	GWAS-SNP	(Demontis et al., 2017)	Synaptogenesis, Glia and Microglia

connections. These include short-range connections with neighbouring neurons in the same region and long-range projections to other regions, for example between thalamic nuclei and the neocortex, which encompasses the motor and sensory cortices and areas responsible for higher-order cognitive functions. This initial pattern of connectivity is later refined through activity-driven pruning, selecting for the strongest synaptic contacts, and reducing the number of neurons. Here we discuss the role of ADHD associated genes (Table 2) in each of these phases of neurodevelopment.

2. Neurogenesis

Neural progenitors in the developing brain undergo different modes of proliferation; symmetrical division to generate two progenitor cells and amplify the progenitor pool, or asymmetrical division; giving rise to a single progenitor cell and a neuron. In the later phases of development, progenitors undergo terminal symmetrical division, generating two neurons and depleting the neurogenic pool. Brain formation depends on a suitable balance between the different division modes to maintain sufficient progenitors whilst generating the appropriate number of neurons. This equilibrium is mediated through cell-cell interactions, for example, the Notch-Delta pathway, which promotes proliferation and inhibits differentiation (Egger et al., 2010). Alteration of this proliferation-differentiation balance has dramatic consequences for brain development and has been implicated in neurodevelopmental cognitive disorders including ASD (Kaushik and Zarbalis, 2016).

The numerous brain structures affected in ADHD, as revealed by MRI studies, (Table 1 and Fig. 1) have distinct developmental origins with the neurons populating them arising from separate neurogenic niches, each with a characteristic pattern of gene expression. Amongst the most studied of the brain structures affected in ADHD (see Table 1) is the neocortex, comprised of a heterogeneous population of locally born glutamatergic excitatory neurons, emerging from the neurogenic zones lining the lateral ventricles, and GABA (gamma aminobutyric acid)-ergic inhibitory interneurons, arising from the subcortical ganglionic eminences and preoptic area. The mechanisms regulating the development of the thalamus, caudate, putamen, and striatum are not as well defined as that of the neocortex but, the neurons populating these regions emerge from neurogenic zones lining the 3rd ventricle (Marin et al., 2000).

Several signalling molecules, such as glutamate, participate in neurogenesis. Given glutamate's role as a positive regulator of neurogenesis (reviewed in Schlett, 2006), it is unsurprising that members of the metabotropic glutamate receptor (*GRM*, *mGluR*) family also play roles in this process. *GRM-1*, *– 5*, *– 7*, and *– 8*, demonstrated association with ADHD in a GWA-CNV study (duplications: *GRM1*, deletions: *GRM-5*, *– 7* and *– 8*; Elia et al., 2012). GRM1 and GRM5 can both induce neurogenesis (Baskys et al., 2005; Zhao et al., 2011), and activation of GRM5 in neural progenitor cells (NPCs) increases expression of *cyclinD1*, known to induce neural proliferation (Sundberg et al., 2006). Knockdown of GRM7 in mouse NPCs increases proliferation by relieving inhibition of cyclic AMP response element-binding protein (CREB) phosphorylation and Yes-associated protein (*Yap*) expression, thereby increasing expression of *cyclinD1* (Xia et al., 2015). This data provides the connection between ADHD-associated glutamate receptor signalling and the control of cell proliferation.

In addition to the neurotransmitter glutamate influencing proliferation, serotonin, and nitrous oxide (NO) may also play a role. NO is a non-synaptic signalling molecule that inhibits dopamine, noradrenaline, and serotonin reuptake by inhibiting transporter function (Asano et al., 1997; Kaye et al., 1997; Lonart and Johnson, 1995, 1994; Pogun et al., 1994). Nitrous oxide synthase 1 (NOS1) is responsible for producing NO (Nathan, 1992) and has demonstrated association with ADHD in a candidate gene study (Reif et al., 2009). Application of NO to developing *Xenopus* embryos decreases neuronal proliferation in the optic tectum, and, conversely, loss of NO increases proliferation (Peunova et al., 2001), which is also seen in *Nos1* knockout mice (Packer et al., 2003). In addition, inhibition of NOS1 increases proliferation in neurogenic regions of the adult mouse brain, such as the subventricular zone and the dentate gyrus, (Matarredona et al., 2004; Zhu et al., 2006). Of particular interest is the interaction between NOS1 and the serotonin transporter (SLC6A4, 5-HTT, SERT; Chanrion et al., 2007). *SLC6A4* is associated with ADHD (Gizer et al., 2009; Manor et al., 2001), and regulates the uptake of serotonin from the synaptic cleft into the pre-synaptic neuron (Lesch and Waider, 2012). The physical interaction between NOS1 and SLC6A4 reduces SLC6A4's cell-surface localisation in HEK293 cells and decreases serotonin uptake in these cells (Chanrion et al., 2007). In addition, application of serotonin to NOS1 and SLC6A4 expressing cells increases NO production (Chanrion et al., 2007). This could then result in decreased

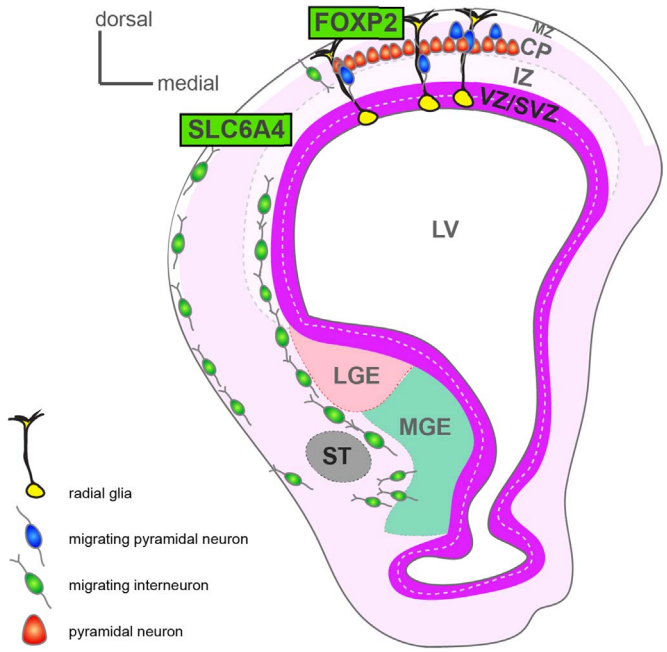

Fig. 2. Neurogenesis in the human embryonic neocortex. Coronal section through a human embryonic brain at 12 weeks post conception illustrating newly born pyramidal neurons (blue), generated locally, migrating radially along glial processes, through preceding generations of neurons (red) and settling over them. Inhibitory interneurons (green) are born ectopically, in subcortical regions and migrate tangentially, forming a deep and a superficial stream to avoid the striatum (ST), which secretes repulsive signals. The interneurons later switch to a radial migratory mode to reach the appropriate cortical layer. ADHD associated genes (boxed) involved in neurotransmitter regulation, participate in the both radial and tangential neuronal migration. **CP** cortical plate; **IZ** intermediate zone; **LGE** lateral ganglionic eminence; **LV** lateral ventricle; **MGE** medial ganglionic eminence; **MZ** marginal zone; **ST** striatum; **VZ/SVZ** ventricular/subventricular zones.

neural proliferation, consistent with decreased brain volume.

Members of the cadherin family are known to play important roles in axon outgrowth, guidance, synaptogenesis, and synapse maintenance (Redies et al., 2012). *CDH13* showed association with ADHD in a quantitative trait GWAS (Lasky-Su et al., 2008), and its expression is consistent with a role in neurodevelopment; peaking at postnatal day 7 in the developing mouse brain, before steadily decreasing into adulthood (Rivero et al., 2015). From GWAS studies it is not possible to determine if an increase or decrease of CDH13 function is associated with ADHD, but neuroblastoma cells expressing CDH13 lose their mitogenic proliferative response when treated with epidermal growth factor, suggesting that CDH13 acts as a negative regulator of proliferation (Takeuchi et al., 2000). In addition, CDH13 is suppressed by DNA methyltransferase 3b (DNMT3b), and release of this suppression, due to *DNMT3b* loss in PC12 cells, prevents nerve growth factor induced neuronal differentiation (Bai et al., 2006), suggesting that CDH13 negatively regulates both proliferation and differentiation.

The E3 ubiquitin ligase parkin (*PARK2*) is another example of an ADHD associated gene that influences both neural proliferation and differentiation. A GWA-CNV study demonstrated an enrichment of *PARK2* CNVs (deletions and duplications) in ADHD (Jarick et al., 2014). E3 ubiquitin ligases are important for the ubiquitination of proteins destined for the 26 S proteasome (Goldberg, 2003), and PARK2 has demonstrated roles in mitophagy, cell survival, and vesicle trafficking (Imai et al., 2002; Kawahara et al., 2008; Staropoli et al., 2003). Park et al. (2017) demonstrated that PARK2 is directly involved in the ubiquitination of p21, a negative regulator of cell-cycle progression. Knockout of *Park2* results in accumulation of p21 in neural stem cells, blocking differentiation. The exact role of PARK2 in the aetiology of ADHD is not yet known, however, in vitro evidence suggests that PARK2 is important for forming dopaminergic neurons (Shaltouki et al., 2015). Given the well-established role for the dopamine system (Kirley, 2002) and reduction in volume of dopaminergic-rich brain regions in ADHD (Schneider et al., 2006), the requirement for PARK2 in dopamine neurogenesis strongly supports its association with the disorder.

Two transcription factors, Myocyte Enhancer Factor 2C (*MEF2C*), and Forkhead box transcription factor P2 (*FOXP2*) also have roles in neural differentiation and have recently been associated with ADHD via GWAS (Demontis et al., 2017). CNVs encompassing *MEF2C* have also been associated with ASD (Yingjun et al., 2017), and conditional brain specific *Mef2c* knockout mice are hyperactive (Adachi et al., 2016). Expression of *Mef2c* in murine embryonic stem cells induces differentiation into neuronal progenitors in vitro, (Z. Li et al., 2008) and conditional brain specific *Mef2c* null mice show impaired neural differentiation, without deficits in proliferation or survival (H. Li et al., 2008). Knockout of *Foxp2* in mice leads to severe motor impairment and premature death (Shu et al., 2005), while knockdown of FOXP2 in mice embryonic stem cells leads to decreased neurogenesis, and expression of human FOXP2 promotes neurogenesis (Tsui et al., 2013). Whilst *MEF2C* haploinsufficiency and knockout of *Foxp2* result in severe mental retardation (Rocha et al., 2016; Zweier and Rauch, 2011) and premature death (Shu et al., 2005) respectively, it is possible that the subtle changes in the function or expression of these genes as a result of ADHD gene variants would result in decreased differentiation, and hence contribute to the decreased brain volume seen in ADHD.

3. Migration

Following the initial proliferative phase, newborn neurons exit the neurogenic zone to populate the developing brain. They are guided along "molecular corridors" consisting of unique combinations of migration cues. The migrating neuron's ability to sense the appropriate cue, and therefore follow the correct path to its predestined location, is determined by the set of receptors it expresses at its surface, which is in turn specified by its lineage. Neural progenitors are characterised by the differential expression of morphogens and transcription factors, which regulate the genes expressed by their neuronal progeny, determining their functional and molecular identities and, ultimately, their fate. Therefore, neurons originating from the same pool of progenitors migrate together, forming large migratory streams across the developing brain. These neurons migrate according to two distinct modes, radial or tangential to the surface of the brain, often switching from one to the other. For example, the glutamatergic excitatory neurons populating the cortex are born locally and migrate radially in the developing cortical plate, along the glial fibres (Fig. 2). Their GABAergic inhibitory counterparts are born ectopically, in the subcortical ganglionic eminences and the preoptic area, and migrate first tangentially along the ventral surface of the brain and switch to a radial migratory mode upon entering the cortical plate, at the level of the marginal zone or the intermediate zone (reviewed in Marín and Rubenstein, 2003). The neurons of the caudate, putamen, and striatum originate from the neurogenic zones lining the 3rd ventricle and migrate laterally to cluster into discrete nuclei. Cell adhesion molecules, including the cadherin family, are critically involved in this process, segregating subpopulations of cells based on their expression. Disruptions to migratory pathways can lead to abnormal brain development, either by delaying the migration of neurons to their final positions, or mislocalisation of neuronal subsets.

The association between variants in neurotransmitter receptors, including glutamate and GABA receptors, and ADHD (Chang et al., 2014; Lasky-Su et al., 2008; Yuan et al., 2017) is particularly interesting as neurotransmitters have been demonstrated to modulate neuronal migration (reviewed in Heng et al., 2007). For example, activation of the glutamate receptors stimulates the migration of glutamatergic excitatory cortical neurons during development, promoting radial migration from the neurogenic zones to the appropriate cortical layer.

Similarly, activation of GABA receptors expressed by inhibitory interneurons is able to modulate both their tangential and radial migration. Therefore, variants affecting GABA and glutamate receptors in ADHD might not only affect neuronal communication but also disrupt the migration of excitatory and inhibitory neurons during development.

In addition to glutamate, the neurotransmitter serotonin also has a role in neuronal migration. The migration of GABAergic interneurons is delayed, and more neurons are found, in the supragranular cortical layers in mice lacking the serotonin transporter gene, *Slc6a4* (Riccio et al., 2009). Knockout mice have increased levels of extracellular serotonin, due to the inability of the serotonin transporter to appropriately reuptake serotonin from the extracellular space into the presynaptic neuron. This increase in extracellular serotonin would lead to elevated activity of the 5HT6 serotonin receptor, which decreases the rate of migration in radially migrating pyramidal neurons (Riccio et al., 2011) and in interneurons (Riccio et al., 2009). Altogether, this evidence suggests that serotonin acts to regulate the rate of neuronal migration to provide correct developmental timing and positioning.

Interestingly, *FOXP2* may play a role in radial neuron migration through the modification of neural progenitor morphology (Garcia-Calero et al., 2016). A gradient of *Foxp2* expression in the developing mouse striatum, with low FOXP2 levels in the SVZ through to high levels in the mantle layer, promotes a change from multipolar (many neurites) to bipolar (two neurites) morphology (Garcia-Calero et al., 2016). Ectopic expression of *Foxp2* in the SVZ induces a change to bipolar morphology (Garcia-Calero et al., 2016), and impairs the radial migration of multipolar cells (Clovis et al., 2012; Garcia-Calero et al., 2016). Migration of radial glial cells is also disrupted in the knockout (Shu et al., 2005). Variants affecting the level of function of FOXP2 could therefore disrupt neuronal morphology and subsequently migration in ADHD.

The evidence for a role of neurotransmitters in neurodevelopment prior to synaptogenesis is building. Considering that neurotransmitters, such as glutamate, can regulate the levels of intracellular Ca^{2+} that are vital for the reorganisation of the cytoskeleton during migration (Doherty et al., 2000; Gordon-Weeks, 2004), it is possible that neurotransmitters influence early stages of neuronal development. Further characterisation of the role of neurotransmitters in development could therefore greatly add to our knowledge of ADHD.

4. Connectivity

The guidance cues and adhesion molecules dispersed across the developing brain not only coordinate neuronal migration, they also direct the pathfinding of neuronal processes (neurites), and the formation of connections. The growth cone located at the tip of extending neurites is enriched in guidance cue receptors and adhesion molecules, which allow it to probe the environment. Interactions between the molecules at the surface of the navigating growth cone and their specific ligands in the extracellular matrix, or on neighbouring cells, triggers intracellular cascades resulting in cytoskeletal rearrangements. These morphological changes promote growth towards the source of the guidance cue (attraction) or away from it (repulsion; Fig. 3). Similar to migration, dysregulation of guidance cues can lead to abnormal distribution of neurons in the developing brain. Delayed establishment of neural connections would result in an underdeveloped brain, consistent with the developmental delay seen in individuals with ADHD.

Short-range cues are membrane bound, acting as guide posts for branching axons. Upon contact with these molecules, growth cones will either continue to extend in the same direction or will be repelled. Two members of the cadherin family, *CDH13* and protocadherin 7 (*PCDH7*, also known as neural fold protocadherin, *NFPC*), act as short-range guidance cues. CDH13 is a negative regulator of neuronal axon projections that acts on spinal motor neurons (Fredette et al., 1996; Fredette and Ranscht, 1994), and infragranular (cortical layers 5 & 6)

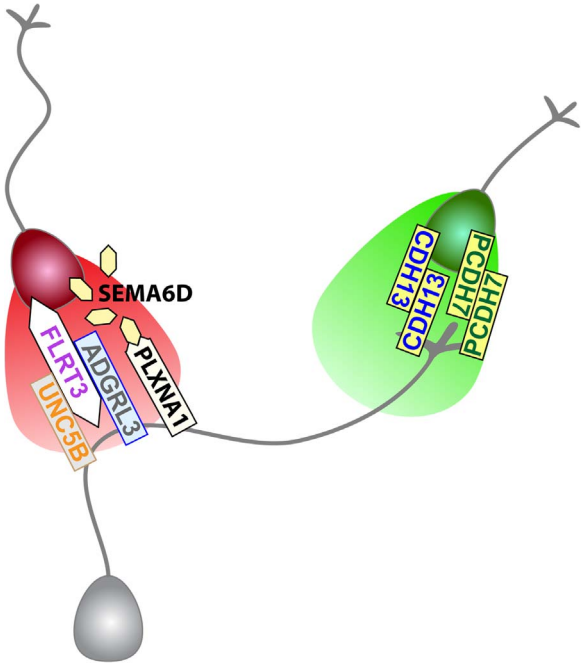

Fig. 3. Axonal outgrowth is directed by short and long range guidance cues. Membrane bound FLRT3 and UNC5B are repulsive cues (red), forming a trimeric complex with ADGRL3. SEMA6D acts as a repulsive cue either as a short range transmembrane cue, or when the extracellular domain is cleaved, over long range. Cleaved FLRT3 could act as a long range repulsion cue. CDH13 and PCDH7 homophilic interactions are short range attractants (green).

neurons of the cortex (Hayano et al., 2014). CDH13 knockdown in infragranular neurons, which send contralateral projections through the corpus callosum and ipsilateral projections through the intermediate zone, results in abnormal projections to the subcortical plate (Hayano et al., 2014). In addition, ectopic expression of CDH13 in the supragranular (layers 2 & 3) neurons results in some neurons projecting into the internal capsule, rather than the corpus callosum as expected, and delays extension (Hayano et al., 2014). Therefore alteration of *Cdh13* expression has dramatic consequences for cortical axonal pathfinding.

PCDH7 was recently identified as a significant GWAS hit (Demontis et al., 2017), and is known to be expressed in the developing rat brain (Kim et al., 2007). Leung et al. (2013) demonstrated that knockdown of Pcdh7 in developing *Xenopus* embryos leads to stalled axonal projection in the optic tract, showing that Pcdh7 acts as a positive cue for axonal guidance. While it is not yet fully understood whether CDH13 acts as a short or long range cue, or both (Ciatto et al., 2010); (Denzel et al., 2010; Hug et al., 2004), both CDH13 and PCDH7 show evidence of homophilic binding (Ciatto et al., 2010; Leung et al., 2013), suggesting that both of these genes can act as short range guidance cues. Overall, *CDH13* and *PCDH7* have important roles in axonal guidance, most likely through roles in cell-cell adhesion and short range signalling, and variants in these genes could lead to disruptions in neuronal localisation, and, as a result, brain structure.

Long range guidance cues are secreted in the neural environment and diffuse to form a gradient to guide growth cones expressing the corresponding receptors. One such guidance cue is formed by the cleavage of the extracellular domain from fibronectin and leucine-rich transmembrane protein-3 (FLRT3), which then acts as a chemorepellent when bound to the uncoordinated-5B (UNC5B) membrane bound protein (Yamagishi et al., 2011). FLRT3 can also form a transmembrane connection with Adhesion G protein coupled receptor L3 (ADGRL3), previously known as latrophilin 3 (LPHN3) (O'Sullivan et al., 2012). Interestingly, studies examining the structure of ADGRL3-FLRT3 binding have demonstrated that FLRT3 can bind to

UNC5B and ADGRL3 proteins simultaneously (Lu et al., 2015; Ranaivoson et al., 2015). *ADRGL3* demonstrates association with ADHD (Arcos-Burgos et al., 2010; Ribases et al., 2011), and is a member of a family of secretin G protein coupled receptors (Matsushita et al., 1999) that localise to the presynaptic terminal (Grishin, 1998). Increased locomotor activity is seen in *Adrgl3* mutant mice (Orsini et al., 2016; Wallis et al., 2012), *Drosophila melanogaster* (van der Voet et al., 2016), and zebrafish (Lange et al., 2012), with the fish phenotype rescued by the most common ADHD medication, methylphenidate (Ritalin). It is important to note that the combination of receptors present on individual axons affects the response to the guidance cues in the environment, therefore it is difficult to ascertain what the effect of this trimeric complex is on axonal guidance. However, variants in *ADGRL3* could potentially modulate growth cone extension and neural connectivity through modulation of the trimeric ADGRL3/FLRT3/UNC5B complex.

Semaphorin 6D (*SEMA6D*), recently associated with ADHD via GWAS (Demontis et al., 2017), is a chemo-repellent during axonal pathfinding (Qu et al., 2002), acting as both a short range transmembrane cue and, when the extracellular domain is cleaved, a long range cue (Toyofuku et al., 2004a, 2004b). *Sema6d* mutant mice show abnormal proprioceptive axon positioning in the spinal cord (Leslie et al., 2011) and recombinant secreted SEMA6D inhibits axon extension and induces growth cone collapse (Qu et al., 2002). In addition, SEMA6D repels retinal ganglion cell axons at the optic chiasm, thereby promoting the crossing of contralateral fibres, however, when SEMA6D is coupled with PLXNA1 and Ng-CAM-related cell adhesion molecule (Nr-CAM), this becomes a growth promotion effect (Kuwajima et al., 2012). *SEMA6D* is an example of how complex even singular guidance cues can be, and how disruptions to such a gene could result in a wide array of neuronal localisation abnormalities.

Considering that axonal branching and extension occurs from early life through to adulthood, an inability to efficiently guide projecting neurites to their targets could potentially delay the establishment of effective neuronal connections. Over time, it is possible that these detrimental effects could become less profound as neuronal pathways are established, consistent with the decline in ADHD symptoms with age.

5. Synaptogenesis

The significant volume reduction in multiple regions of ADHD brains is often attributed to loss of synaptic density rather than actual loss of neurons. The mechanisms controlling neuronal migration and pathfinding are also recruited during the establishment of synaptic contacts between axons and the dendrites of postsynaptic neurons, with local attractive cues determining the sites of synapse formation. The accumulation of guidance cues at a specific location along the dendrites suggests that synapses are pre-patterned. However, the underlying mechanisms remain unknown, although some studies in *C. elegans* suggest control by glial-like cells (reviewed in Shen and Cowan, 2010). In order for synaptic contacts to mature into a functional synapse, the transient contacts require stabilisation through cell-cell interactions mediated by surface proteins, for example ephrin type-B receptor 2 (EPHB2, Kayser et al., 2008) and Cadherins 11 and 13 (Paradis et al., 2007). Surface proteins are also involved in recruiting the machinery necessary for the maturation of a functional synapse, including clustering neurotransmitter receptors (Takasu et al., 2002). A wide array of ADHD associated genes are involved in synaptogenesis, with a reduction in synaptic density potentially contributing to the reduced brain volumes seen in individuals with ADHD.

In addition to their roles in axonal connectivity, intercellular signalling proteins CDH13 and ADRGL3 have a role in synapse formation. Knockdown of CDH13 leads to a reduction in GABAergic and glutamatergic synaptic density (Paradis et al., 2007). In addition, *CDH13* expression overlaps with regions that show volume reductions in ADHD, such as the prefrontal cortex (Takeuchi et al., 2000). It is likely that these volume reductions are a consequence of decreased synaptic densities resulting from disruption of CDH13's role in cell-cell signalling. Knockdown of ADRGL3 in rodents decreases glutamatergic synaptic density in the hippocampus (O'Sullivan et al., 2012) and the cortex (O'Sullivan et al., 2014), and in zebrafish, loss of the ADRGL3 orthologue Lphn3.1 results in a decrease in dopaminergic neurons in the ventral diencephalon (Lange et al., 2012). Given the close relationship between axonal connectivity and synaptogenesis it would be beneficial to determine how variants in *CDH13* and *ADGRL3* affect both of these processes in the same model.

Other ADHD associated genes have roles in synaptogenesis via modulation of glutamate transmission, including *CHRNA7*, *GRM5*, and *BDNF*. Duplications at the 15q13.3 locus, which includes *CHRNA7*, are enriched in ADHD (Williams et al., 2012) and individuals with a deletion encompassing *CHRNA7* and the first exon of *OTUD7A* demonstrate consistent neurological phenotypes such as mental retardation and global developmental delay (Shinawi et al., 2009). *CHRNA7* codes for the α7 subunit of the neuronal nicotinic acetylcholine receptor (α7nAChR), and mice null for the *CHRNA7* orthologue have decreased cortical glutamatergic and GABAergic synapse development, with a decrease in synaptic N-methyl-D-aspartate receptor (*Nmdar*) expression, suggesting dysfunction in glutamate transmission (Lin et al., 2014a, 2014b). Disruption of glutamate transmission by knockout of the postsynaptic receptor *Grm5*, decreases dendritic spine density in younger mice (P21–23; Wijetunge et al., 2008), and increases densities in older mice (P45; Chen et al., 2012). Chen et al. (2012) suggest that this could be due to glutamate's ability to induce *de novo* spine formation (Kwon and Sabatini, 2011), so it is possible that without correct postsynaptic modulation of glutamate signalling, an increased number of spines could form. At the least, the lower dendritic levels seen in younger mice is consistent with a developmental delay, and loss of α7nAChR and GRM5 disrupt synaptogenesis, likely through irregular glutamate signalling. Further examination, particularly into α7nAChR, is needed to elucidate the mechanism behind this phenotype. In addition, BDNF has been shown to stimulate both GABAergic synapse formation (Palizvan et al., 2004; Vicario-Abejón et al., 1998), which is supported by a reduction in GABAergic synapse development in *Bdnf* knockout mice (Kohara et al., 2007), and formation of glutamatergic synapses (Alsina et al., 2001; Hu et al., 2005; Vicario-Abejón et al., 1998). However, in contrast to GABAergic neurons, glutamatergic synapses are not reduced in density in *Bdnf* knockout mice, but their maturation into functional synapses is impaired (Itami et al., 2003; Korte et al., 1995). The loss of inhibitory synapses would be consistent with the impulsive/loss of inhibitions phenotype seen in ADHD.

Roles in synaptogenesis have also been suggested for the newly associated GWA genes, *ST3GAL3*, *PTPRF*, *MEF2C*, and *FOXP2* (Demontis et al., 2017). ST3GAL3 plays a role in the sialylation of glycosphingolipids (also known as gangliosides), a subset of cell-surface glycans which play an important role in cell-cell and cell-environment signalling. Proteoglycans are particularly important in brain maturation as they enwrap neurons, forming a perineuronal net that stabilises mature synapses. Deficits in sialylation due to mutations in *ST3GAL3* lead to intellectual disability and reduced cognitive function (Edvardson et al., 2013; Hu et al., 2011). *St3gal3* null mice also show significantly increased motor activity, and decreased synaptic densities (Yoo et al., 2015). *PTPRF*, encodes the Leukocyte Antigen-Related Protein Tyrosine Phosphatase receptor (LAR-RPTP) and loss of excitatory synapses and dendritic spines is seen following overexpression of dominant-negative mutations or knockdown of LAR (Dunah et al., 2005). Presynaptic LAR expression has also been shown to induce clustering of excitatory postsynaptic proteins (Woo et al., 2009). This is potentially related to a role in axon guidance, as demonstrated for the *Drosophila* orthologue (Johnson and Van Vactor, 2003), however, experiments in *Xenopus* suggest LAR does

not play the same role in vertebrates (Johnson et al., 2001). Lastly, *MEF2C* also negatively regulates excitatory synapse formation, with brain specific loss of *Mef2c* in mice leading to increased synapse and dendritic spine formation in the hippocampus (Adachi et al., 2016; Barbosa et al., 2008). In addition, overexpression of MEF2C-VP16, to create a transcriptional enhancer, decreases excitatory synapse formation (Barbosa et al., 2008). In the cortex, the opposite is seen, with conditional loss of *Mef2c* resulting in decreased excitatory synapses densities and increased inhibitory synapses densities (Harrington et al., 2016), however, this is potentially due to cell-specific effects of *Mef2c* loss. *Mef2c* has also been shown to be repressed by FOXP2 through direct DNA binding, *Foxp2* knockouts having decreased synaptic density as a result of the de-repression of *Mef2c* (Chen et al., 2016). FOXP2 also negatively regulates the sushi repeat-containing protein X-linked 2 (*Srpx2*) gene (Sia et al., 2013). *Srpx2* positively regulates excitatory synapse formation, and transfection of *Foxp2* into rat cortical neurons decreases SRPX2 levels, and as such, decreases excitatory synapse densities (Sia et al., 2013). Together, this evidence supports the associations between these genes and an ADHD phenotype, and while a full loss of these genes is not seen in individuals with ADHD, a subtle phenotype caused by a gene variant could well be contributing to alterations in synaptic densities.

6. Synaptic activity

Other than a small fraction of electrical synapses, which directly transmit the nerve impulse to the post-synaptic neuron, synapses are predominantly chemical with the action potential carried along the axon triggering the release of neurotransmitters. Vesicles containing the neurotransmitter fuse with the membrane of the pre-synaptic element to release their contents into the synaptic cleft enabling neurotransmitter molecules to bind to receptors located at the surface of the post-synaptic element. Therefore, vesicular trafficking, fusion and recycling are critical for neurotransmission, and mutations affecting these processes have deleterious effects on brain function, and as a consequence disrupt development (Fig. 4).

Synaptosomal associated protein 25 (SNAP25) is a member of the family of proteins that make up the soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) complex. This complex is involved in intracellular vesicular trafficking, facilitating neurotransmitter release, and is also important for the maintenance of cell membranes during cell fusion and division (Cupertino et al., 2016). Variants in genes encoding components of the SNARE complex have been implicated in ADHD, ASD, schizophrenia, depression, and bipolar disorder, and defects in this complex disrupt neurodevelopment at multiple stages including axonal growth, synaptic plasticity, and neuronal survival (Cupertino et al., 2016).

SNAP25 makes up two of the four helices that comprise the SNARE complex (Sutton et al., 1998), and variants in *SNAP25* are associated with ADHD (Brophy et al., 2002; Gizer et al., 2009). Deletion of *Snap25* is found in the *Coloboma* mouse, in which homozygotes die embryonically (Theiler et al., 1979), and heterozygotes display hyperactive phenotypes and fail to meet neurodevelopmental milestones (Hess et al., 1992; Heyser et al., 1995). SNAP25A and SNAP25B, are the primary splice isoforms expressed during pre- and post-natal development, respectively (Bark et al., 1995). Adult mice expressing SNAP25A, but not SNAP25B, have decreased spatial learning, higher anxiety, and swollen hippocampal mossy fibres, with some areas showing almost complete loss of synaptophysin immunoreactivity, suggesting a loss of functional presynaptic elements (Johansson et al., 2008). This is most likely due to the disruption of the SNARE complex and hence failure of synaptic membrane maintenance.

The solute carrier family 9 member A9 gene (*SLC9A9*) encodes a sodium/proton exchanger (NHE9), and has shown strong association with both ADHD (de Silva et al., 2003; Kondapalli et al., 2014; Lasky-Su et al., 2008) and ASD (Kondapalli et al., 2014). NHE9 is localised to late recycling endosome membranes, where it acts as a trans-membrane transporter for Na^+ and H^+ ions, controlling endosomal pH (Casey et al., 2010). Mutations in *Slc9a9* have been found in the WKY/NCrl rat strain that primarily displays an inattentive phenotype (Zhang-James et al., 2011), as well as in ADHD cases displaying impulsivity and intellectual disability (de Silva et al., 2003). Downregulation of *Slc9a9* expression is also seen in the spontaneously hypertensive rat (SHR), which is known to display the combined phenotype of ADHD (Zhang-James et al., 2011). The limited work using knockout models of *Slc9a9* has mostly identified traits related to ASD rather than ADHD (Yang et al., 2016). Considering that SLC9A9 has been shown to interact with proteins such as CHP and RACK1 (Lin and Barber, 1996; Ohgaki et al., 2008), known to be involved in Ca^{2+} signalling which is important for the phosphorylation of plasma membrane receptors such as solute carrier family 6 member 3 (SLC6A3, also known as DAT1) and NMDA (Belmeguenai and Hansel, 2005; Lee et al., 2004; Mansuy et al., 1998), this may be how *SLC9A9* variants contribute to ADHD. However, more research into SLC9A9 knockdown animal models is needed to determine if this is the case.

7. Synaptic plasticity

Potentiation and depression of synapses reflect how synaptic activity can modulate neuronal pathways in the context of learning and memory. Potentiation and depression refer to the strengthening and weakening of synapses, respectively, which allow for neuronal pathways to be tuned in an activity dependent manner to improve efficiency. The inability to regulate neural connections can lead to decreased brain volumes and inefficient neural networks. A range of neurotransmitter systems, including dopamine, nitrous oxide, glutamate, and serotonin have been implicated in synaptic plasticity, and could therefore play a role in maintaining these neural connections.

The role of glutamate signalling in the long term depression and potentiation of synapses is demonstrated by members of the GRM family, as well as the newly identified ADHD gene, *SORCS3*. Members of the GRM family localise to pre- and postsynaptic elements, consistent with their role in long term depression and potentiation (Niswender and Conn, 2010). *Grm1* knockout mice exhibit decreased long term potentiation in hippocampal neurons when attempting to learn an associative classical conditioning task, which coincides with an inability to learn the task (Gil-Sanz et al., 2008). GRM7's regulation of both excitatory and inhibitory signalling systems makes it a candidate for the regulation of synapses (Palazzo et al., 2016). Its ability to inhibit excessive neurotransmitter release suggests that GRM7 is important for preventing over-excitation, and as such has a protective effect against neurological disorders, which is exemplified by the seizure susceptibility phenotype seen in *Grm7* null mice (Niswender and Conn, 2010; Sansig et al., 2001). Similarly, an epileptic phenotype is observed in mice lacking the extracellular-leucine-rich repeat (LRR) fibronectin domain 1 (ELFN1) protein, which interacts with, and recruits, GRM7 to synapses in somatostatin-containing interneurons in the hippocampus (Tomioka et al., 2014). *Elfn1* knockouts also show hyperactivity phenotypes and deficits in presynaptic plasticity (Tomioka et al., 2014). An inability to appropriately recruit GRM7 to synapses could be the cause of this phenotype, and the fact that *Grm7* null mice have deficits in working memory as well as short term potentiation supports GRM7's role in synaptic plasticity (Bushell et al., 2002; Goddyn et al., 2008; Hölscher et al., 2004). Given the strong evidence regarding the roles of GRM7 in neurodevelopment, and its interaction with ELFN1, *ELFN1* may also be a promising candidate for ADHD.

In addition to members of the GRM family, Sortilin Related VPS10 Domain Containing Receptor 3 (*SORCS3*), demonstrates a role in long term depression via glutamatergic signalling. *SORCS3* has been associated with ADHD both with rare overlapping CNVs (Lionel et al., 2011), and in a recent GWAS (Demontis et al., 2017). Loss of

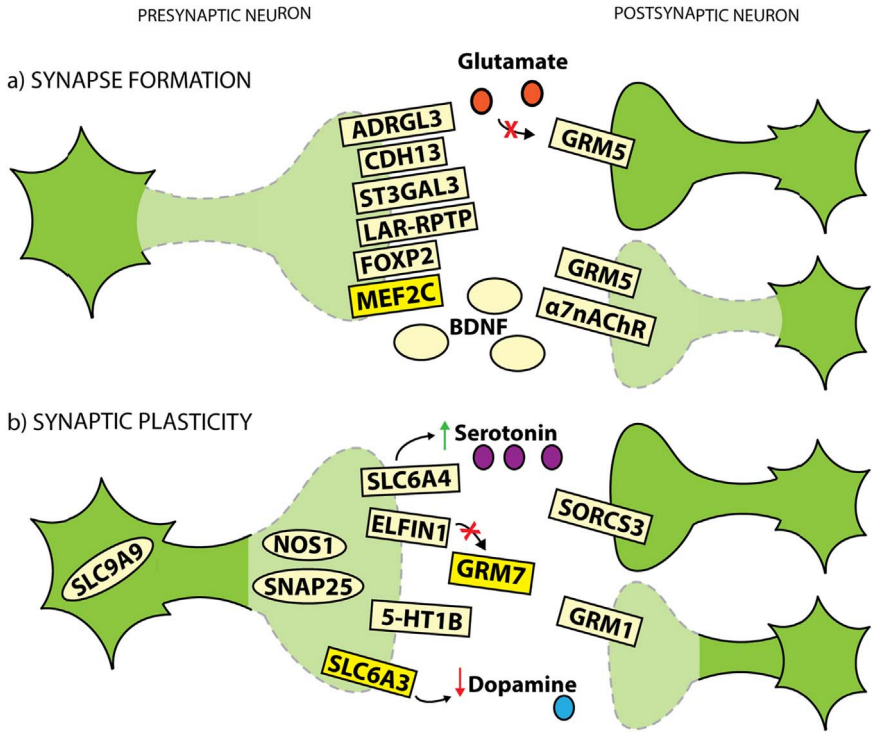

Fig. 4. Impacts of ADHD associated gene knockdowns on synaptogenesis. Gene knockdowns or knockouts are shown in faded yellow, while decreases in synaptic density or plasticity are depicted as faded green. a) Loss of ADGRL3, CDH13, ST3GAL3, LAR-RPTP, FOXP2, MEF2C, α7nAChR, and BDNF all result in decreased synaptic densities. *mGluR5* knockouts show decreased densities in young mice, and increased densities in older mice, potentially due to lack of postsynaptic glutamate regulation. b) Loss of NOS1, SNAP25, and GRM1 all result in decreased synaptic plasticity either through decreased long term potentiation or lack of synapse maintenance. An inability to recruit GRM7 to presynaptic membranes due to *Elfin1* knockout is thought to lead to a decrease in presynaptic plasticity, while SLC6A3 3'UTR 10R could potentially lead to a decrease in potentiation through increased dopamine reuptake. SLC6A4 and 5-HT1B, through modulation of serotonin signalling, and SLC9A9, through endosomal recycling, could also disrupt synaptic plasticity. SORCS3 acts as a negative regulator of synaptic plasticity.

Sorcs3 in mice leads to a loss of NMDA and mGluR dependent long-term depression in the hippocampus, as well as deficits in spatial learning ability (Breiderhoff et al., 2013). Glutamate signalling thus plays a vital role in synaptic plasticity and variants in genes that are involved in glutamate pathways could lead to abnormal neural connections and inefficient brain networks.

Nitrous oxide, serotonin, and dopamine, also play roles in synaptic plasticity. *Nos1* knockout mice show increased impulsivity (Nelson et al., 1995), as well as deficits in spatial learning and memory (Wultsch et al., 2007). Memory deficits are suggestive of dysfunction in synaptic potentiation, as memory is the result of establishing lasting neuronal pathways. Serotonin has long been associated with alterations in synaptic plasticity, and the serotonin receptor 1B (*5-HT1B*) and *SLC6A4* are both associated with ADHD (Gizer et al., 2009; Hawi et al., 2002; Manor et al., 2001) and are known to play a role in this process (Lesch and Waider, 2012). Knockout of *Slc6a4* results in increased serotonin in the synaptic cleft (Lesch and Waider, 2012), and *5-Ht1b* knockout mice display increased activity (Brunner et al., 1999). Further, 5-HT1B activation by serotonin inhibits glutamate release in the thalmocortical somatosensory pathway in the developing rat (Rhoades et al., 1994; Salichon et al., 2001). The decrease in glutamate release following *Slc6a4* knockout would reduce long term potentiation of excitatory synapses, supported by decreased NMDAR-dependent long term potentiation following treatment of rat primary visual cortex slice with serotonin (Kim et al., 2006). It is however, important to note that the effect of serotonin may vary between developmental stages and regions due to difference in the expression of its receptors (Wirth et al., 2017).

SLC6A3 is potentially the best established ADHD-associated gene (Cook et al., 1995; Gizer et al., 2009), and is important for the reuptake of dopamine from the synaptic cleft into the pre-synaptic neuron. ADHD individuals homozygous for the ten repeat (10R) VNTR allele in the *SLC6A3* 3'UTR show significantly decreased cortical thickness in the right prefrontal cortex compared to heterozygotes and homozygotes for the 9 repeat (9R; Fernández-Jaén et al., 2015). In children, the 10R allele is associated with higher levels of the dopamine transporter (Brookes et al., 2007), which would lead to lower levels of dopamine in the synaptic cleft, consistent with a decrease in synaptic potentiation. This evidence further highlights the role of signalling molecules in synaptic plasticity, strengthening the association of neurotransmitter pathway genes with ADHD.

The combined evidence points to disruption of synapses being the most common effect of ADHD associated variants. In addition, decreases in brain volume through decreased synaptic potentiation and synaptic maintenance are consistent with the ADHD phenotype.

8. Selective cell death

In the nervous system, neurons are generated in excess and the brain undergoes a phase of selective cell death to eliminate redundant neurons. Connectivity is one of the main criteria determining if a neuron is to survive or not. This is achieved through the release of trophic factors at the level of the post-synaptic neuron, including nerve growth factor and brain derived neurotrophic factor (BDNF), to promote the survival of the presynaptic neuron. Therefore, the more active connections a neuron establishes, the more likely it is to survive. Nerve growth factor and BDNF bind with tropomyosin receptor kinase A (TrkA) and tropomyosin receptor kinase B (TrkB), respectively. Nerve growth factor binding to TrkA prevents an apoptosis cascade in the peripheral nervous system, but its role in the central nervous system is not clear (Dekkers et al., 2013), although cholinergic neurons in the basal forebrain follow this same form of neuronal survival (Sanchez-Ortiz et al., 2012). In the CNS TrkB does not activate apoptosis, and BDNF binding is not required to prevent apoptosis

from occurring (Nikoletopoulou et al., 2010), suggesting BDNFs role in cell survival is not via TrkB. However, most *Bdnf* null mice die at postnatal day 2 (Jones et al., 1994), and conditional *Bdnf* knockout in the cortex, (Baquet et al., 2004), which is the source of striatal BDNF, or the whole brain (Rauskolb et al., 2010), results in loss of dendritic complexity and neurons in the striatum, as well as loss of dopaminergic neurons in the midbrain-hindbrain region (Baquet et al., 2005). Conditional, forebrain restricted, knockouts also show progressive loss of cortical dendrite complexity (Gorski et al., 2003). There is therefore, substantial evidence that BDNF contributes to neuronal survival in the CNS, consistent with the ADHD phenotype.

The regulation of neuronal survival is also influenced by the ADHD associated genes *SNAP25*, *PTPRF*, *PARK2*, *NOS1*, *SLC6A4*, and *GRMs*, via control of BDNF release, apoptosis, and oxidative stress. Loss of SNAP25 leads to neuronal degeneration, through an inability to maintain protein recycling at the plasma membrane (Peng et al., 2013). In addition to this, SNAP25 demonstrates an important role in neuronal survival through regulation of the exocytosis of BDNF in axons and dendrites of cortical neurons (Shimojo et al., 2015). Given that loss of BDNF results in neuronal loss in the CNS, appropriate regulation of its release would be essential for neuronal survival. Interestingly, BDNF strengthens the interaction of LAR-RPTP with TrkB in mice hippocampal neurons (Yang et al., 2006), and BDNF neurotrophic activity decreases in *Lar* knockouts and knockdowns, and increases following exogenous expression of *Lar* (Yang et al., 2006), connecting BDNF to another ADHD associated factor. PARK2 regulates apoptotic factors, with dopaminergic neurons formed from *PARK2* mutant iPSC lines showing lower pro-apoptotic factors and higher anti-apoptotic factors than controls (Konovalova et al., 2015). However, how this particular imbalance of apoptotic factors alters neuronal survival in ADHD requires further examination.

Signalling molecules such as nitrous oxide, serotonin, and glutamate demonstrate roles in neuronal programmed cell death. Administration of anaesthetics containing high levels of NO to postnatal infant rats causes severe hippocampal neurodegeneration (Head et al., 2009) and activation of the apoptotic proteins caspase-3 and – 9 in the cerebral cortex and thalamus (Lu et al., 2006). *Slc6a4* knockout mice have decreased levels of apoptosis in the striatum, thalamus/hypothalamus, cerebral cortex, and hippocampus, suggesting that serotonin activity can trigger programmed cell death (Persico et al., 2003). In the case of glutamate, excess glutamate can lead to reduced glutathione levels, causing oxidative stress and cell death (Murphy et al., 1989). This glutamate cytotoxicity can be prevented through the activation of group 1 metabotropic glutamate receptors, GRM1 and GRM5, restoring glutathione levels and preventing oxidative stress (Sagara and Schubert, 1998). Considering other members of the GRM family such as GRM7 also regulate glutamate levels, they may also have a role in programmed cell death.

BDNF, NO, serotonin, glutamate, PARK2, SNAP25, and PTPRF, all play important roles in selective cell death. Increases in apoptosis would result in decreased neuronal number, consistent with decreased brain volumes seen in ADHD, while a decrease in apoptosis could result in an inability to clear inefficient neural connections, preventing the establishment of optimal neural networks.

9. Glia and microglia

Glial and microglial cells are essential to the development of a normal functioning brain and genetic variants affecting their organisation have been linked to neurodevelopmental cognitive disorders, including ASD (Zhan et al., 2014). Glial cells, comprising oligodendrocytes and astrocytes, arise from the same pools of progenitors as neurons, and disperse through the developing brain using the same guidance molecules as neurons. The supporting role of glial cells in neurodevelopment cannot be overstated, as they are important in synaptic plasticity, maintaining neural environments, and allowing efficient neural networks through myelination. Disruptions to glial cell processes can therefore have wide reaching effects during neurodevelopment.

Oligodendrocytes are the myelinating cells of the brain, they wrap around segments of the axon, forming a sheath of insulating myelin to accelerate the conduction of action potentials. Myelinated fibres assemble in bundles, forming large white matter tracts easily detected by MRI and are reduced in ADHD (Liston et al., 2011; van Ewijk et al., 2012). The migration of oligodendrocyte precursor cells (OPCs) depends on cues expressed by neurons, including polysialylated neural adhesion molecule (PSA-NCAM), which promotes OPCs survival (Palser et al., 2009) and migration (Decker et al., 2000). PSA-NCAM also prevents the differentiation of OPCs into myelinating oligodendrocytes (Decker et al., 2000) with downregulation of PSA-NCAM on axons coinciding with the onset of myelination in the human fetal forebrain (Jakovcevski et al., 2007). Decreased levels of PSA-NCAM have also been shown in *St3gal3* null mice, which coincides with decreased myelination, myelin basic protein, and oligodendrocyte transcription factor 2 (Yoo et al., 2015). Variants in *NCAM* have demonstrated association with schizophrenia, which shows significant genetic overlap with ADHD (Cristino et al., 2013). In addition to this, NOS1 which promotes the growth and arborisation of oligodendrocytes (Garthwaite et al., 2015) also shows association with ADHD, suggesting that myelination defects might contribute to ADHD symptoms.

Genes implicated in the development and maturation of astrocytes have also been associated with ADHD. Astrocytic functions are essential for the brain's development and activity, providing supportive roles for neurons, clearing the environment of metabolic waste and cell debris following injury. The migration of astrocytes during brain development and maturation depends on GIT1, which promotes cell motility. *Git1* null mice exhibit abnormal astrocytosis in the basal ganglia pathway, altering synaptic transmission in the basal ganglia and, ultimately, impairing the inhibitory modulation of the thalamus (Lim and Mah, 2015). Alteration of these structures in ADHD (Table 1) and genetic studies revealing a correlation between *GIT1* and ADHD (Won et al., 2011), suggest that increased astrocytosis may play a role in the disorder. However, there is conflicting evidence with regards to *GIT1*'s role in ADHD (Klein et al., 2015), and this requires future investigation.

In addition to its neuronal expression, α7nAChR has also been detected on astrocytes, in the rat hippocampus (Shen and Yakel, 2012). Activation of astrocytic α7nAChR results in a greater increase in intracellular calcium in astrocytes compared to that recorded in neurons, suggesting that astrocytic α7nAChR participates in neuroprotection by reducing levels of extracellular calcium. Abnormal astrocytic expression of *CHRNA7* in ADHD could therefore result in increased neuronal cell death.

Astrocytes are responsible for clearing the neurotransmitter at the level of the synaptic space following neurotransmission to prepare the environment for a new release. Therefore neurotransmitter receptors and transporters are expressed in astrocytes, in particular the norepinephrine transporter SLC6A2 (Inazu et al., 2003), which is associated with ADHD (Lasky-Su et al., 2008). It is therefore possible that abnormal norepinephrine signalling by astrocytes may contribute to ADHD. Similarly, the dopamine receptor DRD5 is expressed in striatal astrocytes during development (Brito et al., 2004) and has also been associated with ADHD (Daly et al., 1999; Gizer et al., 2009). Astrocytic expression of *Drd5* is promoted by BDNF, which is pivotal in brain development, accelerating the maturation of newborn neurons and facilitating their survival (Brito et al., 2004). BDNF is expressed by oligodendrocytes and, to a lesser extent, astrocytes, which upregulate the trophic factor's expression following lesion (Dougherty et al., 2000). Altogether, the evidence indicates that abnormal oligodendrogenesis would lead to a reduction of BDNF, impairing the astrocytic expression of *DRD5* and dopamine reuptake.

The brain also contains microglia, the resident myeloid cells found

throughout the mammalian central nervous system. Microglia are critically involved in the immune response in the injured brain but also play essential roles during brain maturation. Microglia promote learning-dependent synapse formation in the juvenile brain through BDNF release at the level of the synapse (Parkhurst et al., 2013). Both spine elimination and formation, part of learning-dependent synaptic turnover, are significantly reduced following loss of microglial BDNF, resulting in severe learning deficits as seen in neurological disorders. Therefore, the symptoms associated with ADHD could result from abnormal secretion of BDNF from microglia. Similar to astrocytes, microglia express neurotransmitter receptors, including the serotonin receptor HTR2B (Kolodziejczak et al., 2015) and α7nAChR (Shytle et al., 2004; Suzuki et al., 2006). The cholinergic activation of α7nAChR that promotes the neuroprotective functions of microglia, and inhibits inflammation, may be another route by which variants in *CHRNA7* contribute to ADHD.

Therefore, whilst research has mainly focussed on the neuronal defects underlying ADHD symptoms, genes associated with ADHD are also involved in the development of the non-neuronal fraction of the brain and abnormal gliogenesis and microgliogenesis could contribute to the disorder.

10. Conclusions

ADHD associated genes participate in all stages of brain development, with those affecting neurotransmission potentially playing a role at every stage. Of course, neurotransmitters have been associated with ADHD for a long time, with the targeting of dopamine reuptake by methylphenidate being the most common medication. The beneficial effects of methylphenidate suggests that neurotransmitter dysregulation contributes to the disorder, but this does not preclude an additional contribution of neurotransmitters during neurodevelopment, and the dysregulation of the dopamine pathway in ADHD may have its origins in the early stages of brain development.

The majority of ADHD associated genes with a known developmental role are involved in the formation and activity of synapses, and disruption of this process is a likely cause of the reduced brain volume observed in ADHD. Furthermore, aberrations in neuronal and axonal migration are consistent with the developmental delay hypothesis. Whilst it is therefore possible to look at cell and animal studies to make a link to the symptoms observed, it is important to note that most of the studies reviewed here involve gene knockout or overexpression systems, while variants detected in ADHD are usually SNPs or variable number tandem repeats. The majority of these variants are found in non-coding regions and, individually, are likely to have very small effects on function. Looking forward, this presents a challenge in modelling ADHD-associated variants, as while it is getting easier to introduce single variants into animal models, we are lacking the necessary assays to detect the small changes in behaviour and physiology that these variants likely cause. Examining multiple variants simultaneously could provide us a way of examining the effects of these variants in a form naturally seen in ADHD, but would not allow dissection of their individual roles. The development of suitable animal models and, importantly, sensitive behavioural assays for these models, will allow further examination of the neurodevelopmental contribution to ADHD, and is a paramount to understanding the disorder as a whole.

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Introduction: Part B

An understanding of neurodevelopmental pathways will help to determine what biological mechanisms might be at play in ADHD. However, we are currently inundated with ADHD-associations whose involvement in neurodevelopment has not been investigated, let alone how they functionally relate to the development of the disorder. Determining which variants to investigate is a challenge, as the associated gene is rarely functionally impacted by the original associated variant. Rather, it is more likely that the original variant is in linkage disequilibrium (LD) with one, or potentially several, variants, that have a functional impact on the associated gene. However, examining all of the variants in LD with the associated variant just isn't feasible. Further complicating the matter, 97% of ADHD-associated variants are mapped to non-coding regions (Tong et al., 2016), the functions of most of which are not understood. Therefore, there is a need to prioritise the array of associated variants, and those in LD with them, for those that show the strongest likelihood of being functional. This would be informative as to which variants and their associated genes are the best candidates for further functional follow up.

Tong et al., (2016), approached this challenge through the use of bioinformatic pathways. They functionally prioritised 2016 non-coding ADHD-associated variants (composed from the published literature, as well as those in strong LD with them), down to a final list of 65 variants that displayed the highest likelihood for functionality. A case-control association analysis was then performed on these variants, and observed that one variant, rs2294123 (G→T), which maps to charged multivesicular body protein 7 (*CHMP7*), showed significant association with ADHD. Homozygotes for the risk allele (T) demonstrated significantly higher ADHD-related symptoms, lower sustained attention, and 67% total *CHMP7* mRNA than homozygotes for the non-risk allele (Tong et al., 2016). Overall, this evidence further strengthened the potential of *CHMP7* as a predisposing factor in the development of ADHD. However, how CHMP7 is functionally involved in the development of ADHD is not understood, and further analysis to determine if a reduction in *CHMP7* mRNA can cause an ADHD phenotype is needed. Thus, this is the focus of the first results chapter. For result chapters two and three, two variants implicated in the ADHD-GWAS by Demontis et al., (2019), which map to dual specificity phosphatase 6 (*DUSP6*) and lysine demethylase 4a (*KDM4A*), were selected for analysis.

CHMP7

The first ADHD-associated variant I will examine in this thesis maps to charged multivesicular body protein 7 (*CHMP7*). The human CHMP family consists of 11 proteins, which fall into 7 sub-families: CHMP1 (A & B), CHMP2 (A & B), CHMP3, CHMP4 (A, B & C), CHMP5, CHMP6 and CHMP7. Members of

the CHMP1-6 sub-families are approximately 200 amino acids in length, possess coiled-coil domains, and have basic N-terminals and acidic C-terminals. CHMP1-6 are subunits of the protein complex endosomal sorting complex required for transport-III (ESCRT-III, (Babst et al., 2002; Teis et al., 2008)), which is a member of the ESCRT protein complex family. This family has four members (ESCRT-0, I, II, III), which play roles in a continually expanding list of cellular processes, including the sorting of membrane bound proteins into membrane bound vesicles for transport to the lysosome for degradation, membrane scission, membrane budding, plasma membrane repair, nuclear envelope formation, and autophagy (Hurley, 2015; Vietri et al., 2020). ESCRT-III plays a core role in many of these processes as it acts as the main piece of scission machinery (Wollert et al., 2009). Thus, it is unsurprising that disruptions to its subunits is detrimental to processes such as neuronal pruning (CHMP2B (Belly et al., 2010); CHMP4 (Loncle et al., 2015; Sweeney et al., 2006)), plasma membrane repair (CHMP2A, CHMP3, CHMP4B (Jimenez et al., 2014)), nuclear envelope formation (CHMP2A (Olmos et al., 2015)), and endosomal sorting (CHMP1 (Howard et al., 2001); CHMP2B (Urwin et al., 2010); CHMP5 (Shim et al., 2006)).

CHMP7 is unique amongst the CHMP family, as it is approximately double the length of the other CHMP members (453 amino acids), and possesses two winged helix domains at the N-terminus (Bauer et al., 2015), while the C-terminus is similar to that of CHMP6, allowing it to interact with CHMP4B (Horii et al., 2006). Also, it isn't a subunit of ESCRT-III, rather it has been shown to aid in the recruitment of ESCRT-III to the nuclear envelope during nuclear envelope formation, through its interaction with CHMP4B (which is the most abundant CHMP protein found in ESCRT-III (Teis et al., 2008))(Olmos et al., 2016; Vietri et al., 2015). Similar to the overexpression of GFP-tagged ESCRT-III proteins (CHMP1B (Reid et al., 2005); CHMP3 (Bache et al., 2006); CHMP4B (Katoh et al., 2003); CHMP6 (Yorikawa et al., 2005)), overexpression of GFP-tagged CHMP7 leads to disruptions to the endosomal sorting pathway, resulting in accumulation of ubiquitinated proteins (Horii et al., 2006). Regulation of endosomal sorting by ESCRTs in the neuron has been demonstrated to be important for regular synaptic development (Lee and Gao, 2012), and, in fact, ESCRTs have been shown to play an increasing number of roles during neurodevelopment as a whole, through their involvement in endosomal sorting, membrane scission, and plasma and nuclear membrane maintenance (Sadoul et al., 2018). Given the roles of CHMP7 in endosomal sorting, and its association with ESCRT-III, it is possible that dysregulation of this gene also leads to disruptions to neurodevelopment, and ADHD phenotypes.

DUSP6

The second ADHD-associated variant that I will examine in this thesis is a significant ADHD-GWAS association, which was linked to dual specificity phosphatase 6 (*DUSP6*). This gene is a member of the dual specificity phosphatase (DUSP) protein family, which is important for the regulation of mitogen activated protein kinases (MAPKs). The signalling cascades of MAPKs are important, biologically conserved, signal transduction pathways with multiple levels of regulation to ensure the appropriate timing and response to intracellular and extracellular signals.

MAPKs are the last part of a three-stage phosphorylation pathway, the first being MAPK kinase kinase (MAPKKK), which activates a MAPK Kinase (MAPKK), which in turn activates a MAPK (Treisman, 1996). Phosphorylation of both a threonine and a tyrosine within the conserved T-X-Y motif activates MAPKs (Marshall, 1994). This activation allows MAPKs to take part in the regulation of a wide array of cellular processes, including cell growth and survival, proliferation, and differentiation (Turjanski et al., 2007; Wada and Penninger, 2004), which influence broader processes such as embryogenesis, immunity, and neurodevelopment (Kyriakis and Avruch, 2012; Lawrence et al., 2008; Rincón and Davis, 2009). The exact levels and timing of MAPK activation is vital to the regulation of the aforementioned processes (Marshall, 1995), and one of the mechanisms contributing to this regulation is the dephosphorylation of MAPKs. The fact that MAPKs require two residues to be phosphorylated also means that dephosphorylation of either residue will inactivate it. The DUSP6 protein family (also known as MAPK phosphatases, or MKPs) is able to dephosphorylate one or both of these residues to inactivate the MAPK, and thus oppose the activation performed by MAPKKs (Caunt and Keyse, 2013).

In mammals, there are ten catalytically active DUSPs, which are sorted into 3 sub-groups: DUSP1, -2, -4, and -5, which are located within the nucleus, DUSP6, -7, and -9, which are located in the cytoplasm, and DUSP8, -10, and -16, which are found in both (Camps et al., 2000; Theodosiou and Ashworth, 2002). All these DUSPs share a non-catalytic region near the N-terminus that plays a role in determining the protein's cellular localisation and enzymatic specificity (Kondoh and Nishida, 2007; Owens and Keyse, 2007), and a phosphatase domain containing the catalytic site near the C-terminus (Dickinson and Keyse, 2006; Keyse and Ginsburg, 1993). The specificity of the substrates targeted by this region differs between the DUSPs, for example, DUSP6 targets MAPK1 & 3.

The downstream regulation of many processes, including those affecting several nervous system disorders, is controlled via the dephosphorylation of MAPK1 & 3, and this process in of itself is controlled via regulation of DUSP6 at transcriptional (Jurek et al., 2009), post-transcriptional (Bermudez et al., 2011), and post-translational levels (Bermudez et al., 2008; Marchetti et al., 2005). The inactivation of MAPK1 & 3 via DUSP6 has been linked to Parkinson's (Brehm et al., 2015),

Alzheimer's (Banzhaf-Strathmann et al., 2014; Liao et al., 2018; Liu et al., 2019), and depression (Labonté et al., 2017). It has also shown protective roles against glutamate induced neurotoxicity (Huang et al., 2017), and is important for dopamine homeostasis (Mortensen, 2013; Mortensen et al., 2008). This last process is of particular interest for ADHD, as the regulation of dopamine signalling has long been implicated in the disorder (Barr and Misener, 2008). Both dopamine receptors and the dopamine transporter (SLC6A3) have shown association with ADHD, and it has been shown that DUSP6 is important for the stabilisation of SLC6A3 at the plasma membrane (Mortensen et al., 2008). Therefore, it is likely that disruptions to DUSP6's regulatory role in the maintenance of dopamine homeostasis will lead to abnormal dopamine signalling, and thus, ADHD.

KDM4A

The third, and final, variant that I will examine in this thesis is also significantly associated with ADHD at the GWAS level, and is linked to lysine demethylase 4a (*KDM4A*). The lysine demethylase (KDM) family of proteins is a highly conserved family, which play roles in transcriptional regulation via posttranslational modification of histones (Labbé et al., 2013). Histones can undergo methylation at specific lysine residues by action of methyltransferases, and demethylases can in turn demethylate them (Trojer et al., 2009). The combination of these two processes allows for gene expression to be controlled through adjusting the level of chromatin compaction. Additional control of chromatin compaction levels is achieved through the type of methyl mark that can be added or removed, either a mono-, di-, or trimethyl.

The KDM family plays an important role in the demethylation of histones. There are eight KDM subfamilies in humans (KDM1-8), with some families containing multiple members. The overarching difference between these subfamilies is that KDM1 (A & B) lacks a Jumonji C (JmjC) domain, which restricts its action to the demethylation of mono- and dimethyl marks (Shi et al., 2004), whereas KDM2-8 possess this domain (Mosammaparast and Shi, 2010). KDM2-8 can be further differentiated from each other based on the other domains they possess, and the particular histone lysine residues that they act upon (Klose et al., 2006; Klose and Zhang, 2007).

The KDM4 subfamily consists of five members in humans. All five members contain JmJC and JmjN domains, however, KDM4A-C are over twice the length of KDM4D & -E, are expressed in all major tissues compared to KDM4D & -E (which are primarily expressed in the testes (Labbé et al., 2013)), and also possess two plant homeodomains (PHD) and two Tudor domains, which are important for recognition of target histone lysine residues (Bock et al., 2011; Lee et al., 2008; Musselman and Kutateladze, 2011, 2009).

Investigations into the functions of the KDM4 subfamily has largely pointed to roles in cell proliferation and differentiation (Labbé et al., 2013). This, similar to other members of the larger KDM family, has implicated KDM4 proteins in the development of cancer. Knockdown of KDM4A results in decreased proliferation in a squamous cell carcinoma mouse model (Ding et al., 2013), and knockdown of either KDM4B or KDM4C leads to reduced proliferation in cell culture and mice breast cancer models, respectively (Kawazu et al., 2011; Luo et al., 2012). In addition to this, expression of all three of these genes is increased in multiple other cancer subtypes (reviewed in Labbé et al., (2013)).

The role of *KDM4* genes in differentiation also extends to neurodevelopment, in particular, *KDM4A*. Loss of KDM4A in chick embryos, and knockdown of KDM4A in human neural stem cells (NSCs) both demonstrate decreased neural differentiation (Cascante et al., 2014; Strobl-Mazzulla et al., 2010). Interestingly, KDM4A has a role in maintaining stems cells in undifferentiated states (Pedersen et al., 2016). It is expressed four times higher in embryonic stem cells (ESCs) compared to NSCs, and it enhances the frequency and efficacy of ESC fusion-induced reprogramming of NSCs (Ma et al., 2008). Overall, the KDM4 family play important roles in the regulation of cell proliferation and differentiation, and both up- and down-regulation of *KDM4A* can lead to disruptions of neural differentiation. It is possible that potential delays in the final determination of neuronal cell fate is contributing to a neurodevelopmental delay commonly seen in individuals with ADHD, and this delay could result in ADHD phenotypes.

Functional examination of ADHD-associated genes

To assess the functional roles that *CHMP7*, *DUSP6*, and *KDM4A* are playing in the development of ADHD, and how this can lead to complex ADHD phenotypes, an animal model is required. Several animal models of well-established ADHD-associated genes exist. For example, mice mutant for SLC6A3 (Gainetdinov et al., 1999; Giros et al., 1996), or synaptosomal-associated protein 25kDa (SNAP25, (Wilson, 2000)) both display hyperactivity phenotypes. Similar hyperactivity phenotypes are seen in *Drosophila melanogaster* knockdowns of the *SLC6A3* or latrophilin (*LPHN3*) orthologues (van der Voet et al., 2016), and knockdown of *lphn3.1* in zebrafish (*Danio rerio*) was reported to cause hyperactivity that can be reduced through the application of methylphenidate (Lange et al., 2012). Overall, this demonstrates that the use of mice, fly, or fish models allows the examination of behavioural phenotypes of a complex psychiatric disorder such as ADHD.

In order to examine the ADHD-associated genes chosen for study in this thesis, the zebrafish model was adopted. The zebrafish is an excellent model for examining ADHD-associated genes, as it has been successfully used to examine several neurological disorders (Fontana et al., 2019, 2018). In particular,

zebrafish have been used to examine ADHD-associated genes such as *lphn3.1* (Lange et al., 2012), period1b (*per1b,* (Huang et al., 2015)), and MICAL like 2b (*micall2b,* (Yang et al., 2018)). However, of these models, two utilised morpholinos (*lphn3.1* and *micall2b*), and one used retroviral insertion (*per1b*), meaning the use of CRISPR-Cas9 genome editing has not yet been explored for examining ADHD-associated genes. In addition, the identity between human and zebrafish genomes is relatively high, with up to 82% of human disease-related genes having an orthologue in the fish (Howe et al., 2013), as compared to 75% in *Drosophila* (Reiter et al., 2001). Neurochemistry is also highly conserved, with the zebrafish possessing all major neuromodulator systems (Kaslin and Panula, 2001; Maximino and Herculano, 2010; Panula et al., 2006; Sallinen et al., 2009; Sundvik and Panula, 2012). Furthermore, zebrafish undergo rapid development, with a precursor to all major organs present by 1 day post-fertilisation (dpf), and reaching sexual maturity within three months. They produce hundreds of external offspring in one mating, which is especially beneficial when dealing with variants of small effect size, as this allows for greater statistical power. Zebrafish are transparent up to 2 dpf, which allows for live imaging of internal organs via fluorescently tagged proteins. They are also amenable to a wide array of reverse genetic techniques, including the powerful CRISPR/Cas9 genome editing system. In regards to examining ADHD in particular, phenotypes such as hyperactivity can be measured via locomotion assays, and there is potential to examine attention and impulsivity phenotypes (Choo and Shaikh, 2018; Echevarria et al., 2011). Sophisticated techniques for examining decreased volumes of individual and whole brain regions are also available (Gupta et al., 2018). The combination of these advantages makes the zebrafish an excellent model for the functional examination of ADHD-associated genes.

In this thesis, I aimed to functionally investigate three ADHD-associated genes to determine their impact on the development of ADHD: *CHMP7*, *DUSP6*, and *KDM4A*. Using zebrafish models, I have demonstrated that the loss of mRNA, as well as the disruption of functional protein, can lead to both increased and decreased activity phenotypes, as well as reduction in brain volume. The examples I provide illustrate how zebrafish models can be used to efficiently test if ADHD-associated genes are important for the development of ADHD phenotypes. In addition, I have successfully examined whether or not methylphenidate is an effective treatment in these models.

The framework that I have used in this thesis can confirm if ADHD-associated genes play functional roles in the development of ADHD phenotypes. Further, I have provided evidence suggesting how genotypic differences in ADHD-associated genes contribute to multiple aspects of ADHD, such as the persistence of symptoms into adulthood, variability in drug response, and homeostasis of neurotransmitter systems such as dopamine.

Overall, I have demonstrated the first instances of functionally testing newly associated ADHD-GWAS genes, as well as functionally predicted ADHD associated variants. The work in this thesis will hopefully pave the way for the functional examination of other ADHD-associated genes, to build a functional understanding of how associated variants are contributing to ADHD phenotypes.

Functional validation of *CHMP7* as an ADHD risk gene, using a CRISPR/Cas9 zebrafish model

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Abstract

Attention deficit hyperactivity disorder (ADHD) is a neurodevelopmental disorder of childhood with a strong genetic component. Despite the success of mapping ADHD risk loci, little work has been done to experimentally verify their contribution to ADHD phenotypes. Meta-analysis of four genome wide association studies in ADHD reported *CHMP7* as a predisposing factor for ADHD. A DNA variant mapped to *CHMP7* has been shown (via bioinformatic analysis) to have a high likelihood for functionality. We used CRISPR/Cas9 genome editing to generate a zebrafish line as an animal model for ADHD with a mutation in *chmp7*. *chmp7*$^{+/-}$ fish showed comparable reductions in mRNA levels to individuals homozygous for the *CHMP7* ADHD risk allele. *chmp7*$^{+/-}$ fish displayed significantly higher activity over a 24-hour period at 6 days post-fertilisation than *chmp7*$^{+/+}$ fish, an effect that did not persist into juvenile and adulthood stages. In addition, the increased activity at 6 days post-fertilisation was significantly reduced through application of methylphenidate, a mainstay pharmacological treatment for ADHD. Finally, *chmp7*$^{+/-}$ fish had significantly smaller total brain volumes than *chmp7*$^{+/+}$ fish. Overall, this study highlights a role for *CHMP7* in the neurodevelopment of ADHD, and demonstrates the utility of zebrafish mutant lines for modelling the functional effects of genes conferring risk to ADHD.

Introduction

Attention deficit hyperactivity disorder (ADHD) is a highly prevalent neuropsychiatric disorder affecting ~5% of school age children (Polanczyk et al., 2007). Abnormally high levels of activity, inattention, and impulsivity define the disorder, all of which can contribute to deficits in academic functioning and interpersonal relationships (Faraone et al., 2015). ADHD can persist well into adulthood (Faraone et al., 2006; Faraone and Biederman, 2005). In addition, changes in brain volume are common, with reductions in several regions often associated with ADHD (Hoogman et al., 2017).

The development of ADHD is strongly influenced by genetic factors. Heritability rates support the notion that around 80% of ADHD aetiology can be attributed to genetic factors (Faraone et al., 2005; Levy et al., 1997a). A number of significantly associated DNA variants have been identified via candidate gene studies (Faraone and Larsson, 2019; Hawi et al., 2015). Meta-analyses of multiple independent genome wide association studies (GWAS) pointed towards several variants showing evidence for association with ADHD , including rs2294123, which maps to charged multivesicular body protein 7 (*CHMP7* (Neale et al., 2010)). However, functional validation of the contribution of these variants to the development of the disorder is lacking. Most of the reported ADHD-associated variants detected via candidate gene and GWAS map to non-coding regions (Tong et al., 2016). Given the wide range of roles non-coding regions can play in gene expression, including post-transcription, and post-translational modification (Hill et al., 2010; Hoogendoorn et al., 2003; Mill et al., 2002; Moser et al., 2008; Németh et al., 2013), separating neutral non-coding ADHD-associated variants from potentially linked causative variants is a major challenge.

To tackle this, Tong et al., (2016) utilised a bioinformatic pipeline to functionally prioritise non-coding single nucleotide polymorphisms (SNPs) from the ADHD genetic literature, and performed a case-control association analysis on the prioritised SNPs. Tong and colleagues identified one SNP that was significantly associated with ADHD (G→T, rs2294123). This variant is mapped 14 bp upstream of the translational start site of *CHMP7*. Further, ADHD individuals homozygous for the ADHD risk allele (T), as well as heterozygous individuals, had significantly lower neurocognitive function than homozygous G individuals. In addition, healthy homozygous T individuals had significantly higher levels of ADHD symptoms than homozygous G individuals. Furthermore, quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis from post-mortem healthy brain samples showed that *CHMP7* transcript levels were reduced to 67% in homozygous T individuals, compared to homozygous G individuals (Tong et al., 2016). Overall, these findings suggest that the reduction in *CHMP7* transcripts contributes to ADHD phenotypes and warrants further investigation.

The functional role of CHMP7 in ADHD is not characterised. What is known about CHMP7, is that it plays an important role in the endosomal sorting pathway (Horii et al., 2006), nuclear envelope formation (Olmos et al., 2016), and has recently been implicated in spinal and bulbar muscular atrophy (Malik et al., 2019). CHMP7 also interacts with a member of the endosomal sorting complex required for transport (ESCRT) family, ESCRT-III. Several CHMP family members are part of ESCRT-III and have roles in cellular processes which are important in neurodevelopment (Sadoul et al., 2018), and have been implicated in neuropsychiatric disease (Chidambaram et al., 2019; Lau and Zukin, 2007; Mathews and Levy, 2019). These processes include the endosomal sorting pathway (CHMP1, CHMP2B, CHMP7 (Horii et al., 2006; Howard et al., 2001; Urwin et al., 2010)), dendritic branching and synaptic density (CHMP2B (Chassefeyre et al., 2015)). Further, ESCRT-III proteins are important for nuclear envelope formation (CHMP2A, CHMP4B, CHMP7 (Olmos et al., 2015, 2016)), and mice lacking CHMP5 (also a component of the ESCRT-III complex), die embryonically (Shim et al., 2006). Together, this evidence suggests that further characterisation of members of this family could help explain the mechanism of genetic risk for ADHD.

In order to examine the functional relevance of *CHMP7* to ADHD, we have adopted an animal model approach to examine if a reduction in *CHMP7* mRNA levels is sufficient to cause an ADHD phenotype, hyperactivity. Due to the large number of progeny, ease of genetic manipulation, conserved neurochemistry, and establishment of behavioural assays, zebrafish are becoming increasingly popular for the examination of neuropsychiatric disorder (Fontana et al., 2019, 2018; Sakai et al., 2018; Vaz et al., 2019). We generated a *chmp7* zebrafish mutant line using CRISPR/Cas9 genome editing, and hypothesised that *chmp7* heterozygous animals will mimic the reduction in transcripts caused by the ADHD-associated SNP, rs2294123. We demonstrate that *chmp7*$^{+/-}$ fish are more active than wildtype (*chmp7*$^{+/+}$) fish, and have decreased total brain volumes. Thus, we provide experimental validation for the association of *CHMP7* with ADHD. We also show that the increased activity levels in *chmp7*$^{+/-}$ fish can be significantly reduced through the application of the commonly used ADHD medication, methylphenidate.

Results

Zebrafish possess an orthologue of CHMP7, and it is expressed throughout early development

In order to examine how *CHMP7* could be functionally relevant to the development of ADHD, the zebrafish was selected as an animal model. The CHMP family is well conserved between humans and zebrafish. Zebrafish possess orthologues of all members of the human CHMP family (Figure 1), and zebrafish Chmp7 has a sequence identity of 51% and similarity of 70% to human CHMP7.

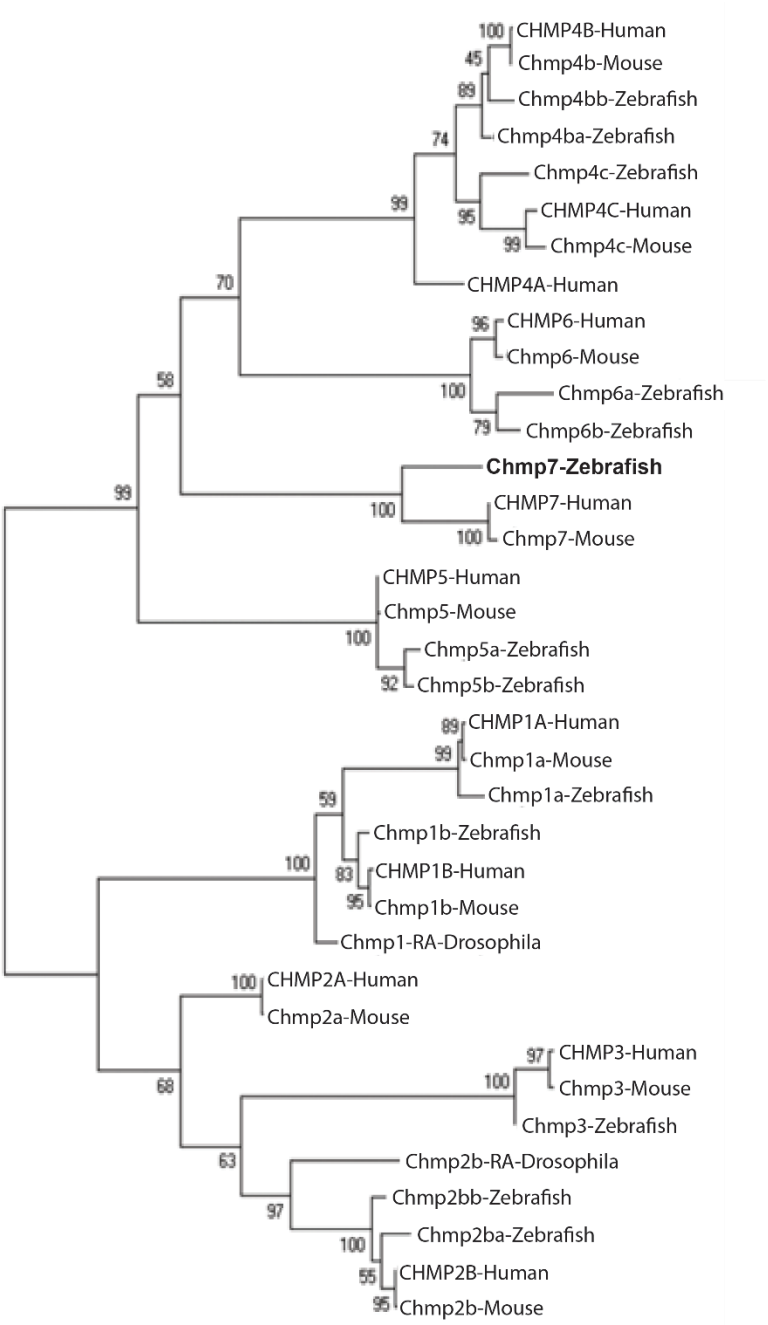


Figure 1. Phylogenetic tree of the CHMP family in humans, mice, *Drosophila*, and zebrafish. Zebrafish possess all seven members of the CHMP family known in humans and mice. Zebrafish Chmp7 is bolded. Evolutionary analyses were conducted in MEGA (Version 6 (Tamura et al., 2013)), using a Maximum Likelihood method based on the Le Gascuel 2008 model (Le and Gascuel, 2008). The tree with the highest log likelihood (-10065.9096) is shown.

To identify where and when *chmp7* is expressed in the zebrafish, *in situ* hybridisations and RT-PCR were performed on wildtype (*Tübingen*, TU) embryos. *In situ* hybridisations demonstrated that *chmp7* was expressed ubiquitously in the zebrafish embryo at 1 day post-fertilisation (dpf), with higher levels of expression in the brain, becoming more restricted to the head by 2 dpf. It remained visible only in the head and kidney at 6 dpf (Figure 2A). RT-PCR showed that *chmp7* was expressed at the 8-somite stage through to at least 5 dpf (Figure 2B).

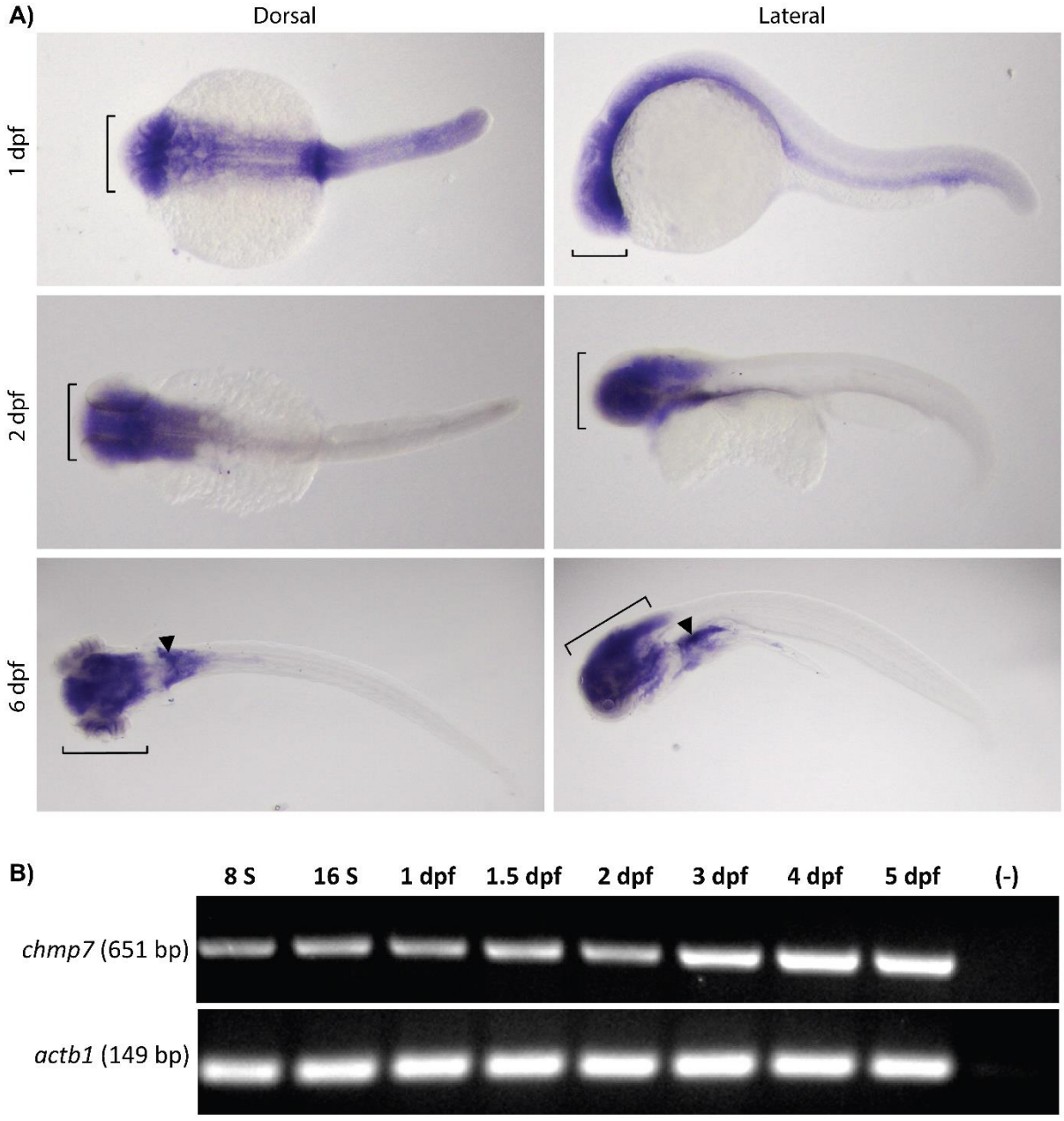


Figure 2. Characterisation of *chmp7* expression. **A)** Whole-mount *in situ* hybridisation on zebrafish embryos at 1 dpf, 2 dpf and 6 dpf, using DIG-labelled RNA probes specific to zebrafish *chmp7*. *chmp7* expression is ubiquitous with stronger expression in the head ([ ) at 1 dpf. Expression becomes more restricted to the head ([ ) by 2 dpf, and by 6 dpf is restricted to the head ([ ) and kidney (▼). **B)** RT-

PCR for *chmp7* at the 8-somite stage (8 S), 16-somite stage (16 S), 1 dpf, 1.5 dpf, 2 dpf, 3 dpf, 4 dpf, and 5 dpf. *chmp7* is expressed throughout early zebrafish development from the 8-somite stage through to 5 dpf. *actb1* was amplified as a positive control.

***chmp7* heterozygotes have reduced mRNA levels**

After confirming that *chmp7* was present and detectable during early zebrafish development, CRISPR/Cas9 genome editing was used to mutate *chmp7*, resulting in a 7 bp deletion mapped to exon 2 (Figure 3A). This resulted in the addition of 20 amino acids, and a stop codon following the 142nd amino acid (Figure 3B). This is predicted to result in the removal of the Snf7 domain, which is the main catalytic domain for CHMP7 and is responsible for its interaction with CHMP4B and thus, ESCRT-III (Horii et al., 2006).

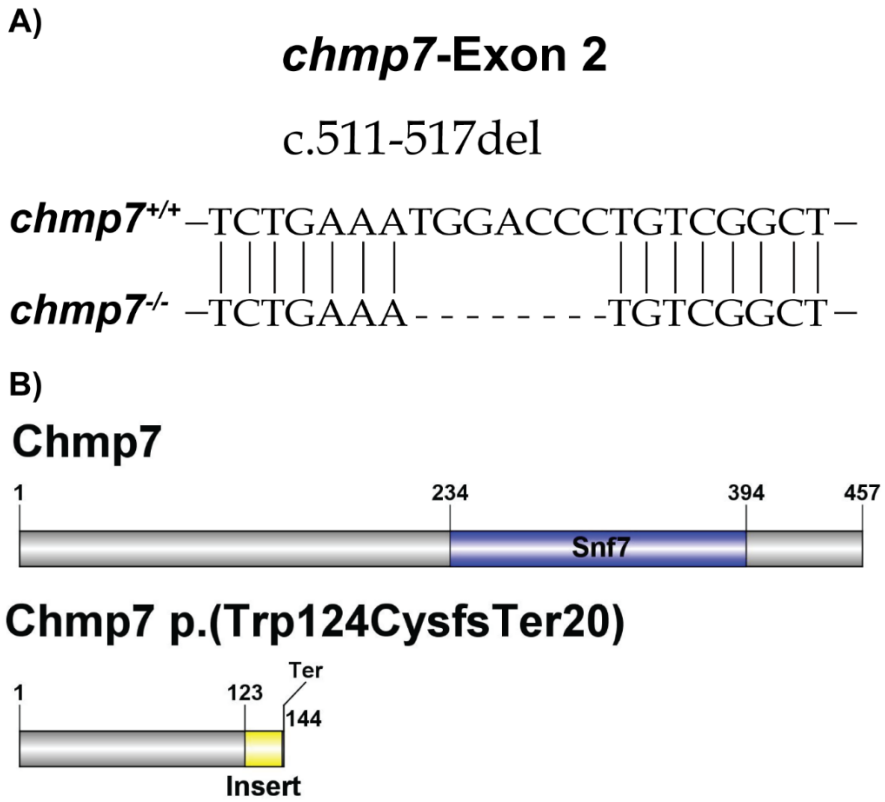


Figure 3. A) CRISPR/Cas9 genome editing was used to induce a mutation in *chmp7*, resulting in a 7 bp deletion at positions 511-517 in exon 2. **B)** Schematics of the Chmp7 wildtype and mutant proteins. Insertion of 20 amino acids at position 123 is followed by the addition of a premature STOP codon. This is predicted to result in the complete removal of the Snf7 domain from the Chmp7 mutant protein.

This truncation would be predicted to trigger nonsense mediated decay, and a loss of protein function, rather than the production of a truncated protein. In order to determine if *chmp7*$^{+/-}$ fish have a reduction in *chmp7* mRNA, thereby mimicking the reduction observed in individuals homozygous for the risk allele (T) of the ADHD-associated rs2294123 SNP, quantification was performed using qRT-PCR on cDNA from *chmp7*$^{+/+}$, *chmp7*$^{+/-}$, and *chmp7*$^{-/-}$ 6 dpf fish. One-way ANOVA analysis demonstrated a significant difference in mRNA levels between genotypes ($F = 14.41$ (2, 6), $p = .005$, two tailed, Figure 4). *chmp7*$^{+/-}$ fish had 53% of the total *chmp7* mRNA compared to *chmp7*$^{+/+}$ fish. Thus, this supports the use of *chmp7*$^{+/-}$ fish as a model of the rs2294123 homozygous risk allele.

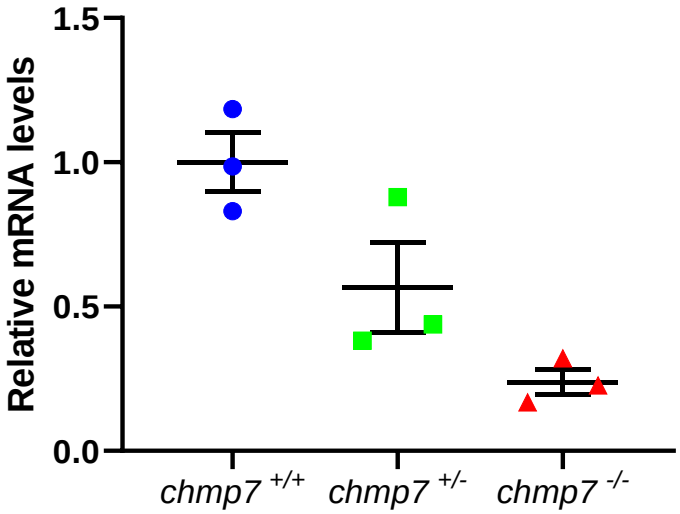


Figure 4. *chmp7* qRT-PCR on *chmp7*$^{+/+}$, *chmp7*$^{+/-}$, and *chmp7*$^{-/-}$ embryos. Heterozygotes had 53% of the total *chmp7* mRNA levels compared to wildtype. Comparison of mRNA levels using a one-way ANOVA demonstrated a significant difference between genotypes ($p = .005$). *actb1*, *18srRNA,* and *eef1α1* were used as reference genes. Data is from three biological replicates, and is normalised to *chmp7*$^{+/+}$ values. Centre lines = mean, error bars = +/- standard error of the mean (SEM).

chmp7 heterozygous embryos are hyperactive compared to wildtype siblings

Given that *chmp7*$^{+/-}$ fish possess similar reductions in *chmp7* mRNA levels as individuals homozygous for the *CHMP7* ADHD risk allele, we examined if a reduction in *chmp7* mRNA levels leads to a hyperactivity phenotype in developing zebrafish. The activity of *chmp7*$^{+/+}$ ($n = 153$) and *chmp7*$^{+/-}$ ($n = 131$) zebrafish embryos were tracked over a period of 24 hours from 158 hours post-fertilisation (hpf). *chmp7*$^{+/-}$ fish show increased activity compared to *chmp7*$^{+/+}$ fish over the entire experimental period

(Figure 5). To investigate this further, a mixed linear model analysis was performed. A significant main effect of genotype was observed (F = 4.69 (1, 291.11), p = .031, two tailed). The main effect of Zebrabox tracking system was significant, and was thus kept in the model. There was no significant interaction effect of genotype and time (F = .31 (23, 3438.08), p = 1.00, two-tailed). This demonstrates that *chmp7*$^{+/-}$ fish are consistently more active than *chmp7*$^{+/+}$ fish over the 24-hour period.

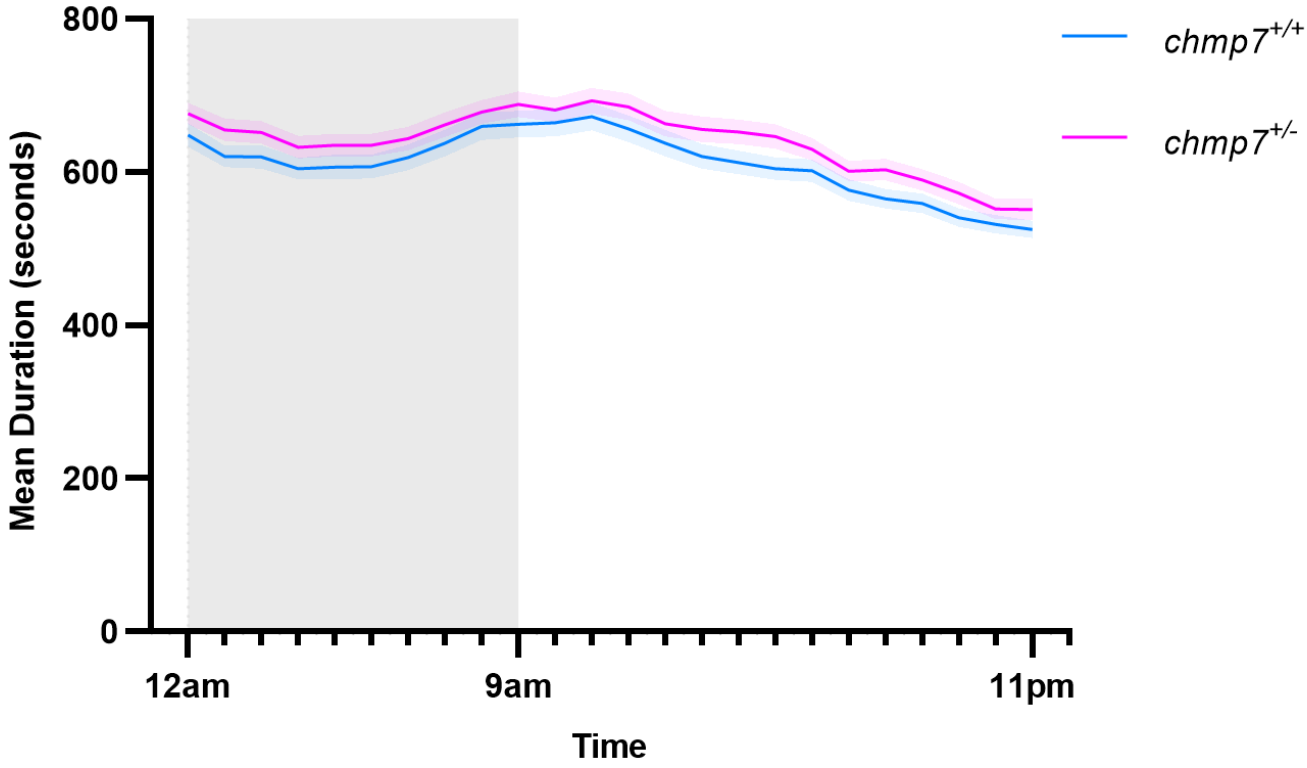


Figure 5. Activity analysis of *chmp7*$^{+/+}$ (n = 153) and *chmp7*$^{+/-}$ (n = 131) zebrafish 6 dpf embryos over a 24-hour period. *chmp7*$^{+/-}$ fish demonstrated significantly higher activity than *chmp7*$^{+/+}$ fish over the whole time period. The average time spent per genotype moving in each hour time period is displayed on the Y axis. Data is from three biological replicates. Error bars = +/- SEM.

Methylphenidate significantly reduces hyperactivity in *chmp7* heterozygotes

To determine if the increased activity seen in *chmp7*$^{+/-}$ fish could be ameliorated through the application of methylphenidate, the activity of *chmp7*$^{+/+}$ + dH_2O (n = 179), *chmp7*$^{+/-}$ + dH_2O (n = 160), *chmp7*$^{+/+}$ + methylphenidate (n = 171), *chmp7*$^{+/-}$ + methylphenidate (n = 166) zebrafish embryos was tracked over a period of 24 hours from 158 hpf. *chmp7*$^{+/-}$ + dH_2O fish demonstrated increased activity compared to *chmp7*$^{+/+}$ + dH_2O fish over the night period (Figure 6). However, this effect was

diminished in the *chmp7*$^{+/-}$ + methylphenidate fish. Mixed linear modelling demonstrated a significant interaction between genotype, drug treatment, and time (F = 1.60 (69, 8038.37), p = .001, two-tailed). The main effect of Zebrabox tracking system was significant, and was thus kept in the model. Given the significant interaction of genotype and treatment over time, we then investigated the differences between groups across time.

chmp7$^{+/-}$ + dH_2O fish demonstrated significantly higher activity than *chmp7*$^{+/+}$ + dH_2O fish across the majority of the night period (hour 3, p = .002; hour 4, p = .006; hour 5, p = .013; hour 6, p = .020; hour 7, p = .024; hour 8, p = .014). Application of methylphenidate gradually reduced the activity of *chmp7*$^{+/-}$ + methylphenidate fish until it was significantly less than *chmp7*$^{+/-}$ + dH_2O fish (hour 8, p = .044). In addition, *chmp7*$^{+/-}$ + methylphenidate fish were not significantly different from *chmp7*$^{+/+}$ + dH_2O fish for the majority of the night period, with the exception of hour 3 (p = .038). All pairwise comparisons were two tailed, performed using Bonferroni adjustments for multiple comparisons. Together this demonstrates that the application of methylphenidate was sufficient to significantly reduce the hyperactivity seen in *chmp7*$^{+/-}$ fish to levels comparable to that of wildtype.

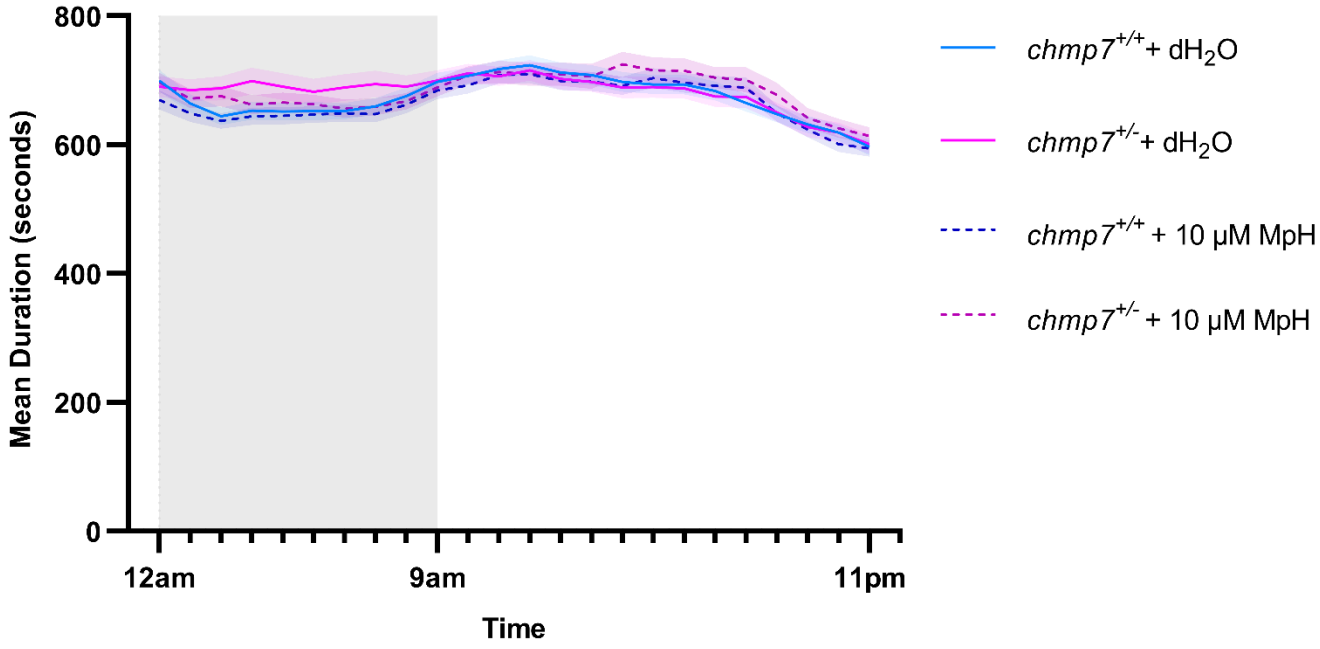


Figure 6. Activity analysis of *chmp7*$^{+/+}$ and *chmp7*$^{+/-}$ zebrafish 6 dpf embryos, both treated and untreated with 10 µM methylphenidate (MpH) or dH_2O over a period of 24 hours. *chmp7*$^{+/-}$ + dH_2O fish demonstrated significantly increased activity compared to *chmp7*$^{+/+}$ + dH_2O fish during the night period, but this difference was reduced in the methylphenidate treated *chmp7*$^{+/-}$ fish. The average

time spent per genotype moving in each hour time point is displayed on the Y axis. Data is from six biological replicates. Error bars = +/- SEM. MpH: Methylphenidate.

No difference between *chmp7* genotypes in juvenile and adult fish

ADHD diagnoses often persist into adulthood (Faraone and Biederman, 2005). To examine if reduction of *chmp7* leads to a hyperactivity phenotype in juvenile and adult zebrafish, the activity of *chmp7*$^{+/+}$ and *chmp7*$^{+/-}$ zebrafish was tracked over a period of 24 hours, from 41 days and 14 hours post-fertilisation for juveniles, and 83 days and 14 hours post-fertilisation for adults. There were no significant differences between genotypes over the entire experimental period for either juveniles (*chmp7*$^{+/+}$, *n* = 41, *chmp7*$^{+/-}$, *n* = 50, Figure 7A) or adults (*chmp7*$^{+/+}$, *n* = 30, *chmp7*$^{+/-}$, *n* = 36, Figure 7B).

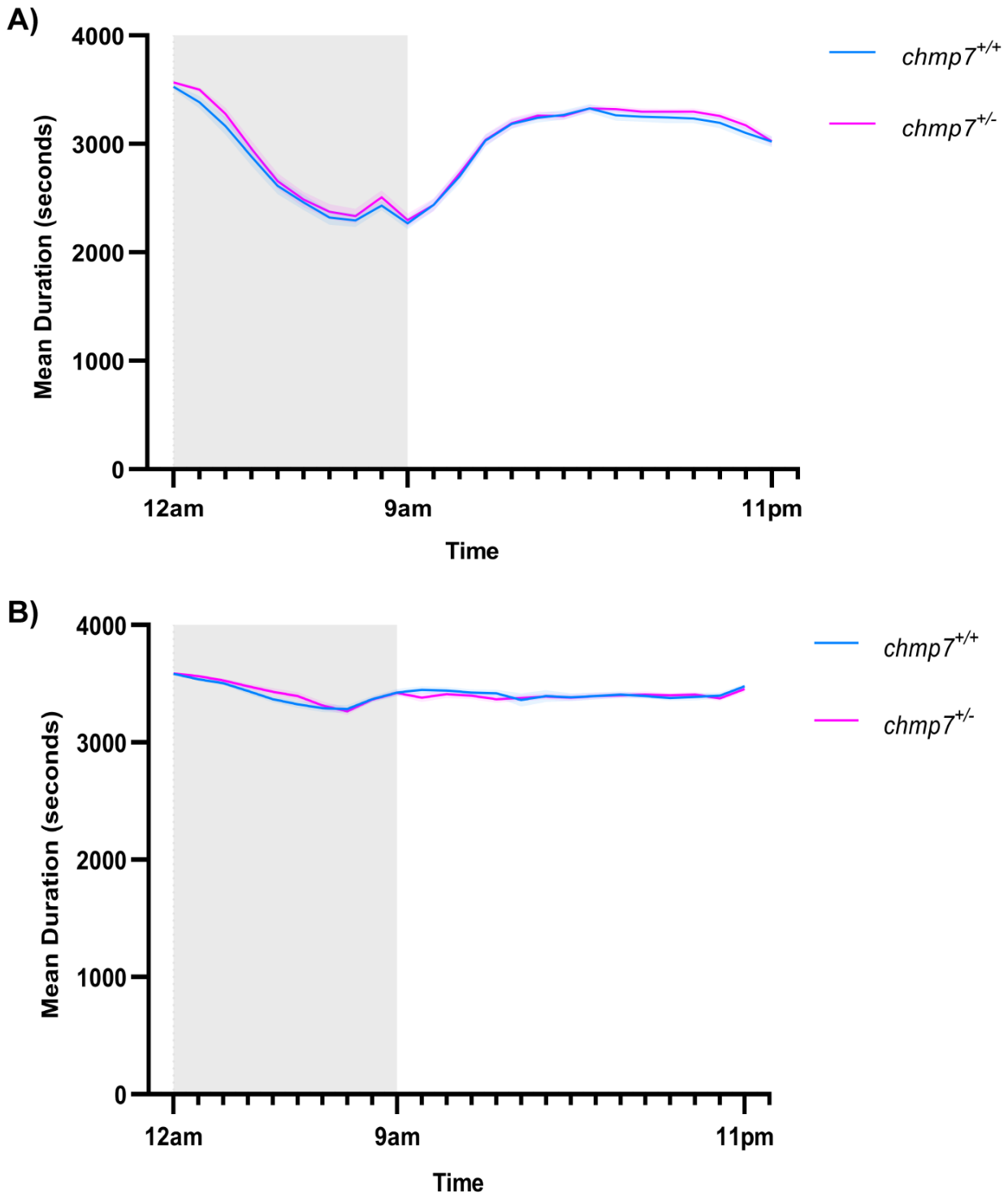
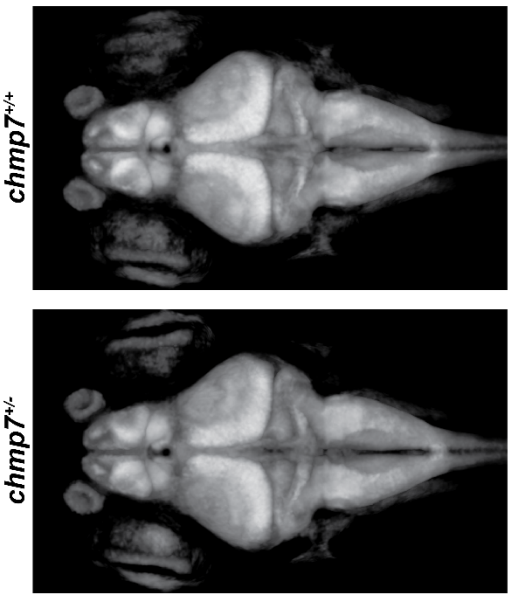


Figure 7. Activity analysis of *chmp7*$^{+/+}$ and *chmp7*$^{+/-}$ zebrafish at **(A)** 42 dpf (*chmp7*$^{+/+}$, *n* = 41; *chmp7*$^{+/-}$, *n* = 50) and **(B)** 84 dpf (*chmp7*$^{+/+}$, *n* = 30; *chmp7*$^{+/-}$, *n* = 36) over a period of 24 hours. No significant differences were seen between genotypes at both time points. The average time spent per genotype moving in each hour time point is displayed on the Y axis. Data is from five biological replicates. Error bars = +/- SEM.

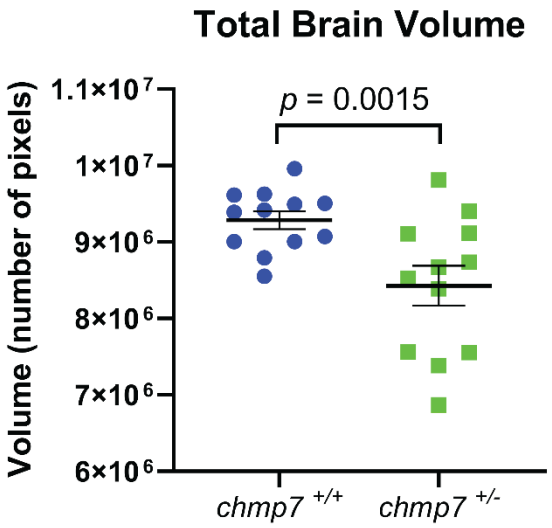
Analysis of brain volume in *chmp7* mutant lines

In order to investigate if a loss of *chmp7* leads to anatomical changes to the zebrafish brain, the heads of *Tg(HuC:eGFP);chmp7*$^{+/+}$ (*n* = 12) and *Tg(HuC:eGFP);chmp7*$^{+/-}$ (*n* = 12) zebrafish at 6 dpf were imaged live using confocal microscopy (Figure 8A). Confocal stacks were registered to a reference brain, then brain volumes were compared using cobraZ software (Gupta et al., 2018). Given the reductions in brain volumes reported in ADHD individuals (Hoogman et al., 2017), we expected decreased brain volume in *chmp7*$^{+/-}$ fish compared to *chmp7*$^{+/+}$ fish. After Bonferroni corrections for multiple comparisons, we observed a 9.2% total brain volume reduction in *chmp7*$^{+/-}$ fish compared to *chmp7*$^{+/+}$ fish (*t* = 3.01 (22), *p* = .0033, Cohen's *d* = 1.23, one-tailed, Figure 8B).

A)



B)



C)

Region	Average: *chmp7*$^{+/+}$	Average: *chmp7*$^{+/-}$	*p* value (one-tailed)
Total Brain Volume	9286411.42 (115960.56)	8427591.92 (261207.92)	0.0033*
Pallium (r005)	100701 (2137.95)	95892.92 (3019.91)	0.10
Pallium (r037)	76365.92 (1424.93)	71808.42 (2394.97)	0.058
Pallium (r076)	99504.58 (2134.86)	93779.08 (3345.13)	0.082
Pallium (r133)	36432.92 (620.33)	33704.08 (1138.39)	0.023
Pallium (r180)	65179.33 (1472)	61414.67 (2163.25)	0.082
Subpallium (r026)	79100.83 (1589.65)	76443 (2368.08)	0.18
Subpallium (r075)	67461.67 (1295.72)	63329.34 (2137.78)	0.056
Ventral Thalamus (r048)	84930.17 (1403.96)	80069.08 (2746.55)	0.065
Ventral Thalamus (r129)	63853.33 (1123.07)	58089.42 (1978.8)	0.0095
Thalamus (r138)	58817.75 (952.45)	53701.08 (1880.9)	0.012
Anterior commissure (r137)	80268.67 (1808.83)	77571.42 (2258.61)	0.18

Figure 8. A) Z projections of the average of whole brains from *Tg(HuC:eGFP);chmp7*$^{+/+}$ (*n* = 12) and *Tg(HuC:eGFP);chmp7*$^{+/-}$ (*n* = 12) fish. **B)** *chmp7*$^{+/-}$ fish had significantly reduced total brain volumes when compared to *chmp7*$^{+/+}$ fish. Data is from three biological replicates, and normalised to the

average of *chmp7*$^{+/+}$ fish. Centre lines = mean, error bars = +/- SEM. **C)** Average volumes of chosen brain volumes for *chmp7*$^{+/+}$ and *chmp7*$^{+/-}$ 6 dpf fish. Volumes are given in total number of pixels per region, with SEM in brackets. An asterisk indicates significance after corrections for multiple comparisons (corrected α = .0042). Brain regions in brackets can be visualised at http://vis.arc.vt.edu/projects/zbb/ (Tabor et al., 2019).

Discussion

This study is the first of its kind to functionally examine *CHMP7* using an animal model. It is also the first example of using CRISPR/Cas9 in a zebrafish model to functionally validate an ADHD-associated gene identified through a case-control GWAS. We show that *chmp7*$^{+/-}$ fish are an appropriate model of the *CHMP7* ADHD-associated SNP, with a similar reduction in mRNA levels to individuals homozygous for the *CHMP7* ADHD risk allele. *chmp7*$^{+/-}$ fish demonstrated consistently higher activity than *chmp7*$^{+/+}$ which was significantly ameliorated following the application of methylphenidate. In addition, *chmp7*$^{+/-}$ fish displayed a significant reduction in total brain volume when compared to *chmp7*$^{+/+}$ fish. These findings demonstrate that the decrease in *chmp7* mRNA levels can lead to common ADHD phenotypes in a zebrafish model.

Zebrafish are emerging as a promising model for neuropsychiatric disorders (Fontana et al., 2018). We demonstrate here the utility and versatility of zebrafish models to validate ADHD associations through analysis of swimming activity and brain volume. Firstly, the use of 24-hour locomotion assays at embryo, juvenile and adult stages allowed us to determine if the activity phenotype seen in 6 dpf *chmp7*$^{+/-}$ fish persisted into adulthood, demonstrating the use of zebrafish for testing the progression of ADHD phenotypes. Secondly, we applied methylphenidate in our 24-hour locomotion assays to determine if hyperactivity in *chmp7*$^{+/-}$ fish could be rescued by a mainstay ADHD drug treatment, which in turn provides insight into what neuromodulator systems are involved in this phenotype. We observed that methylphenidate rescued the hyperactive phenotype in *chmp7*$^{+/-}$ fish, suggesting decreased dopamine, or noradrenaline, signalling could be contributing to this phenotype. Given the fact that individuals have varying responses to methylphenidate due to genetic differences (Polanczyk et al., 2010), the use of zebrafish for testing ADHD-associated gene models for their response to drugs is beneficial for understanding drug response variability. Finally, zebrafish can be used to examine changes in brain volume commonly seen in ADHD individuals (Hoogman et al., 2017), providing anatomical evidence for ADHD-associations. Overall, our use of a zebrafish model to validate *CHMP7* showcases the strengths of zebrafish for understanding ADHD genetic associations.

The activity assays presented in Figure 5 and Figure 6 both identified a significant increase in activity in *chmp7*$^{+/-}$ fish compared to *chmp7*$^{+/+}$. However, in the vehicle treated control, essentially replicating the previous activity experiment, the increased activity was restricted to the majority of the night period. This could indicate that a loss of *chmp7* mRNA has stronger, more consistent impacts on sleep patterns rather than waking cognition. This is an interesting finding and is consistent with sleep impairments often seen in ADHD, as well as inter- and intra-subject variability in circadian rhythms (Becker, 2020). In addition, hyperactivity during night periods has been observed in both *DAT* and *latrophilin* pan-neuronal knockdown in *Drosophila*, which was suggested to be characteristic of dysregulation of dopamine signalling (van der Voet et al., 2016). Given our findings of differences in activity between genotypes during the night period, this is suggestive that the hyperactivity seen in *chmp7*$^{+/-}$ fish is in at least part due to disruptions to the dopamine signalling pathway.

The exact mechanism behind the increased activity and decreased total brain volume in the heterozygotes is of great interest. CHMP7's known interactions with ESCRT-III proteins is suggestive for number of roles. Defects in neuronal pruning are seen in knockdown (Loncle et al., 2015), loss of function (Sweeney et al., 2006), and dominant negative mutations (Belly et al., 2010), of ESCRT-III proteins. This provides strong evidence that the ESCRT-III complex is important for pruning, and functioning CHMP proteins are required for this complex to work appropriately. The increased activity phenotype of *chmp7*$^{+/-}$ fish could be attributed to a reduction of mature neural networks, which is consistent with the neurodevelopmental delay seen in ADHD individuals (reviewed in Dark et al., 2018). The results of the brain volume analysis suggest a global reduction in brain volume, as opposed to specific regional reductions. This is consistent with reductions in total volume seen in individuals with ADHD (Castellanos et al., 2002; Hoogman et al., 2017), and may be indicative of the disruption of early neurodevelopmental processes affecting development of the brain as a whole.

Previous work has also demonstrated that CHMP7 is involved in the endosomal sorting pathway (Horii et al., 2006). Disruption of the ESCRT-III complex leads to abnormal recycling of glutamate receptor subunits (Lee et al., 2011). A decrease in CHMP7 could therefore lead to retention of neurotransmitter membrane bound proteins in endosomes, or the inability to sort these proteins into multivesicular bodies. This could disrupt long-term depression (LTD) or long-term potentiation (LTP) of synapses (Park, 2018; Park et al., 2004), a common feature of many psychiatric disorders (Martella et al., 2018). The activation of LTP and LTD has been associated with increased and lowered synaptic density respectively (Engert and Bonhoeffer, 1999; Nägerl et al., 2004; Zhou et al., 2004), and decreased synaptic density is associated with reduced brain volume (Henstridge et al., 2016; Kovalenko et al., 2018). Although speculative, it is possible that the inability to maintain appropriate levels of receptors

present at the postsynaptic membrane could explain the decreased total brain volume seen in *chmp7*$^{+/-}$ fish.

This study does demonstrate some strengths and weaknesses of the use of zebrafish to model ADHD-associated genes. The use of behavioural assays in a vertebrate species, coupled with the large sample size produced by zebrafish, allows us to uncover subtle behavioural differences likely to be seen in ADHD-associations with small effect size. In addition, we can examine morphological differences relevant to ADHD, such as brain volume, and drug treatments are easily performed, once again in large numbers. This ability to test multiple phenotypes, with large power, demonstrates the utility of zebrafish for modelling ADHD associations.

One limitation of this study is that the lack of protein analyses on the mutant line means we cannot be certain that the reduction in *chmp7* transcript corresponds to a similar reduction in Chmp7 protein levels. Several antibodies were tested, and none found to be specific, a common issue when using zebrafish models as most epitopes are mammalian. Without an appropriate antibody, we have to predict the effects on the protein through sequencing of the DNA and quantification of RNA levels. While having protein analyses in addition would allow a more complete picture of the *chmp7* genetic model, our model demonstrates that a loss of mRNA is sufficient to result in ADHD-related phenotypes. In addition, analyses on human samples by Tong et al., (2016) only examined reduction in *CHMP7* mRNA, not protein. Therefore, we believe that a reduction in *chmp7* mRNA in zebrafish is sufficient to model what is seen in humans.

Another limitation is the lack of a second *chmp7* allele. A second allele in the *chmp7* gene, which led to a reduction in *chmp7* transcript to around 50%, would allow us to determine if the phenotypes we observed were in fact due to a reduction in *chmp7* mRNA rather than a potential off-target effect, or the result of an unknown interaction with the mutated protein produced in the original model. We can be fairly confident that the phenotypes we have observed stem from the *chmp7* mutation, as the use of F_3 and subsequent generation animals should eliminate the majority of non-linked off-target mutations. However, the use of a second allele would add another degree of certainty.

This study is the first to functionally examine the ADHD-associated gene *CHMP7* using an animal model. We have demonstrated, through the use of a CRISPR/Cas9 generated *chmp7* mutant line, that a reduction of *chmp7* mRNA can result in a hyperactivity phenotype in an animal model and, as such, functionally validates the association of *CHMP7* with ADHD. Additionally, this study demonstrates the utility of zebrafish models for validating future ADHD-associated variants, as well as for testing the efficacy of prescribed ADHD drug treatments.

Materials and Methods

Ethics

All fish were maintained in the Fish Core facility at Monash University under breeding colony license MARP/2015/004/BC. The creation of transgenic lines was approved by the School of Biological Sciences Animal Ethics Committee (BSCI/2015/07). All experiments were carried out on embryos of wildtype (*Tübingen*, TU) background.

Generation and genotyping of the *chmp7* mutant line

A guide RNA targeting exon 2 of *chmp7* (ENSDARG00000041362) was generated according to Gagnon et al., (2014). A 2.5 µl injection mixture containing 150 ng/µl of guide RNA, 5 µg/µl of Cas9 protein (PNA Bio), 20 µM of STOP cassette, 0.25 µl Phenol Red, 0.25 µl Cascade Blue (Molecular Probes), and ultra-pure H_2O up to a final volume of 2.5 µl, was injected into embryos at the one cell stage. Embryos were screened for successful injections at 24 hours post-fertilisation (hpf) using UV light to visualise Cascade Blue. Cascade Blue positive embryos were raised to adulthood. F_0 founders were identified by outcrossing to TU fish, DNA was collected from 15-20 offspring, then the pooled DNA was used as a template for amplification of the region surrounding the mutation via PCR. Polyacrylamide gel electrophoresis (PAGE) was used to visualise any heterodimers formed due to differences in the DNA sequence. Identified founders were then outcrossed to TU wildtype fish, and F_1 individuals were screened for the presence of mutations using PCR and gel electrophoresis. The mutation sequence was confirmed using Sanger sequencing. Experiments were carried out on fish of the F_3 and subsequent generations. Guide RNAs and primers for generating the *chmp7* mutant line are presented in Supplementary Table 1. Genotyping was performed using allele specific KASP fluorescence assays (Geneworks) once the mutation was sequenced.

Phylogenetic tree

CHMP protein sequences from human, mouse, zebrafish, and *Drosophila* were aligned using multiple sequence alignment software, ClustalX (Version 2.1 (Larkin et al., 2007))(Supplementary Table 2). The evolutionary history was inferred by using the Maximum Likelihood method based on the Le Gascuel 2008 model (Le and Gascuel, 2008). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using a Jones-Taylor-Thornton (JTT) model (Jones et al., 1992). A discrete Gamma distribution was used to model

evolutionary rate differences among sites (5 categories (+G, parameter = 5.0837)). The tree was calculated from the alignment using Molecular Evolutionary Genetics Analysis (MEGA) software version 6 (Tamura et al., 2013), and bootstrap values taken from 1000 repetitions using the Le Gascuel 2008 model.

Whole-mount *in situ* hybridisation

The *chmp7* template for the *in situ* probe was amplified from genomic DNA using the following primers: forward 5'-GGACTTCATCCTGCTGCTTC-3' and reverse 5'-TGTCGCACAGCTCCTGTATC-3', and cloned into pGEM-T Easy (Promega). Sequence orientation was determined via PCR using combinations of the above primers as well as pGEM-T Easy M13 forward 5'-TGTAAAACGACGGCCAGT-3' and reverse 5'-CAGGAAACAGCTATGACCATG-3' primers. The presence or absence of a band on a 1% Tris-acetate-EDTA agarose gel for each combination of primers indicated the orientation of the insert. Probe templates were amplified from the plasmid using the *chmp7* reverse and M13 forward primers, and digoxygenin riboprobes were generated using T7 RNA polymerase as previously described (Broadbent and Read, 1999). Whole-mount *in situ* hybridisations were carried out as outlined by Ruparelia et al., (2012).

Reverse transcription-PCR to examine expression of *chmp7*

RNA was extracted from wildtype embryos at the 8-somite stage, 16-somite stage, 1 day post-fertilisation (dpf), 1.5 dpf, 2 dpf, 3 dpf, 4 dpf and 5 dpf. Total RNA was isolated using TRIzol® reagent as described by the manufacturer (Sigma) and treated with DNAse (Promega) to remove genomic DNA. One µg of total RNA was reverse transcribed using the Superscript III first-strand synthesis kit (Invitrogen). PCR was performed using the following primers: forward *chmp7* 5'-GTGCGACACTCAGGATGAAG-3' and reverse 5'-TAATGGGGTGTGTCGGGACT-3', and actin beta 1 (*actb1*) was amplified as a positive control, using forward 5'-GCATTGCTGACCGTATGCAG-3' and reverse 5'- GATCCACATCTGCTGGAAGGTGG-3'. The PCR cycles were as follows: initial DNA denaturing step at 96°C for two minutes, 30 cycles of 96°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds, followed by a final 72°C step for 5 minutes. Twenty-five µl of the PCR product was run on a 1% Tris-acetate-EDTA agarose gel for visualisation.

Quantitative RT-PCR

RNA pooled from 20-25 embryos per genotype was extracted from *chmp7*$^{+/+}$, *chmp7*$^{+/-}$, and *chmp7*$^{-/-}$ embryos at 6 dpf, with a constant number of fish per genotype within each biological replicate. cDNA was prepared as described in the RT-PCR section above. qRT-PCR was performed using a Lightcycler 480 (Roche) and SYBR Green Master mix (Roche). An average of *actb1*, 18s ribosomal RNA (*18SrRNA*), and eukaryotic translation elongation factor 1 alpha 1 (*eef1α1*) expression values was used as a reference, as these genes are considered to be stably expressed throughout the body. qRT-PCR primers were as follows: *chmp7* forward 5'-GTGCGACACTCAGGATGAAG-3' and reverse 5'-TAATGGGGTGTGTCGGGACT-3', *actb1* forward 5'-GCATTGCTGACCGTATGCAG-3' and reverse 5'-GATCCACATCTGCTGGAAGGTGG-3', *18srRNA* forward 5'-TCGCTAGTTGGCATCGTTTATG-3' and reverse 5'-CGGAGGTTCGAAGACGATCA-3', *eef1α1* forward 5'-CTGGAGGCCAGCTCAAACAT-3', and reverse 5'-ATCAAGAAGAGTAGTACCGCTAGCATTAC-3'. Three technical replicates were completed for each biological replicate.

24-hour locomotion assay: 6 dpf

Embryos were collected in the morning between 9:00 am and 10:00 am and raised in petri dishes in a 14-hour day (9:00 am-11:00 pm) and 10-hour night (11:00 pm-9:00 am) cycle until 6 dpf to entrain the embryos to a day/night cycle. The tracking itself was performed in full darkness, to avoid confounding effects of light during the tracking, while still being able to record behavioural differences between day and night due to entrainment. Light intensity during the day was 300 lux ± 20 lux, while night was in full darkness. Embryos were fed 0.5 ml concentrated paramecium between 9:00 am and 10:00 am on day 5 and 6, and the water was changed between 2:00 pm and 4:00 pm each day. Between 2:00 pm and 4:00 pm on day 6, embryos were transferred to 24-well plates containing 1.5 ml of E3 embryo medium (5 mm NaCl, 0.17 mm KCl, 0.33 mm CaCl, 0.33 mm MgSO4 in water) per well to acclimatise to their new environment. Between 10:30 pm and 10:50 pm on day 6, plates were transferred to Zebraboxes (Viewpoint). At 10:50 pm the Zebraboxes were closed to allow the fish to habituate to the darkness for 10 minutes, and tracking began at 11:00 pm. The experiment ran for 24 hours and 30 minutes, after which videos of the tracking were collected for analysis, and embryos were then sacrificed and genotyped.

24-hour locomotion assay: drug treatment at 6 dpf

Locomotion assays for examining the effect of methylphenidate on 6 dpf fish were performed as above. However, at 10:00 pm on day 6, 150 µl of dH_2O (used as a vehicle control) or 100 µM of Threo-

methylphenidate hydrochloride (Tocris Bioscience) was added to wells containing 1.35 ml of E3 and the fish, to yield a final volume of 1.5 ml per well and a concentration of 10 µM of methylphenidate, as described by Lange et al., (2012). For each experiment, drug treatment and vehicle control application were randomised across the 24-well plate, and the investigator was blinded by a third party as to which solution was drug and which was control. Blinding was removed after initial mixed model tests were performed.

24-hour locomotion assay: 6 & 12 weeks post-fertilisation

Fish were fin clipped at 3 dpf, and DNA was extracted from the clipped tissue using 50 mM NaOH and 1 M Tris-HCl (pH 7.5), then the extracted DNA was used for genotyping. After which, fish were sorted according to genotype. Fish were then raised with less than 10 fish per tank under a day-night cycle of 12 hours per day (8:00 am-8:00 pm) and night (8:00 pm-8:00 am). Between 12:00 pm and 2:00 pm on day 41 and day 83, fish were transferred to individual tanks to acclimatise to their new environment. Between 7 pm and 7:50 pm on day 41 and 83, tanks were transferred to Zebracubes (Viewpoint, 9 tanks per system). At 7:50 pm the Zebracubes were closed to allow the fish to habituate to the darkness for 10 minutes, and tracking began at 8:00 pm. Positions of genotypes were randomised, and the investigator was blinded to genotype. Video tracking ran for 24 hours and 30 minutes in full darkness, after which videos of the tracking were collected for analysis, and fish were returned to their tanks.

Video analysis

Fish locomotion videos were analysed using Ethovision software (Noldus, version 14). Movement thresholds for all assays were: Moving, 1 mm/sec; Stopping, 0.75 mm/sec; Detection threshold, Dynamic Subtraction, Darker, 9. Voxel smoothing was used to remove small video jitters and errors in detection in 6 dpf embryos, with movements smaller than 0.04 mm and larger than 12 mm per frame excluded.

Locomotion assay statistical analysis

Locomotion data was processed using Microsoft Excel 2013 and analyses were performed using SPSS Statistics 26 (IBM). Data was ordered chronologically into 10-minute bins. Any time points at the end of videos that were less than 300 seconds were excluded. For each fish, activity data was summed by hour. Then, a normalised value for each hour was determined by comparing activity per hour to the

average activity value of all fish (calculated as the average activity of all fish from the respective replicate, for that respective hour). Genotyping data was then assigned to individual fish, and fish with ambiguous genotypes were removed from analysis. The data was then imported into SPSS. Data points from the 30 minutes past the initial 24 hours were excluded. Data was visualised using a line graph in GraphPad Prism Version 8.

To examine differences in activity between genotypes, a mixed linear model was used. For the 6 dpf, 42 dpf, and 84 dpf locomotion assays, main effects of time and genotype, and an interaction effect of time by genotype were used. A main effect of Zebrabox tracking system was used to determine if there were differences between Zebraboxes. If a significant ($p < .05$) main effect of Zebrabox tracking system was observed, it was kept in the model to account for any contributing variation. Repeated measures of time (hour) were modelled using a first order autoregressive variance structure. Random effects were defined as individual animals, grouped by genotype. A natural log transformation was applied to the normalised data to meet assumptions of normality which were checked by inspection of the residuals. F tests were performed using a maximal likelihood model, with Satterthwaite estimated degrees of freedom. For the drug treatment assays main effects of time, Zebrabox tracking system, treatment and genotype, and an interaction effect of time by genotype by treatment were used.

Confocal microscopy live imaging

chmp7$^{+/-}$ fish were crossed to a GFP-tagged HuC reporter (*HuC:eGFP* (Park et al., 2000)) and raised to adulthood. *Tg (HuC:eGFP);chmp7*$^{+/-}$ fish were then crossed to *chmp7*$^{+/-}$ fish, and embryos were raised in E3 medium containing 200 µM N-Phenylthiourea (PTU, Sigma) from 6 hours to suppress the formation of melanocytes, with changes in medium every 48 hours. Embryos were sorted for fluorescence at 2 dpf. At 3 dpf, fish were anesthetized using Tricaine methanesulfonate (Sigma) at a final concentration of 0.0016% in E3 embryo medium, and their tails were clipped. DNA was then extracted from the clipped tissue, and fish were sorted by genotype. At 6 dpf embryos were again anesthetised and set in 1% low melting agarose in clear E3 medium containing tricaine in 0.8 mm fluorinated ethylene propylene (FEP) tubing (Bola). Images were taken using a Thorlabs confocal microscope with an Olympus 20x water dipping NA 1.0 objective, pinhole 25 µm, 2.005 µm/pixel, step size = 1 µm, averaging = 16 frames.

Brain image registration and analysis

Image registration of live confocal stacks was done using Advanced Normalization Tools (ANTs) registration software (3.0.0.0), running on Monash University's MASSIVE computing cluster. Registered images were then analysed using cobraZ brain volume analysis software as described by Gupta et al., (2018). Zebrafish brain regions homologous to human regions known to have volume differences in ADHD individuals (Hoogman et al., 2017) were selected for analysis. Compared regions were the telencephalon (pallium, subpallium, anterior commissure), thalamus, ventral thalamus, and whole brain volume. Individual regions can be visualised at http://vis.arc.vt.edu/projects/zbb/ (Tabor et al., 2019).

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Conflict of Interest

The authors declare no conflicts of interest.

Functional investigation of an ADHD-GWAS associated gene, *DUSP6,* using a CRISPR/Cas9 zebrafish model

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Abstract

Attention deficit hyperactivity disorder (ADHD) is a highly prevalent neurodevelopmental disorder that has strong, lasting impacts on educational and interpersonal functioning in the life of affected individuals. A recent genome wide association study of ADHD has uncovered a significant association mapped to dual specificity phosphatase 6 (*DUSP6*). Using post mortem brain tissue we demonstrated that individuals homozygous for the *DUSP6* ADHD risk allele have significantly higher mRNA levels compared to those homozygous for the non-risk allele. In order to functionally examine *DUSP6*'s involvement in the development of ADHD phenotypes, we have used CRISPR/Cas9 genome editing to create a *dusp6* mutant zebrafish line, in which the mutant form of Dusp6 is expected to be non-functional. We observed that *dusp6*$^{-/-}$ fish did not show hyperactivity over 24 hours, nor any gross neurological changes. Overall, we demonstrate that the loss of Dusp6 function is not associated with activity or brain phenotypes in the zebrafish, however, investigation into the impact of the loss of Dusp6 on inattention phenotypes is recommended.

Introduction

Attention deficit hyperactivity disorder (ADHD) is a neuropsychiatric disorder that predominantly presents in childhood, with characteristic symptoms such as hyperactivity, inattention, and impulsiveness often leading to substantial deficits in academic functioning, and disruptions to interpersonal relationships (Faraone et al., 2015). The high prevalence of ADHD and its negative impacts on developing children have highlighted the importance of understanding the underlying causes of the disorder.

ADHD is a multifactorial disorder with a strong genetic component. The identification of both high heritability (76% (Faraone et al., 2005)) and high concordance rates of 80% (Levy et al., 1997b), has led to the search for genetic variants associated with ADHD. Meta-analysis of large case control genome wide association studies (GWAS), has allowed the discovery of ADHD risk loci (Demontis et al., 2019). Understanding how these variants play roles in biological mechanisms underlying ADHD symptoms is important for understanding the disorder. This therefore leads us to examine how ADHD-associated genes contribute to the development of common ADHD phenotypes, such as hyperactivity and reduced brain volume.

A common feature seen in ADHD individuals is abnormal neurodevelopment. Functional magnetic resonance imaging studies have demonstrated that individuals with ADHD have associated brain volume decreases in a number of regions, including, but not limited to, the accumbens, amygdala, hippocampus, caudate, and putamen, as well as intracranial brain volumes (Hoogman et al., 2017). These reductions are suggestive of disruptions to genes acting in neurogenesis and synaptogenesis, for which a number of ADHD-associated genes have been shown to play roles (Dark et al., 2018). In particular, recent significant ADHD-GWAS associations have known neurodevelopmental roles (Demontis et al., 2019), including *FOXP2*, *MEF2C*, *SEMA6D*, *PCDH7*, *PTPRF*, *SORCS3*, and *ST3GAL3* (reviewed in Dark et al., (2018)). This indicates that newly discovered associations require investigation for potential roles in neurodevelopment, which in turn provides excellent avenues for determining how these genes are contributing to the underlying mechanisms of ADHD.

One recent ADHD-GWAS association was mapped to dual specificity phosphatase 6 (*DUSP6* (Demontis et al., 2019)). The DUSP family of proteins are inhibitors of the mitogen-activated protein (MAP) kinase superfamily (Owens and Keyse, 2007), and have roles in cell proliferation and differentiation (Bermudez et al., 2010). *DUSP6* in particular has previously been associated with bipolar disorder (Lee et al., 2006), supporting its involvement in neurodevelopmental disorders. Expression of FLAG-tagged DUSP6 in Madin-Darby canine kidney (MDCK) cells leads to a stabilisation of the dopamine transporter (SLC6A3, also known as DAT1) at the plasma membrane, preventing its internalisation and degradation

(Mortensen et al., 2008). *SLC6A3* has been well established as an ADHD risk gene, and is thought to contribute to ADHD phenotypes through its regulation of dopamine levels in the synaptic cleft (Barr and Misener, 2008). In addition, a missense mutation in DUSP6 (rs13480726) in mice has been associated with decreased forebrain weight and a reduction in the area and length of the hippocampal and anterior commissures (Bin Liu, 2008). A reduction in forebrain size is consistent with ADHD, with decreases in brain volume in the prefrontal cortex associated with ADHD cases (Mostofsky et al., 2002; Sowell et al., 2003). Given the evidence supporting *DUSP6*'s role in neurodevelopment, and now it's association with ADHD, investigations into if disruptions to *DUSP6* can lead to a common ADHD phenotype such as hyperactivity are needed.

This study has functionally examined the potential role of *DUSP6* as an ADHD risk gene, using an animal model. We have adopted the use of zebrafish, as it is a promising model for examining behavioural and neurological phenotypes such as those seen in ADHD (Fontana et al., 2019, 2018; Sakai et al., 2018; Vaz et al., 2019). We first analysed *DUSP6* mRNA levels in post mortem human brain tissue with respect to the GWAS associated *DUSP6* single nucleotide polymorphisms (SNPs), identifying higher *DUSP6* mRNA levels associated with the ADHD risk allele. We predicted that higher DUSP6 expression contributes to ADHD development, potentially via increasing SLC6A3 stability and thus increasing dopamine reuptake from the synaptic cleft as described in Mortensen et al., (2008). We also hypothesised that a loss of DUSP6 could lead to a substantial increase of dopamine in the synaptic cleft, as a result of a destabilisation of SLC6A3 at the plasma membrane. This loss of SLC6A3 at the plasma membrane could also lead to a hyperactivity phenotype, similar to SLC6A3 knockout mice (Giros et al., 1996). In order to test if this hypothesis was true, we generated a *dusp6* mutant zebrafish line using CRISPR/Cas9 genome editing. Analysis of both *dusp6*$^{+/-}$ and *dusp6*$^{-/-}$ fish, demonstrated that *dusp6*$^{+/-}$ and *dusp6*$^{-/-}$ fish are not significantly different in activity to their *dusp6*$^{+/+}$ siblings. We have also analysed the potential effects of methylphenidate on *dusp6*$^{+/+}$, *dusp6*$^{+/-}$, and *dusp6*$^{-/-}$ fish to test if a synergistic increased activity phenotype is seen in *dusp6*$^{-/-}$ fish, but no significant differences were observed. Finally, we examined the brain size of *dusp6*$^{+/+}$ and *dusp6*$^{-/-}$ fish to examine if loss of Dusp6 function could lead to any neurodevelopmental changes, however, no significant differences were observed between genotypes.

Results

ADHD risk alleles of *DUSP6* associated SNPs demonstrate increased *DUSP6* mRNA levels

A bioinformatic prioritisation pipeline (Tong et al., 2016), was used to determine whether the *DUSP6* ADHD risk allele of rs1427829 (A) or another variant (in linkage disequilibrium (LD) with rs1427829)

was most likely to be functional. We observed that the G allele of rs10506971 (A→G) is in very strong LD with the A allele of rs1427829. rs10506971 also has strong functional prediction scores, above the described thresholds for likelihood of functionality (Kircher et al., 2014; Ritchie et al., 2014), using Genome Wide Annotation of Variants (GWAVA, TSS score = .64) and Combined Annotation Dependent Depletion (CADD, C-score = 16.22).

We then aimed to determine if there was an association between either of the two SNPs and *DUSP6* mRNA levels. Using cDNA synthesised from post mortem human brain samples, quantification of *DUSP6* mRNA levels was performed using qRT-PCR. Kruskal-Wallis tests demonstrated that both rs1427829 and rs10506971 are significantly associated with changes in *DUSP6* mRNA levels (χ^2 (2) = 7.92, p = .019, two-tailed, Figure 1A; χ^2 (2) = 7.54, p = .023, two-tailed, Figure 1B, respectively). Pairwise comparisons of the rs1427829 genotype demonstrated that AA individuals (n = 22, mean rank = 27.02) had significantly higher *DUSP6* mRNA levels, than GG individuals (n = 14, mean rank = 45.9; p = .015, two-tailed), using a Bonferroni adjustment for multiple comparisons (Figure 1A). Pairwise comparisons of the rs10506971 genotype demonstrated that GG individuals (n = 22, mean rank = 27.57) had significantly higher *DUSP6* mRNA levels, than AA individuals (n = 17, mean rank = 45.03; p = .019, two-tailed), using a Bonferroni adjustment for multiple comparisons.

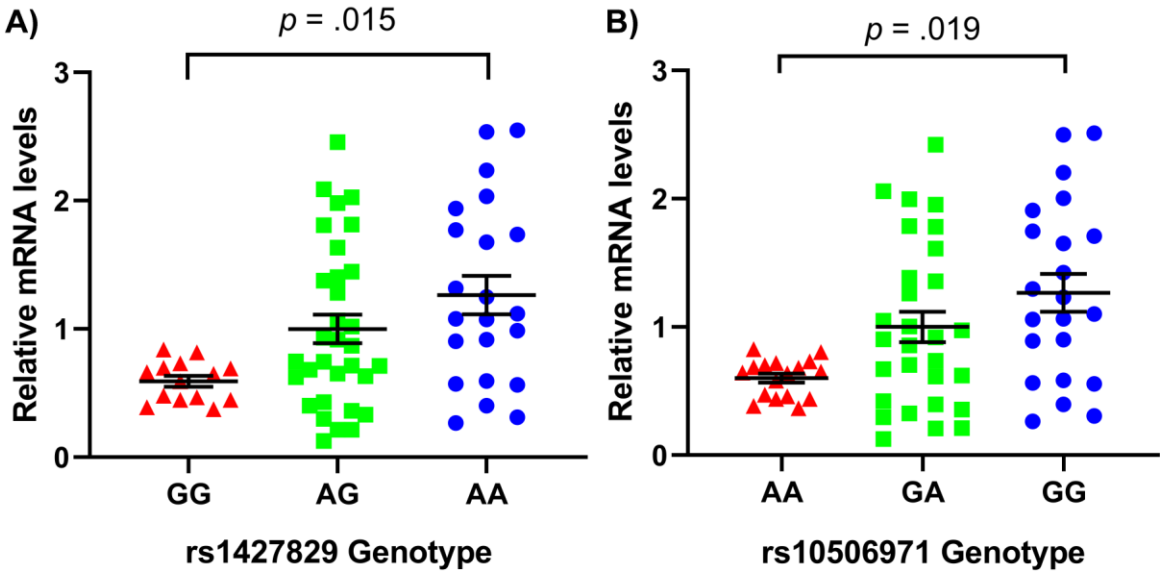


Figure 1. qRT-PCR examining *DUSP6* mRNA levels in human brain samples. **A)** Individuals homozygous for the A allele of the *DUSP6* ADHD-associated SNP rs1427829 demonstrated significantly higher mRNA levels than GG individuals. **B)** Individuals homozygous for the G allele of the functionally predicted SNP rs10506971 demonstrated significantly higher mRNA levels than AA homozygotes. The rs10506971 G allele is in very strong LD with the rs1427829 ADHD risk allele, A. *B2M* and *ACTB* were used as reference genes. Centre lines = mean, error bars = +/- standard error of the mean (SEM).

Zebrafish *dusp6* is expressed primarily in the brain during early development

In order to examine how *DUSP6* could be functionally relevant to the development of ADHD, the zebrafish was selected as an animal model. The DUSP family is well conserved between humans and zebrafish (Figure 2). The zebrafish DUSP6 orthologue (Dusp6), has a sequence identity of 80% and similarity of 86% to human DUSP6.

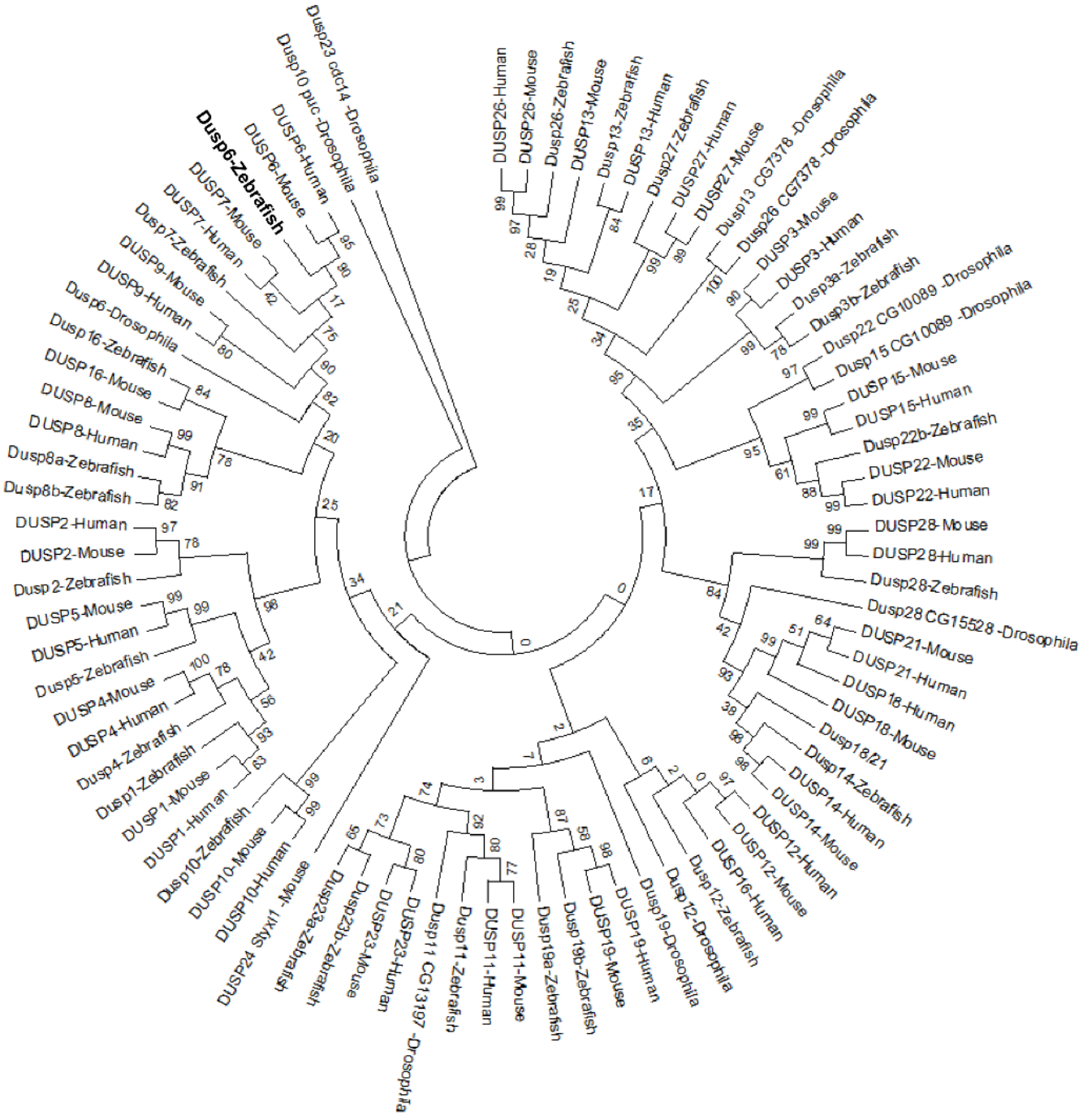


Figure 2. Phylogenetic tree of the DUSP family in humans, mice, *Drosophila*, and zebrafish. Zebrafish possess an orthologue of most members of the DUSP family known in humans and mice. The zebrafish Dusp6 is bolded. Evolutionary analyses were conducted in MEGA (Version 6 (Tamura et al., 2013)), using a Maximum Likelihood method based on the Jones-Taylor-Thornton (JTT) matrix-based model (Jones et al., 1992). The tree with the highest log likelihood (-13414.3500) is shown.

To determine where and when *dusp6* is expressed in the zebrafish, *in situ* hybridisations and RT-PCR were performed on wildtype (*Tübingen*, TU) embryos. *In situ* hybridisations demonstrated that *dusp6* is expressed in the forebrain, hindbrain (rhombomere 0 and 1), and the tip of the tail at 1 day post-fertilisation (dpf). The expression pattern becomes restricted to the head and the liver by 6 dpf (Figure 3A). RT-PCR shows that *dusp6* is expressed at the 8-somite stage through to at least 5 dpf (Figure 3B).

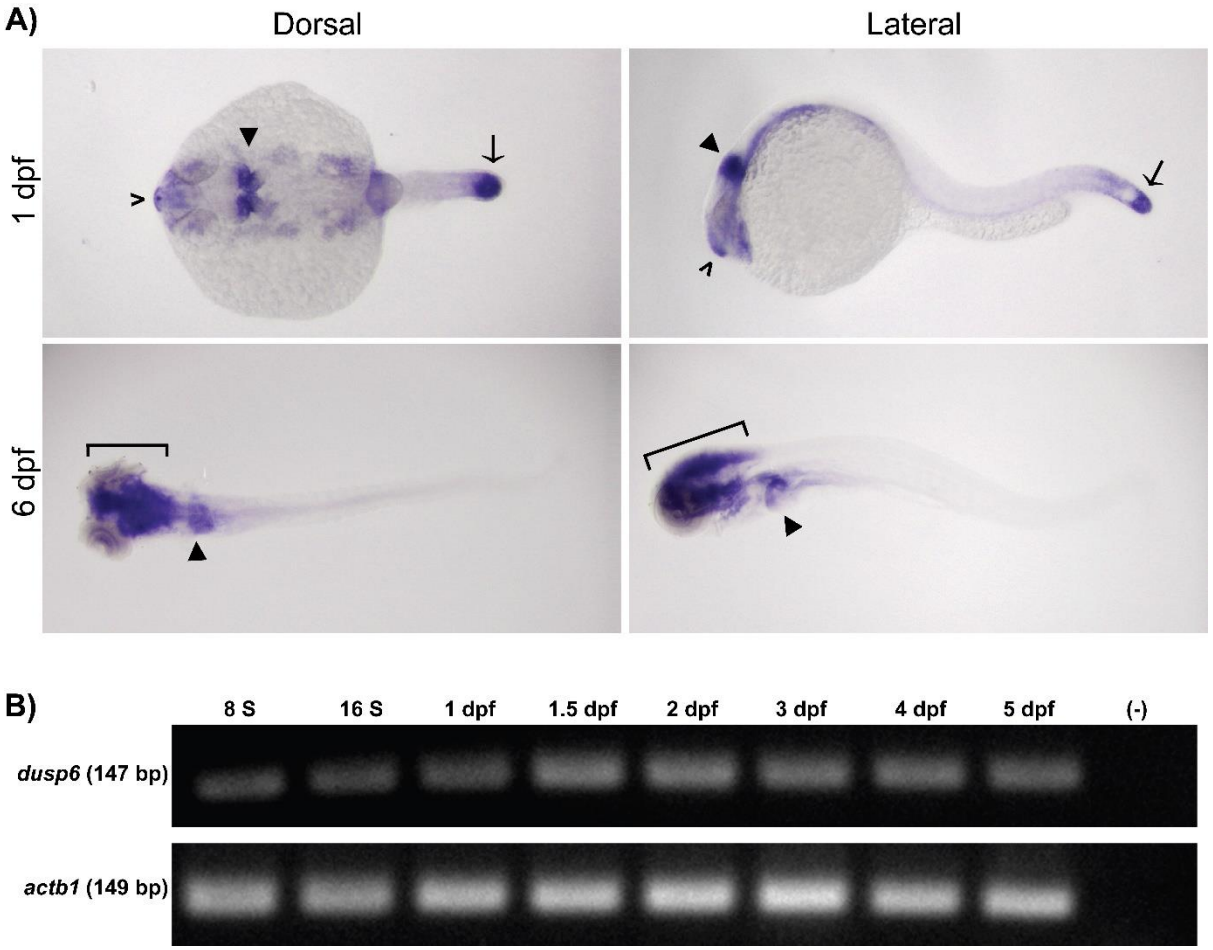


Figure 3. Characterisation of *dusp6* expression. **A)** Whole-mount *in situ* hybridisation on zebrafish embryos at 1 dpf, and 6 dpf, using DIG-labelled RNA probes specific to zebrafish *dusp6*. Expression is localised to the forebrain (ᵛ), hindbrain (rhombomere 0 and 1, ▼), tip of the tail (↓)at 1 dpf, and becomes restricted to the head ([ ) and liver (▲) by 6 dpf. **B)** RT-PCR for *dusp6* performed using zebrafish cDNA at the 8-somite stage (8 S), 16-somite stage (16 S), 1 dpf, 1.5 dpf, 2 dpf, 3 dpf, 4 dpf, and 5 dpf. *dusp6* is expressed as early as 8 S through to 5 dpf. *actb1* was amplified to act as a positive control.

Generation of a *dusp6* mutant line using CRISPR/Cas9

After confirming that *dusp6* is expressed and detectable during early zebrafish development, CRISPR/Cas9 genome editing was used to mutate *dusp6*. This resulted in a 13 bp deletion within exon 3 (Figure 4A) and consequently a frameshift resulting in the addition of 17 amino acids, and a stop codon after the 298th amino acid (Figure 4B). This would truncate the protein, interrupting the tyrosine-protein phosphatase domain which is the main catalytic domain of Dusp6, and would be expected to render it non-functional if the protein is produced. However, it would also be expected to trigger nonsense mediated decay, which would result in a loss of protein rather than the production of a truncated protein. In order to determine if *dusp6*$^{+/-}$ and *dusp6*$^{-/-}$ fish have a reduction in *dusp6* mRNA levels, indicative of nonsense mediated decay, we carried out qRT-PCR, and demonstrated that *dusp6*$^{+/-}$ and *dusp6*$^{-/-}$ fish have 58% and 40% of the total *dusp6* mRNA levels seen in *dusp6*$^{+/+}$ fish respectively, however One-way ANOVA demonstrated no significant differences between genotypes (F = 1.03 (2, 5), p = .42, two-tailed, Figure 5).

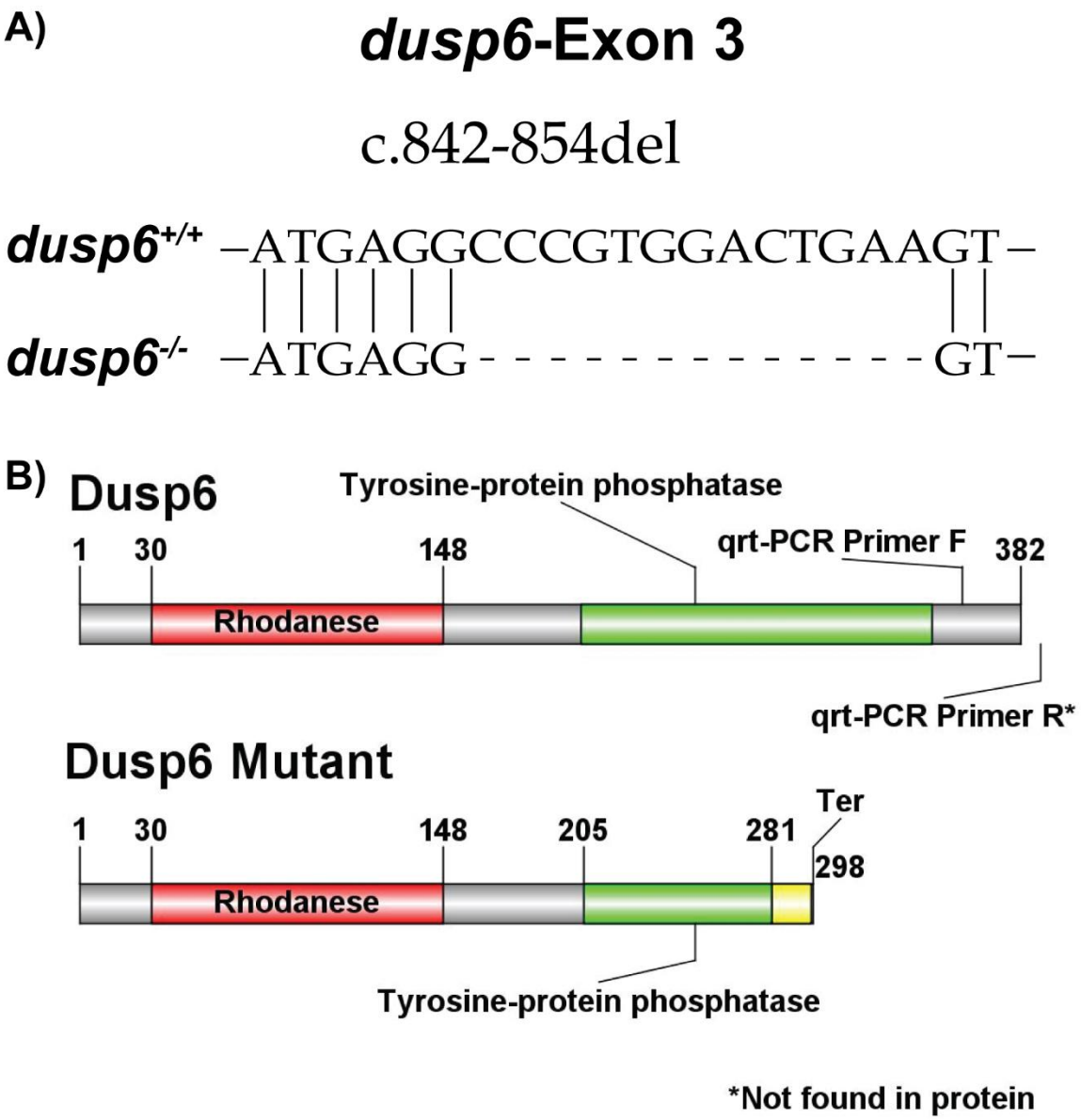


Figure 4. A) CRISPR/Cas9 genome editing was used to induce a mutation in *dusp6*, resulting in a 13 bp deletion at positions 842-854, in exon 3. **B)** Schematic representation of the Dusp6 mutant and wildtype proteins. Insertion of 17 amino acids at position 281 is followed by the addition of a premature STOP codon. This results in the tyrosine-protein phosphatase domain being truncated in the Dusp6 mutant protein.

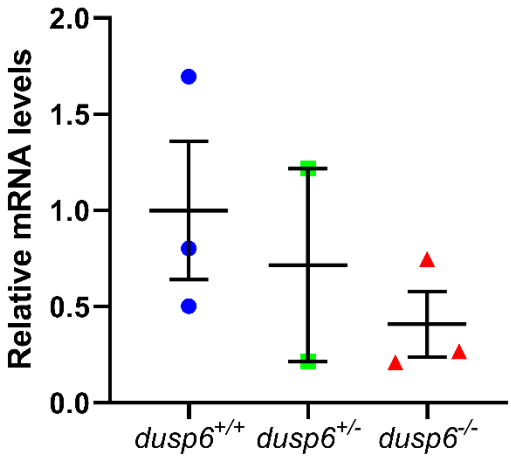


Figure 5. *dusp6* qRT-PCR of *dusp6*$^{+/+}$, *dusp6*$^{+/-}$, and *dusp6*$^{-/-}$ 6 dpf embryos. Heterozygotes demonstrated 58% of the total *dusp6* mRNA levels compared to wildtype, while mutants demonstrated 40% mRNA compared to wildtype. *actb1*, *18SrRNA,* and *eef1α1* were used as reference genes. Data is from two biological replicates for *dusp6*$^{+/-}$ embryos, and three biological replicates for *dusp6*$^{+/+}$, and *dusp6*$^{-/-}$ embryos. All data is normalised to *dusp6*$^{+/+}$ values. Centre lines = mean, error bars = +/- SEM.

dusp6 mutant and heterozygous fish have no significant differences in activity compared to wildtype siblings

Given that changes in *DUSP6* mRNA levels were significantly associated with the ADHD risk alleles in post mortem brain samples (Figure 1A & 1B), we decided to examine if a loss of Dusp6 function results in an activity phenotype. We tracked the activity of *dusp6*$^{+/+}$ (*n* = 182), *dusp6*$^{+/-}$ (*n* = 284), and *dusp6*$^{-/-}$ (*n* = 167) embryos over a period of 24 hours, from 158 hours post-fertilisation (hpf, Figure 6). To investigate any differences between genotypes, a mixed linear model analysis was performed. No significant main effect of genotype was found (F = .71 (2, 391.23), p = .49, two-tailed). The main effect of Zebrabox tracking system was significant, and was thus kept in the model. There was no significant interaction effect of genotype and time (F = .80 (46, 7385.82), p = .84, two-tailed), suggesting that loss of Dusp6 function does not result in a detectable activity phenotype.

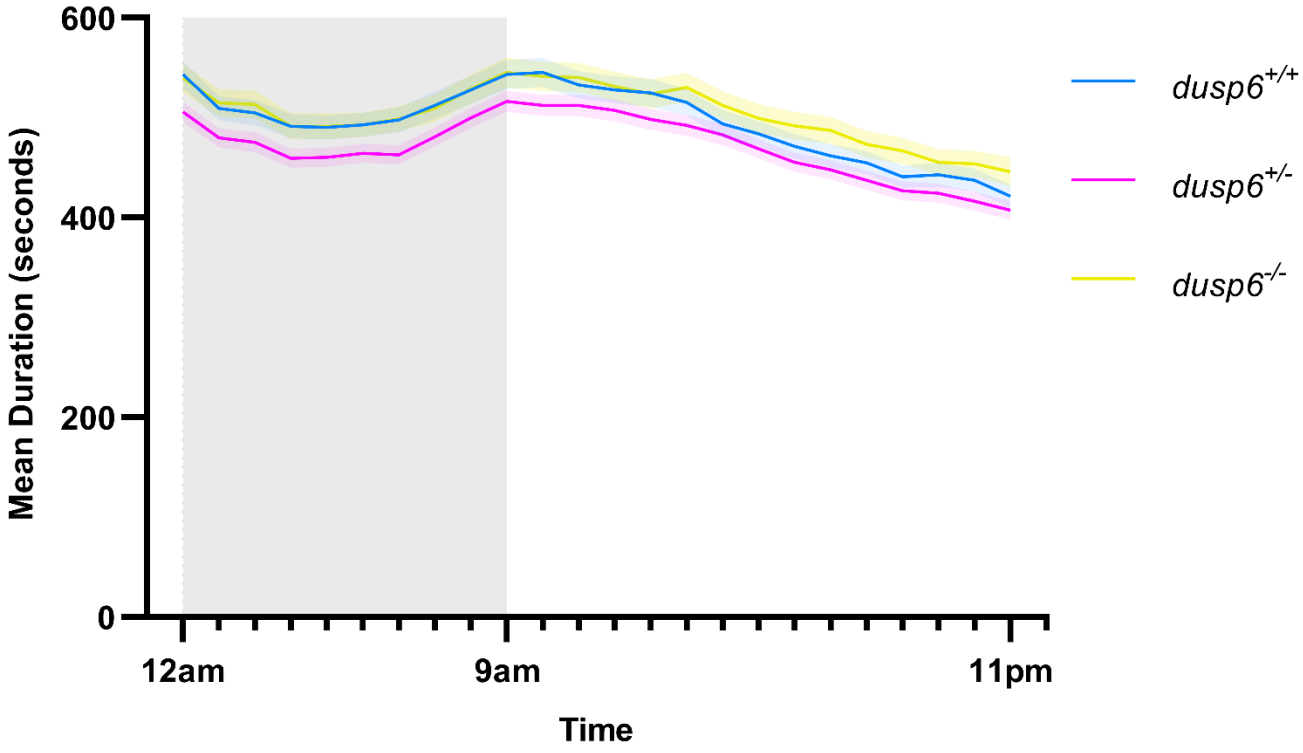


Figure 6. Activity analysis of *dusp6*$^{+/+}$ (*n* = 182), *dusp6*$^{+/-}$ (*n* = 284), and *dusp6*$^{-/-}$ (*n* = 167) zebrafish 6 dpf embryos over a period of 24 hours. There were no significant differences between genotypes over the whole time period. The average time spent per genotype moving in each hour time point is displayed on the Y axis. Data is from seven biological replicates, required to provide sufficient fish based on power calculations performed using data from the *chmp7* 24-hour 6 dpf locomotion assay (results chapter 1, Figure 5). Error bars = +/- SEM.

Methylphenidate does not show a significant synergistic effect with *dusp6* mutants

It has been shown that the overexpression of FLAG-tagged DUSP6 prevents internalisation of SLC6A3 in MDCK cells (Mortensen et al., 2008). Therefore, a reduction in functional Dusp6 could lead to reduced stability of SLC6A3 at the plasma membrane, and consequently less reuptake of dopamine at the synaptic cleft. It is possible that the loss of Dusp6 activity, plus the application of the SLC6A3 blocking drug methylphenidate could cause a synergistic increase in activity of *dusp6*$^{-/-}$ 6 dpf fish, due to an exacerbated reduction in dopamine reuptake. To determine if this was the case, the activity of *dusp6*$^{+/+}$ + dH_2O (*n* = 139), *dusp6*$^{+/-}$ + dH_2O (*n* = 318), *dusp6*$^{-/-}$ + dH_2O (*n* = 159), *dusp6*$^{+/+}$ + methylphenidate (*n* = 137), *dusp6*$^{+/-}$ + methylphenidate (*n* = 316) and *dusp6*$^{-/-}$ + methylphenidate (*n* = 145) zebrafish embryos was tracked over a period of 24 hours, from 158 hpf.

dusp6$^{-/-}$ + methylphenidate fish showed slightly increased activity compared to all other genotype + drug combinations during the day period of the experiment (Figure 7). However, using a mixed linear model demonstrated no significant interaction between genotype, drug treatment, and time (F = 1.056 (69, 14674.36), p = .38, two-tailed). The main effect of Zebrabox tracking system was significant, and was thus kept in the model. Overall, this demonstrated that the application of methylphenidate did not have a significant synergistic increase on the activity of *dusp6*$^{-/-}$ fish.

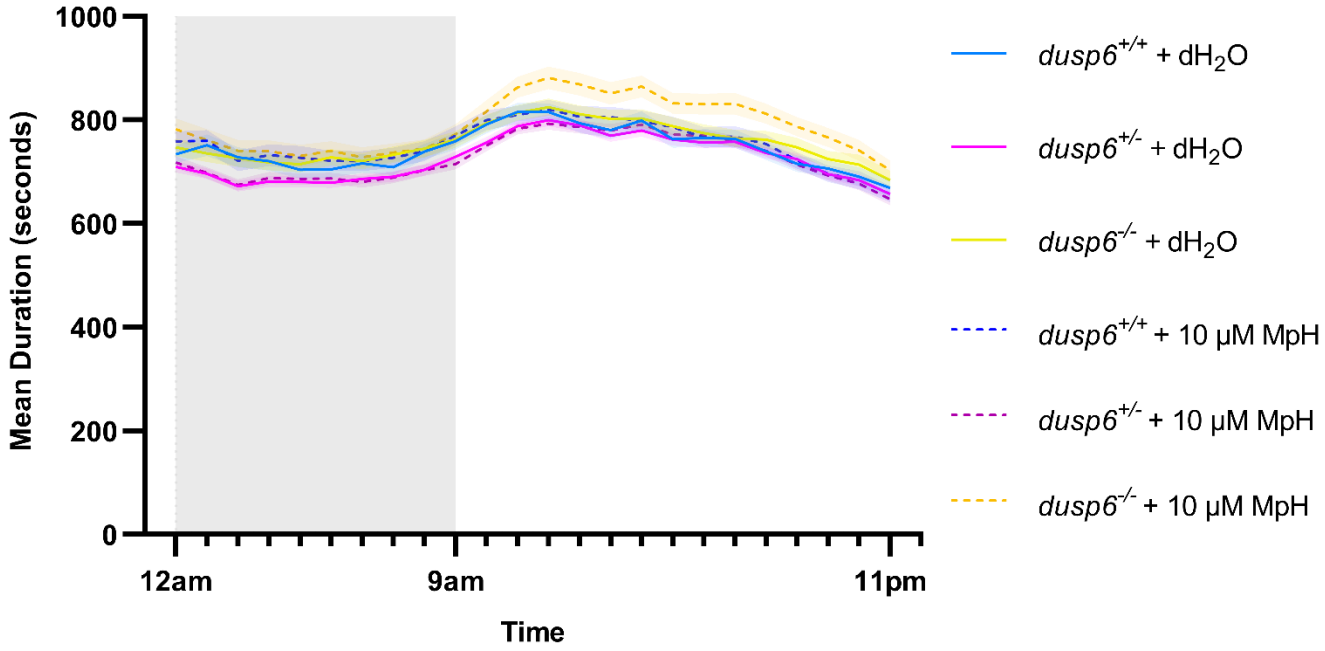


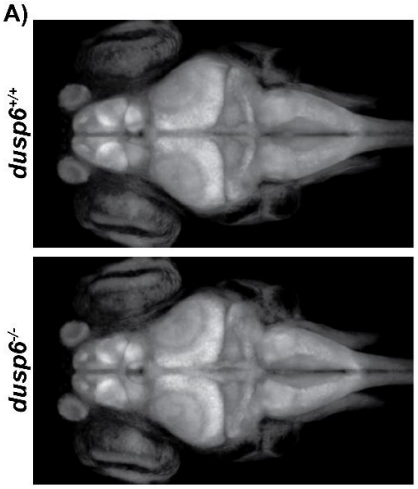
Figure 7. Activity analysis of *dusp6*$^{+/+}$, *dusp6*$^{+/-}$, and *dusp6*$^{-/-}$ zebrafish 6 dpf embryos, both treated and untreated with 10 µM methylphenidate or dH_2O over a period of 24 hours. There were no significant differences between genotype and treatment groups over the whole time period. The average time spent per genotype moving in each hour time point is displayed on the Y axis. Data is from eleven biological replicates, based on power calculations performed using data from the *chmp7* 24-hour 6 dpf locomotion assay (results chapter 1, Figure 5). Error bars = +/- SEM. MpH: Methylphenidate.

Analysis of brain volume in the *dusp6* mutant line

In order to investigate if the loss of Dusp6 activity leads to volume changes in the zebrafish brain, the heads of *Tg(HuC:GFP);dusp6*$^{+/+}$ (n = 12) and *Tg(HuC:GFP);dusp6*$^{-/-}$ (n = 12) zebrafish at 6 dpf were imaged live using confocal microscopy (Figure 8A). Confocal stacks were registered to a reference brain using ANTS software, then analysed using cobraZ software (Gupta et al., 2018). Given the established reductions in brain volume reported in ADHD individuals (Hoogman et al., 2017),

decreased brain volume would be expected in *dusp6*$^{-/-}$ fish compared to *dusp6*$^{+/+}$ fish. However, there were no significant differences between *dusp6*$^{+/+}$ and *dusp6*$^{-/-}$ fish for any of the brain regions analysed after Bonferroni corrections for multiple comparisons (Figure 8B).

A)



B)

Region	Average: *dusp6*$^{+/+}$	Average: *dusp6*$^{-/-}$	*p* value (one-tailed)
Total Brain Volume	8714177	8969383	0.19
Pallium (r005)	1.1052306	1.1144282	0.28
Pallium (r037)	0.8244671	0.8440303	0.06
Pallium (r076)	1.1019094	1.0925153	0.32
Pallium (r133)	0.4046711	0.3985727	0.14
Pallium (r180)	0.7075654	0.7129868	0.33
Subpallium (r026)	0.8745659	0.8630352	0.24
Subpallium (r075)	0.7388633	0.7361072	0.39
Ventral Thalamus (r048)	0.9271026	0.9382811	0.25
Ventral Thalamus (r129)	0.683161	0.6911818	0.20
Thalamus (r138)	0.629397	0.6325379	0.40
Anterior commissure (r137)	0.8789577	0.8881859	0.33

Figure 8. A) Z projections of the average of whole brains from *Tg(HuC:GFP);dusp6*$^{+/+}$ (*n* = 12) and *Tg(HuC:GFP);dusp6*$^{-/-}$ (*n* = 12) fish. **B)** Volume analysis of zebrafish brain regions homologous to those found to be decreased in ADHD individuals. No significant differences were detected between *dusp6*$^{+/+}$ and *dusp6*$^{-/-}$ fish. Values are percentage of total brain, except for total brain volume, which is total number of pixels. Brain regions in brackets can be visualised at http://vis.arc.vt.edu/projects/zbb/ (Tabor et al., 2019).

Discussion

This study is the first of its kind to functionally examine a significant ADHD-GWAS association. We demonstrated that the ADHD risk alleles of two SNPs, rs1427829 and rs10506971, are significantly correlated with increased expression of *DUSP6* mRNA from post mortem human brain tissue. However, a *dusp6* mutant model showed that loss of Dusp6 function is not associated with an activity phenotype. In addition, the application of methylphenidate did not lead to a significant synergistic increase in the activity levels of *dusp6*$^{-/-}$ fish. Finally, analysis of telencephalon, thalamus, ventral thalamus, as well as whole brain volume in *dusp6*$^{-/-}$ fish revealed no significant differences.

This study demonstrates that disruption of Dusp6 does not have a detectable impact on neurodevelopment that is manifested through changes in activity or brain volume. This is somewhat surprising given the reductions in forebrain volume and hippocampal and anterior commissure lengths

in mice with the missense mutation, rs13480726 (Bin Liu, 2008), which maps to the kinase interaction motif (KIM) in exon one of DUSP6 (Owens and Keyse, 2007). The KIM is responsible for the enzymatic specificity of DUSP proteins (Nichols et al., 2000; Tanoue et al., 2000). We did not observe any significant alterations to brain volume, and in our mutant line the KIM was intact, though the phosphatase domain was disrupted and nonsense mediated decay induced. This suggests that disruption of DUSP6's KIM could have a greater impact on brain volume than loss of the protein.

However, while loss of Dusp6 function in the mutant did not lead to any detectable ADHD phenotypes it is possible that an overexpression of Dusp6 could demonstrate an activity phenotype, seeing as higher *DUSP6* expression was associated with the *DUSP6* ADHD risk allele (Figure 1). In support of this, when DUSP6 is expressed alongside SLC6A3 in *Xenopus* oocytes, there is increased dopamine reuptake compared to expression of SLC6A3 alone (Mortensen et al., 2008). Therefore, it is possible that increased DUSP6 could lead to an ADHD phenotype through increasing dopamine reuptake, and thus, less synaptic dopamine. This would fit well with the hypodopaminergic hypothesis of the disorder (Levy, 1991). If increased DUSP6 does reduce levels of synaptic dopamine, then use of methylphenidate could rescue this effect, and be an effective treatment for individuals with higher DUSP6 expression.

The lack of an activity phenotype in our zebrafish model does not rule out *DUSP6* as being functionally relevant to the development of ADHD. In this study, we focused on examining an activity phenotype via a 24-hour locomotion assay. However, individuals with ADHD can present with predominantly inattentive phenotypes, rather than hyperactive phenotypes. Despite the lack of consensus over a true measure of attention in zebrafish (Choo and Shaikh, 2018), there are options for using zebrafish to examine learning as correlates of attention (Echevarria et al., 2011), as well as assays looking at visual attention (Braida et al., 2014), and complex operant tasks (Parker et al., 2012). Investigating potential deficits in attention in *dusp6* mutant adults could indicate if loss of Dusp6 function contributes to a different ADHD subtype.

There were some limitations with this study. Similar to the previous chapter, without protein analyses we cannot be certain that the reduction in *dusp6* transcript corresponds to a similar reduction in Dusp6 protein levels. While the Rhodanese domain was expected to be intact, the tyrosine-protein phosphatase domain, which is the main catalytic domain of Dusp6, would be disrupted by the truncation. Thus, we would expect the protein to be non-functional."

Another limitation is the lack of a *dusp6* overexpression model to model the changes seen in the human samples. An overexpression model would not trigger genetic compensation from other members of the Dusp family (such as Dusp3 (Todd et al., 1999)), which is potentially the cause of the

lack of phenotype in the *dusp6* mutant line. However, the random integration into the genome seen in transgenic overexpression can lead to non-specific effects In addition, the endogenous *dusp6* promoter would be required, to ensure the gene's time and region-specific expression. Without this, the transgenic expression of *dusp6* could result in unintended, non-specific timing that would not correctly model the expression changes seen in human samples. Through reducing Dusp6 function and mRNA, we can be more certain that we are examining the effects of influencing *dusp6*, rather that other potential unintended targets.

Overall, we have functionally examined the significant ADHD-GWAS hit mapped to *DUSP6* using a zebrafish model, and have demonstrated that the loss of Dusp6 function is not associated with changes in activity or brain volume. However, our analysis was restricted to exploring the effect of Dusp6 disruption on the hyperactivity phenotype and not attention deficits. Therefore, further work is required to examine attention, which will allow us to fully determine if a *dusp6* mutation could lead to common ADHD phenotypes.

Materials and Methods

Ethics

Fish maintenance and handling were carried out as per standard operating procedures approved by the Monash Animal Services Ethics Committee and the creation of transgenic lines approved by the School of Biological Sciences Animal Ethics Committee (BSCI/2015/07). All experiments were carried out on embryos of wildtype (*Tübingen*, TU) background. All fish were maintained in the Fish Core facility at Monash University under breeding colony license MARP/2015/004/BC.

Prioritisation of *DUSP6* functional SNPs

Determination of the SNP in linkage disequilibrium with the ADHD-GWAS associated SNP that showed the highest likelihood for functionality was completed using the bioinformatic pipeline previously described in Tong et al., (2016).

Generation and genotyping of the *dusp6* mutant line

A guide RNA targeting exon 3 of *dusp6* (ENSDARG00000070914), was generated according to the protocol outlined by Gagnon et al., (2014). A 2.5 µl injection mixture containing 150 ng/µl of guide RNA, 5 µg/µl of Cas9 protein (PNA Bio), 20 µM of STOP cassette, 0.25 µl Phenol Red, 0.25 µl Cascade

Blue (Molecular Probes), and ultra-pure H_2O up to a final volume of 2.5 µl, was injected into embryos at the one cell stage. Embryos were screened for successful injections at 24 hours post-fertilisation (hpf) using UV light to visualise Cascade Blue. Cascade Blue positive embryos were raised to adulthood. F_0 founders were identified by outcrossing to TU fish, DNA was collected from 15-20 offspring, then the pooled DNA was used as a template for amplification of the region surrounding the mutation via PCR. Polyacrylamide gel electrophoresis (PAGE) was used to visualise any heterodimers formed due to differences in the DNA sequence. Identified founders were then outcrossed to TU wildtype fish, and F_1 individuals were screened for the presence of mutations using PCR and gel electrophoresis. The mutation sequence was confirmed using Sanger sequencing. Experiments were carried out on fish of the F_3 and subsequent generations. Guide RNAs and primers for generating the *dusp6* mutant line are presented in Supplementary Table 1. Genotyping was performed using allele specific KASP fluorescence assays (Geneworks) once the mutation was sequenced.

Phylogenetic tree

DUSP protein sequences from human, mouse, zebrafish, and *Drosophila* were aligned using multiple sequence alignment software, ClustalX (Version 2.1 (Larkin et al., 2007))(Supplementary Table 2). The evolutionary history was inferred by using the Maximum Likelihood method based on the Jones-Taylor-Thornton (JTT) matrix-based model (Jones et al., 1992). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 4.0111)). All positions with less than 80% site coverage were eliminated. That is, fewer than 20% alignment gaps, missing data, and ambiguous bases were allowed at any position. The tree was calculated from the alignment using Molecular Evolutionary Genetics Analysis (MEGA) software version 6 (Tamura et al., 2013) and bootstrap values taken from 1000 repetitions using the JTT model.

Whole-mount *in situ* hybridisation

The *dusp6* template for the *in situ* probe was amplified from genomic DNA, using the following primers: forward 5'- TGCTTTTGCAATCGACATTC-3' and reverse 5'- CGTCCTTCATTCTCCTCAGC-3', and cloned into pGEM-T Easy (Promega). Sequence orientation was determined via PCR using combinations of the above primers as well as pGEM -T Easy M13 forward 5'-TGTAAAACGACGGCCAGT-

3' and reverse 5'-CAGGAAACAGCTATGACCATG-3' primers. The presence or absence of a band on a 1% Tris-acetate-EDTA agarose gel for each combination of primers indicated the orientation of the insert. Probe templates were amplified using the *dusp6* reverse and M13 forward primers, and digoxygenin riboprobes were generated using T7 RNA polymerase as previously described (Broadbent and Read, 1999). Whole-mount *in situ* hybridisations were carried out as outlined by Ruparelia et al., (2012).

Reverse transcription-PCR to examine expression of *dusp6*

RNA was extracted from wildtype embryos at the 8-somite stage, 16-somite stage, 1 day post-fertilisation (dpf), 1.5 dpf, 2 dpf, 3 dpf, 4 dpf and 5 dpf. Total RNA was isolated using TRIzol® reagent as described by the manufacturer (Sigma) and treated with DNAse (Promega) to remove genomic DNA. One µg of total RNA was reverse transcribed using the Superscript III first-strand synthesis kit (Invitrogen). PCR was performed using the following primers: *dusp6* forward 5'-CTGGAGCCAGAACCTCTCAC-3', and reverse 5'- AGCTTCTGCATGAGGTACGC-3'; and actin beta 1 (*actb1*) was amplified as a positive control, using forward 5'-GCATTGCTGACCGTATGCAG-3', and reverse 5'-GATCCACATCTGCTGGAAGGTGG-3'. The PCR cycles were as follows: initial DNA denaturing step at 96°C for two minutes, 30 cycles of 96°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds, followed by a final 72°C step for 5 minutes. Twenty-five µl of the PCR product was run on a 1% Tris-acetate-EDTA agarose gel for visualisation.

Quantitative RT-PCR: Zebrafish

RNA pooled from 20-25 embryos per genotype was extracted from *dusp6*$^{+/+}$, *dusp6*$^{+/-}$, and *dusp6*$^{-/-}$ embryos at 6 dpf, with a constant number of fish per genotype within each biological replicate. cDNA was prepared as described in the RT-PCR section above. qRT-PCR was performed using a Lightcycler 480 (Roche) and SYBR Green Master mix (Roche). The average of the expression values from three reference genes was used, including, *actb1*, 18s ribosomal RNA (*18SrRNA*), and eukaryotic translation elongation factor 1 alpha 1 (*eef1α1*), as these genes are considered to be stably expressed throughout the body. qRT-PCR primers for *dusp6* were: forward 5'- CCAACCCAGCCACTGTACTT-3', reverse 5'-GTCGTCTCAAGCCAACATCA-3'. Primers for *actb1* were: forward 5'-GCATTGCTGACCGTATGCAG-3', reverse 5'-GATCCACATCTGCTGGAAGGTGG-3'. Primers for *18SrRNA* were: forward 5'-TCGCTAGTTGGCATCGTTTATG-3', reverse 5'-CGGAGGTTCGAAGACGATCA-3'. Primers for *eef1α1* were: forward 5'-CTGGAGGCCAGCTCAAACAT-3', reverse 5'- ATCAAGAAGAGTAGTACCGCTAGCATTAC-3. Three technical replicates were completed for each biological replicate.

Quantitative RT-PCR: Human

qRT-PCR was performed on post mortem brain samples from 81 unaffected Caucasian individuals obtained from the Australian Brain Bank. Seventy one percent of the samples were male, with a mean age of all subjects of 51.9 years and post mortem interval of 28.1 years. The pH range of the brain samples was 5.75-7.02. qRT-PCR analysis was performed using tissue obtained from the inferior frontal gyrus (IFG) as it is a key node of the frontostriatal system that has been implicated in attention (Durston et al., 2006), and was shown to be dysfunctional in ADHD (Cortese, 2012). RNA was extracted from the IFG samples using TRIzol® reagent as described by manufacturers (Invitrogen/Life Technologies), treated with DNASE-1 (Qiagen) to remove genomic DNA, and purified with the RNeasy Mini Kit (Qiagen). A standard Invitrogen/Life Technologies procedure was used to synthesize first cDNA strands of the samples. qRT-PCR was performed using a Lightcycler 480 (Roche) using SYBR Green Master mix (Roche). The average of the expression values from two reference genes was used, *β*-2-microglobulin (*β2M*), and *β* actin (*ACTB*), as these genes are considered to be stably expressed throughout the body. Primers for *DUSP6* were: forward 5'- AAGCAAATCCCCATCTCGG-3', reverse 5'-TGTCATAGGCATCGTTCATCG-3'. Primers for *β2M* were: forward 5'-GGCATTCCTGAAGCTGACAG-3', reverse 5'-TGGATGAAACCCAGACACATAG-3'. Primers for *ACTB* were: forward 5'-ACCACACCTTCTACAATGAGC-3', reverse 5'-GCGTACAGGGATAGCACAG-3'. Three technical replicates were completed for each brain sample. Values outside a 1.5 x interquartile range were flagged as outliers and were removed.

24-hour locomotion assay: 6 dpf

Embryos were collected in the morning between 9:00 am and 10:00 am and raised in petri dishes in a 14-hour day (9:00 am-11:00 pm) and 10-hour night (11:00 pm-9:00 am) cycle until 6 dpf to entrain the embryos to a day/night cycle. The tracking itself was performed in full darkness, to avoid confounding effects of light during the tracking, while still being able to record behavioural differences between day and night due to entrainment. Light intensity during the day was 300 lux ± 20 lux, while night was in full darkness. Embryos were fed 0.5 ml concentrated paramecium between 9:00 am and 10:00 am on day 5 and 6, and the water was changed between 2:00 pm and 4:00 pm each day. Between 2:00 pm and 4:00 pm on day 6, embryos were transferred to 24-well plates containing 1.5 ml of E3 embryo medium (5 mm NaCl, 0.17 mm KCl, 0.33 mm CaCl, 0.33 mm MgSO4 in water) per well to acclimatise to their new environment. Between 10:30 pm and 10:50 pm on day 6, plates were transferred to Zebraboxes (Viewpoint). At 10:50 pm the Zebraboxes were closed to allow the fish to habituate to the darkness for 10 minutes, and tracking began at 11:00 pm. The experiment ran for 24 hours and 30

minutes, after which videos of the tracking were collected for analysis, and embryos were then sacrificed and genotyped.

24-hour locomotion assay: drug treatment at 6 dpf

Locomotion assays for examining the effect of methylphenidate on 6 dpf fish were performed as above. However, at 10:00 pm on day 6, 150 µl of dH_2O (used as a vehicle control) or 100 µM of Threo-methylphenidate hydrochloride (Tocris Bioscience) was added to wells containing 1.35 ml of E3 and the fish, to yield a final volume of 1.5 ml per well and a concentration of 10 µM of methylphenidate, as described by Lange et al., (2012). For each experiment, drug treatment and vehicle control application were randomised across the 24-well plate., and the investigator was blinded by a third party as to which solution was drug and which was control. Blinding was removed after initial mixed model tests were performed.

Video analysis

In order to analyse the movements of each fish over the experimental period, videos collected from the locomotion assays were analysed using Ethovision software (Noldus, version 14). Movement thresholds used were: Moving, 1 mm/sec; Stopping, 0.75 mm/sec; Detection threshold, Dynamic Subtraction, Darker, 9. Voxel smoothing was used to remove small video jitters and large errors in detection, with Direct movements smaller than 0.04 mm and larger than 12 mm per frame excluded.

Locomotion assay statistical analysis

Locomotion data was processed using Microsoft Excel 2013 and analyses were performed using SPSS Statistics 26 (IBM). Data was ordered chronologically into 10-minute bins. Any time points at the end of videos that were less than 300 seconds were excluded. For each fish, activity data was summed by hour. Then, a normalised value for each hour was determined by comparing activity per hour to the average activity value of all fish (calculated as the average activity of all fish from the respective replicate, for that respective hour). Genotyping data was then assigned to individual fish, and fish with ambiguous genotypes were removed from analysis. The data was then imported into SPSS. Data points from the 30 minutes past the initial 24 hours were excluded. Data was visualised using a line graph in GraphPad Prism Version 8.

In order to examine the differences in activity between genotypes, a mixed linear model was used. For the 6 dpf locomotion assays, main effects of time and genotype, and an interaction effect of time by genotype were used. To account for known differences in Zebraboxes, a main effect of Zebrabox tracking system was used. If a significant ($p < .05$) main effect of Zebrabox tracking system was observed, it was kept in the model to account for any contributing variation. Repeated measures of time (hour) were modelled using a first order autoregressive variance structure. Random effects were defined as individual animals, grouped by genotype. A natural log transformation was applied to the normalised data to meet assumptions of normality which were checked by inspection of the residuals. F tests were performed using a maximal likelihood model, with Satterthwaite estimated degrees of freedom. For the drug treatment assays main effects of time, Zebrabox tracking system, treatment and genotype, and an interaction effect of time by genotype by treatment were used.

Confocal microscopy live imaging

dusp6$^{+/-}$ fish were crossed to a GFP-tagged HuC reporter (*HuC:eGFP* (Park et al., 2000)) and raised to adulthood. *Tg(HuC:eGFP);dusp6*$^{+/-}$ fish were then crossed to *dusp6*$^{+/-}$ fish, and embryos were raised in E3 medium containing 200 µM N-Phenylthiourea (PTU, Sigma) from 6 hours to supress the formation of melanocytes, with changes in medium every 48 hours. Embryos were sorted for fluorescence at 2 dpf. At 3 dpf, fish were anesthetized using Tricaine methanesulfonate (Sigma) at a final concentration of 0.0016% in E3 embryo medium, and their tails were clipped. DNA was then extracted from the clipped tissue using 50 mM NaOH and 1 M Tris-HCl (pH 7.5), and fish were sorted by genotype. At 6 dpf embryos were again anesthetised and set in 1% low melting agarose in clear E3 medium containing tricaine in 0.8 mm fluorinated ethylene propylene (FEP) tubing (Bola). Images were taken using a Thorlabs confocal microscope with an Olympus 20x water dipping NA 1.0 objective, pinhole 25 µm, 2.005 µm/pixel, step size = 1 µm, averaging = 16 frames.

Brain image registration and analysis

Image registration of live confocal stacks was done using Advanced Normalization Tools (ANTs) registration software (3.0.0.0), running on Monash University's MASSIVE computing cluster. Registered images were then analysed using cobraZ brain volume analysis software as described by Gupta et al., (2018). Zebrafish brain regions homologous to human regions known to have volume differences in ADHD individuals (Hoogman et al., 2017) were selected for analysis. Compared regions were the telencephalon (pallium, subpallium, anterior commissure), thalamus, ventral thalamus, and

whole brain volume. Individual regions can be visualised at http://vis.arc.vt.edu/projects/zbb/ (Tabor et al., 2019).

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Conflict of Interest

The authors declare no conflicts of interest.

Functional validation of an ADHD-GWAS risk gene, KDM4A, using a zebrafish model

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Abstract

Attention deficit hyperactivity disorder (ADHD) is a highly heritable disorder of childhood, commonly associated with hyperactivity/impulsivity, and an inability to maintain attention. Recent success of genome wide association studies (GWAS) in the identification of ADHD-associated DNA variants, has led to a need to functionally validate these associations and determine their relevance to the development of the disorder. We utilised a zebrafish model to examine *KDM4A*, a gene implicated in ADHD aetiology through a large GWAS meta-analysis. We identified rs112984125 as being in strong linkage disequilibrium with the ADHD-associated variant, rs11420276, and that it demonstrated a higher likelihood of being functional. Using post mortem brain tissue, we demonstrated that individuals carrying the ADHD risk allele of rs112984125 show significantly higher *KDM4A* mRNA levels compared with non-risk allele individuals. Zebrafish possess two orthologues of KDM4A, and we have created a double mutant model that is expected to have lost the function of both proteins. We showed that *kdm4aa*$^{-/-}$;*kdm4ab*$^{-/-}$ 6 day post-fertilisation fish have significantly reduced activity. Overall, this study shows that disruptions to *kdm4a* contribute to the development of activity phenotypes in zebrafish, demonstrating the utility of zebrafish for validating future ADHD-GWAS associations, and that *KDM4A* could be contributing to the development of ADHD as a whole.

Introduction

Attention deficit hyperactivity disorder (ADHD) is a highly heritable neuropsychiatric disorder that presents in ~5% of school-aged children worldwide (Polanczyk et al., 2007). Individuals diagnosed with ADHD present with abnormally high levels of inattention and/or hyperactivity/impulsivity, which is associated with detrimental impacts on academic achievement (Faraone et al., 2015). Further, the disorder often persists into adulthood (Faraone et al., 2006; Faraone and Biederman, 2005).

ADHD's high degree of heritability has driven a wide search for DNA variants associated with the disorder (Biederman, 2005; Faraone and Larsson, 2019). Earlier ADHD genetic research used hypothesis driven, candidate gene, approaches mostly focused on genes involved in monoamine transmission, with a number of variants mapped to these genes showing significant association with ADHD (Gizer et al., 2009). However, the findings of candidate gene studies were largely underpowered and used single or limited numbers of genetic markers. More importantly, the findings from candidate genes in ADHD were not consistently replicated. More recently, hypothesis free genome wide association studies (GWAS) have been used to scan the whole genome for ADHD-associated variants. A recent GWAS identified the first 12 significant ADHD-GWAS associations (Demontis et al., 2019). Despite the plethora of ADHD-associated variants from candidate gene studies, and now GWAS, at our disposal, extremely limited work has been done to determine whether these variants, and the genes they are mapped to, contribute to the development of ADHD. Especially now that the hunt for ADHD-associated genes is becoming more and more fruitful, it is more important than ever to functionally validate these variants so that we can confirm their functional relevance to the development of ADHD.

The most significant association reported from the ADHD-GWAS by Demontis and colleagues was rs11420276 (G→GT, G is the risk allele, $p = 2.14 \times 10^{-13}$). It is mapped to a genomic region including several genes, one of these being lysine demethylase 4a (*KDM4A*). KDM's are a family of proteins important for the demethylation of histones to regulate gene expression (Labbé et al., 2013). Members of the KDM4 subfamily contain Jumonji C (JmjC) domains, which are important for the demethylation of trimethyl lysine residues, in addition to the mono- and dimethyl lysine residues KDM1A and -1B are limited to. KDM4A has been shown to alter differentiation in neural cells, as loss of KDM4A down regulates neural crest specifier genes in chick embryos (Strobl-Mazzulla et al., 2010), knockdown of *KDM4A* leads to decreased neural differentiation in human adult neural stem cells (NSCs (Cascante et al., 2014)), and overexpression of KDM4A promotes activation of pathways important in the de-differentiation of human adult NSCs (Ma et al., 2008). Overall, this demonstrates the potential for KDM4A to have a functional impact on the development of ADHD, as dysregulation of KDM4A can prevent both correct differentiation of neural cell populations during embryogenesis,

and proper maintenance to ensure neural cells remain in their differentiated states. Therefore, investigation into whether DNA variants in KDM4A can lead to an ADHD phenotype such as hyperactivity is needed.

In this study, we functionally validated the association of *KDM4A* with ADHD. We demonstrated that the ADHD risk allele of rs112984125, which is in very strong linkage disequilibrium (LD) with the most significant ADHD-associated GWAS variant, rs11420276, and predicted to be strongly functional, is associated with significantly higher *KDM4A* mRNA levels than the non-risk allele. To examine the consequence of changes in KDM4A levels we adopted the zebrafish as an animal model. The use of zebrafish models to examine genes associated with neurodevelopmental disorders has been quite successful (Fontana et al., 2018; Sakai et al., 2018), making them a great model for examining ADHD-associated genes. The nature of requiring two upregulated genes in a *kdm4a* zebrafish overexpression model would mean that two transgenes with the appropriate endogenous promoters would need to be created, potentially integrating in random regions of the genome leading to potential effects not specific to the overexpression of *kdm4aa* or *kdm4ab*. Therefore, we opted for a downregulatory approach, knowing that the effect would be specific to *kdm4a*, and would allow us to examine if a reduction in *kdm4a* mRNA levels could also lead to ADHD phenotypes, similar to the upregulation seen in humans. In addition, the degree to which *kdm4aa* and *kdm4ab* would need to be individually upregulated is not known, while the removal of both functional proteins allows for a simpler approach. We used a double mutant zebrafish model to examine if loss of Kdm4aa and Kdm4ab function leads to an activity phenotype. We demonstrated that *kdm4aa*$^{-/-}$;*kdm4ab*$^{-/-}$ fish have significantly lower activity than *kdm4aa*$^{+/+}$;*kdm4ab*$^{+/+}$ fish. This indicates that the level of functional KDM4A contributes to the development of activity phenotypes in zebrafish, and potentially ADHD.

Results

A functionally prioritised *KDM4A* SNP is associated with higher *KDM4A* mRNA levels

The most likely variant to be functional in the region identified by Demontis et al (2019) was determined by using the bioinformatic pipeline for prioritising functional variants described in Tong et al., (2016). rs112984125 (G→A, mapped to *KDM4A*) is a single nucleotide polymorphism (SNP), that showed strong functional prediction scores from the Combined Annotation Dependent Depletion (CADD, C-score = 10.02 (Kircher et al., 2014)), and RegulomeDB (2b (Boyle et al., 2012) programs, and the rs112984125 G allele is in very strong LD (R^2 = .98, D' = 1) with the rs11420276 risk allele, G.

We then examined the correlation between rs112984125 genotype and mRNA levels of *KDM4A.* Quantification via qRT-PCR using cDNA from post-mortem human brain samples demonstrated that

rs112984125 is significantly associated with changes in *KDM4A* mRNA levels ($F = 4.93$ (2, 54), $p = .011$, two-tailed, Figure 1). Post-hoc tests demonstrated significantly lower mRNA levels, in individuals homozygous for AA ($n = 6$) compared to AG individuals ($n = 25$, $p = .027$, two-tailed) and those homozygous for the ADHD risk allele, G ($n = 26$, $p = .008$, two-tailed).

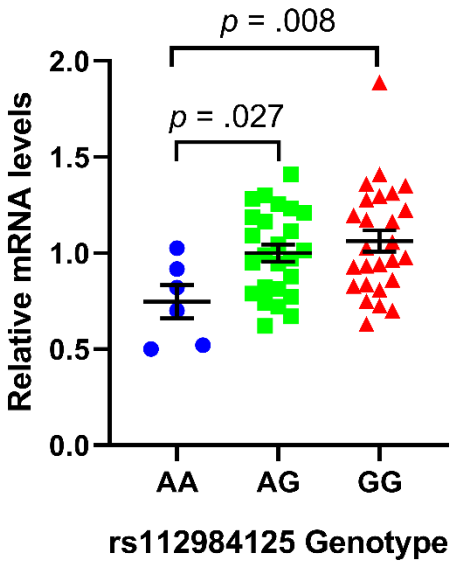

Figure 1. qRT-PCR examining *KDM4A* mRNA levels in human brain samples with reference to a *KDM4A* functionally predicted SNP, rs112984125. Significantly higher mRNA levels were observed in GG and AG individuals than AA individuals. G rs112984125 is in very strong LD with the rs11420276 ADHD risk allele. Data is normalised to heterozygotes. *B2M* and *ACTB* were used as reference genes. Centre lines = mean, error bars = +/- standard error of the mean (SEM).

Zebrafish possess two KDM4A orthologues, which are expressed during early zebrafish development

Zebrafish are an emerging model for examining neuropsychiatric disorders (Fontana et al., 2018), and as such were adopted for validating the association of *KDM4A* with ADHD. The KDM family is well conserved between humans and zebrafish, zebrafish possessing orthologs for almost all members of the human KDM family (Figure 2). Zebrafish possess two orthologous copies of KDM4A, Kdm4aa (sequence identity to human KDM4A is 82%, similarity is 83.5%) and Kdm4ab (sequence identity to human KDM4A is 77%, similarity is 81%). To determine that *kdm4aa* and *kdm4ab* are expressed during embryonic zebrafish development, RT-PCR was performed on wildtype (*Tübingen*, TU) embryos, which showed that both genes are expressed as early as the 8-somite stage through to 5 days post-fertilisation (dpf, Figure 3A & B).

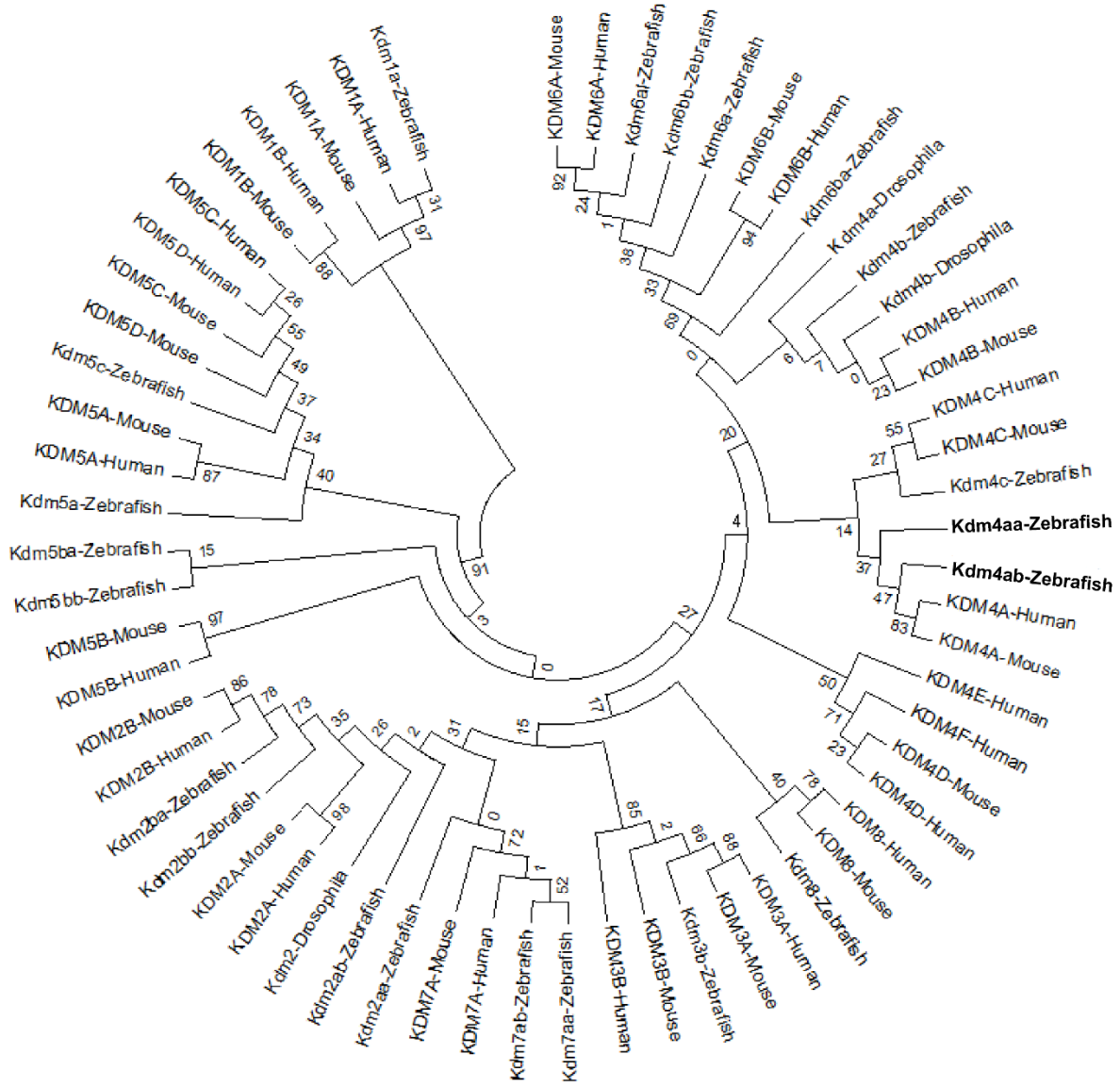

Figure 2. Phylogenetic tree of the KDM family in humans, mice, *Drosophila*, and zebrafish. Zebrafish possess orthologues of most KDM proteins known in humans and mice. Zebrafish possess two orthologues of KDM4A, which are bolded. Evolutionary analyses were conducted in MEGA (Version 6 (Tamura et al., 2013)), using a Maximum Likelihood method based on the Jones-Taylor-Thornton (JTT) matrix-based model (Jones et al., 1992). The tree with the highest log likelihood (-842.2596) is shown.

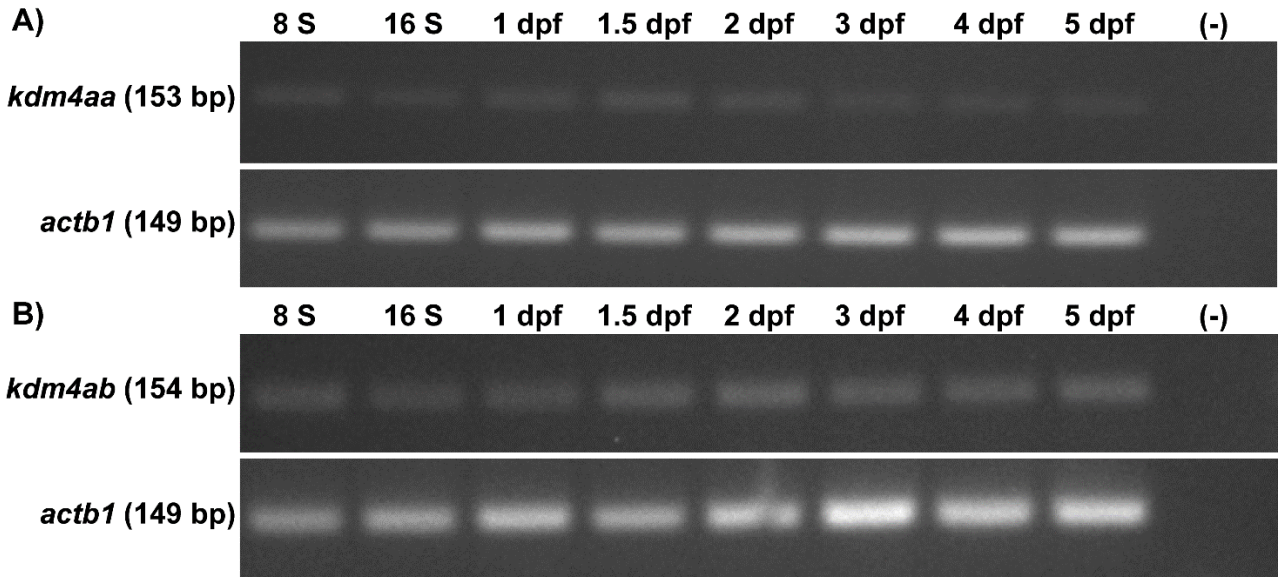

Figure 3. RT-PCR for **A)** *kdm4aa* and **B)** *kdm4ab* performed using zebrafish cDNA at 8-somite stage (8 S), 16-somite stage (16 S), 1 dpf, 1.5 dpf, 2 dpf, 3 dpf, 4 dpf and 5 dpf. Both *kdm4aa* and *kdm4ab* are expressed from as early as 8 S through to 5 dpf. *actb1* was amplified as a positive control.

Fish mutant for *kdm4aa* and *kdm4ab* show reductions in *kdm4aa* and *kdm4ab* mRNA, and show no gross morphological defects

Having identified that *kdm4aa* and *kdm4ab* expression was detectable during early zebrafish development, *kdm4aa* and *kdm4ab* mutant lines were obtained from ZIRC. We do not yet understand what combination of overexpression of *kdm4aa* and *kdm4ab* is required to mimic the level of overexpression seen in human samples. However, the loss of both functional proteins is something that can be made with a higher degree of certainty. Therefore, we utilised two mutant lines that resulted in a downregulation of *kdm4a* mRNA, to examine if a reduction in mRNA transcript could be associated with ADHD phenotypes similar to the upregulation in human samples. *kdm4aa*^{sa40621} had a premature STOP codon at position 134 (Figure 4A), and *kdm4ab*^{sa11870} had a premature STOP codon at position 106 (Figure 4B). Both mutations are predicted to result in the loss of the majority of the respective protein, including the loss of the JmjC and PHD-type domains, and as such is predicted to result in a non-functional protein. In addition, these mutations are expected to result in nonsense-mediated decay, and thus a loss of protein, rather than production of a truncated protein.

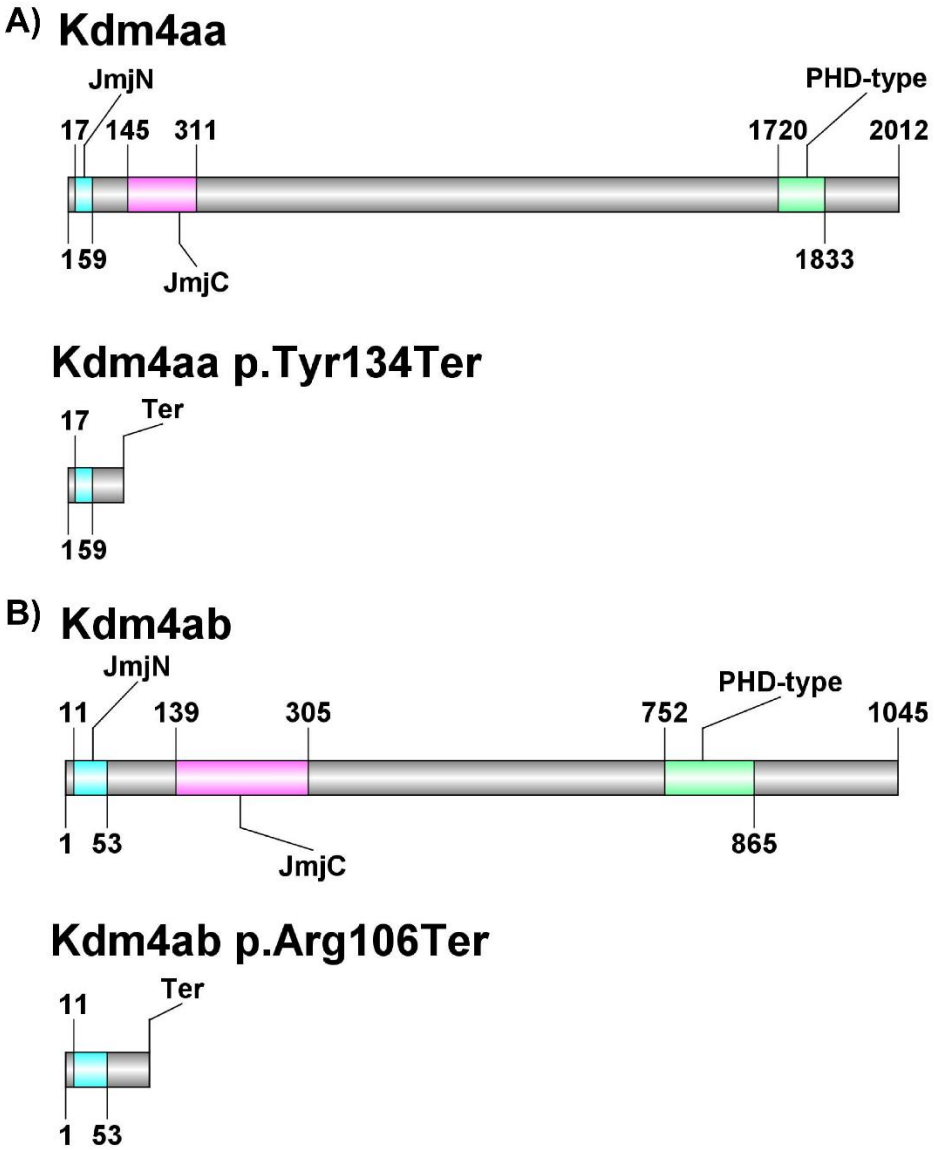

Figure 4. Schematic representations of **A)** the Kdm4aa wildtype and mutant proteins. A premature STOP codon at position 134 results in the loss of the JmjC and PHD-type domains of the protein. **B)** The Kdm4ab wildtype and mutant proteins. A premature STOP codon at position 106 results in the loss of the JmjC and PHD-type domains of the protein.

kdm4aa$^{+/-}$ and *kdm4ab*$^{+/-}$ fish were crossed to create double heterozygotes, which were in-crossed to produce single and double mutants. Offspring from *kdm4aa*$^{+/-}$; *kdm4ab*$^{+/-}$ in-crosses were examined using brightfield images for any gross morphological defects, but no obvious differences were seen between genotypes (Figure 5).

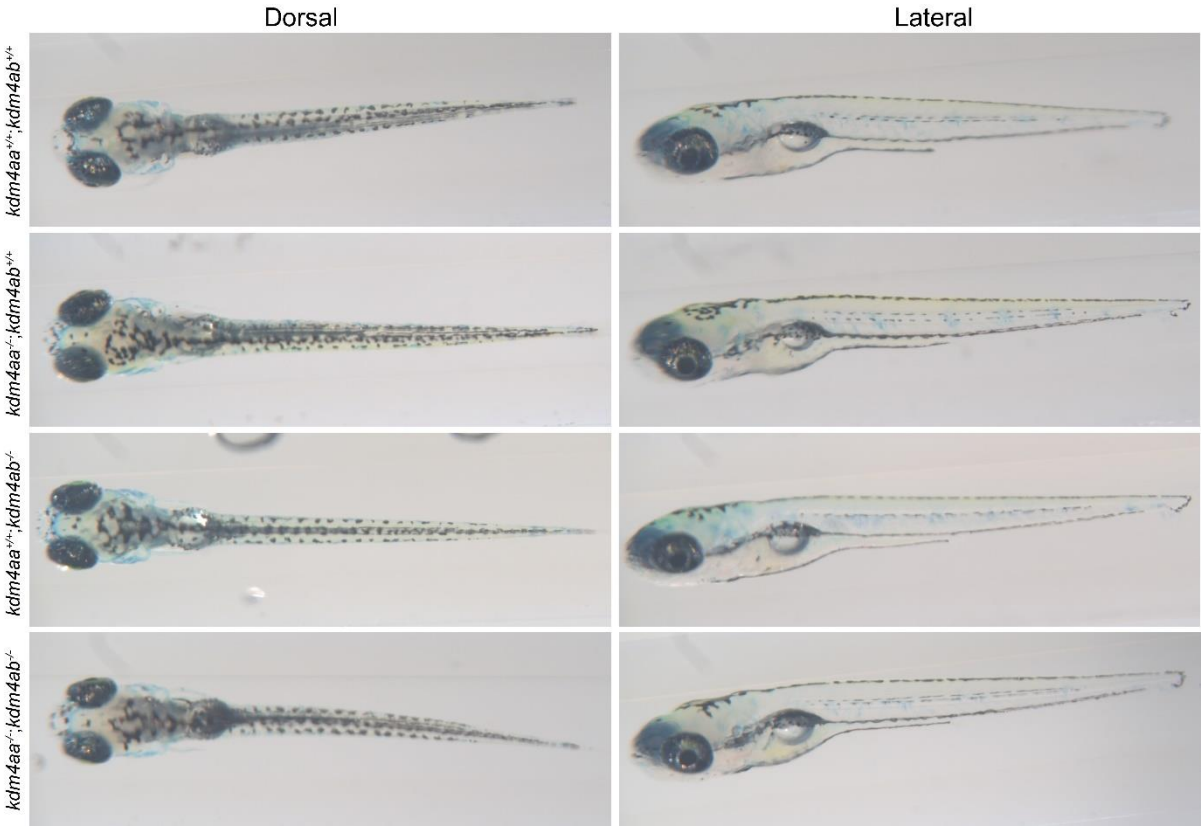

Figure 5. Brightfield images of typical *kdm4aa*$^{+/+}$;*kdm4ab*$^{+/+}$, *kdm4aa*$^{-/-}$;*kdm4ab*$^{+/+}$, *kdm4aa*$^{+/+}$;*kdm4ab*$^{-/-}$ and *kdm4aa*$^{-/-}$;*kdm4ab*$^{-/-}$ 6 dpf embryos. No gross morphological differences were observed between genotypes.

To determine if nonsense mediated decay of both *kdm4aa* and *kdm4ab* mRNA was occurring, RNA was collected from *kdm4aa*$^{+/+}$;*kdm4ab*$^{+/+}$, *kdm4aa*$^{-/-}$;*kdm4ab*$^{+/+}$, *kdm4aa*$^{+/+}$;*kdm4ab*$^{-/-}$ and *kdm4aa*$^{-/-}$;*kdm4ab*$^{-/-}$ 6 dpf embryos. Single mutants were examined to determine if any upregulation of either *kdm4aa* or *kdm4ab* was occurring due to loss of protein from the other respective gene. Quantification using qRT-PCR demonstrated that both *kdm4aa* (F = 13.16 (3, 12), p < .001, two-tailed) and *kdm4ab* (F = 29.21 (3, 12), p < .001, two-tailed) mRNA levels were significantly altered (Figure 6A and 6B). *kdm4aa* mRNA levels were significantly decreased in *kdm4aa*$^{-/-}$;*kdm4ab*$^{+/+}$ and *kdm4aa*$^{-/-}$;*kdm4ab*$^{-/-}$ fish compared to *kdm4aa*$^{+/+}$;*kdm4ab*$^{+/+}$ fish (p = .005 and p = .001 respectively, two-tailed, Figure 6A). Similarly, *kdm4ab* mRNA levels were significantly decreased in *kdm4aa*$^{+/+}$;*kdm4ab*$^{-/-}$ and *kdm4aa*$^{-/-}$;*kdm4ab*$^{-/-}$ fish compared to *kdm4aa*$^{+/+}$;*kdm4ab*$^{+/+}$ fish (both p < .001, two-tailed, Figure 6B). Neither gene demonstrated any suggestion of upregulation when the other protein was lost. Therefore, the predicted loss of function and reduction of mRNA transcript of both *kdm4aa* and *kdm4ab* in combination allows us to examine the near-to-full loss of *kdm4a* function in the zebrafish.

In human samples, there were significant differences in the levels of *KDM4A* mRNA between genotypes for the ADHD functionally predicted SNP (Figure 1). Higher levels of *KDM4A* mRNA were associated with individuals who possessed the ADHD-associated allele, suggesting that increased levels of KDM4A is a risk factor predisposing to ADHD phenotypes, potentially hyperactivity. We hypothesised that a reduction in *kdm4a* mRNA levels would have an opposite, protective effect, potentially reducing activity levels. Therefore, through the use of Kdm4aa and Kdm4ab mutant lines, we aimed to examine whether a reduction in mRNA levels of both genes could affect activity in these animals.

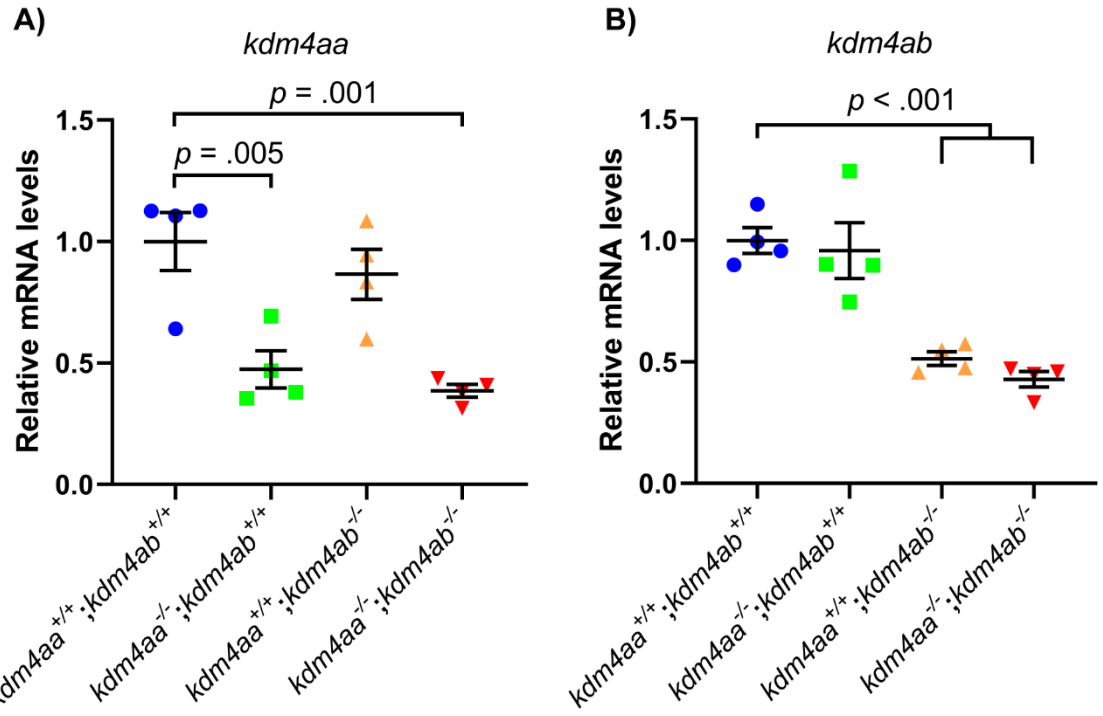

Figure 6. qRT-PCR examination of **A)** *kdm4aa* mRNA levels of *kdm4aa*$^{+/+}$;*kdm4ab*$^{+/+}$, *kdm4aa*$^{-/-}$;*kdm4ab*$^{+/+}$, *kdm4aa*$^{+/+}$;*kdm4ab*$^{-/-}$ and *kdm4aa*$^{-/-}$;*kdm4ab*$^{-/-}$ 6 dpf embryos. *kdm4aa*$^{-/-}$;*kdm4ab*$^{+/+}$ and *kdm4aa*$^{-/-}$;*kdm4ab*$^{-/-}$ fish showed significantly decreased mRNA levels compared to *kdm4aa*$^{+/+}$;*kdm4ab*$^{+/+}$ fish. **B)** *kdm4ab* mRNA levels of *kdm4aa*$^{+/+}$;*kdm4ab*$^{+/+}$, *kdm4aa*$^{-/-}$;*kdm4ab*$^{+/+}$, *kdm4aa*$^{+/+}$;*kdm4ab*$^{-/-}$ and *kdm4aa*$^{-/-}$;*kdm4ab*$^{-/-}$ 6 dpf embryos. *kdm4aa*$^{+/+}$; *kdm4ab*$^{-/-}$ and *kdm4aa*$^{-/-}$; *kdm4ab*$^{-/-}$ fish showed significantly decreased mRNA levels compared to *kdm4aa*$^{+/+}$;*kdm4ab*$^{+/+}$ fish. Data is from four biological replicates, and is normalised to *kdm4aa*$^{+/+}$;*kdm4ab*$^{+/+}$ values. *eef1α1*, *mobk13*, and *lsm12b* were used as reference genes. Centre lines = mean, error bars = +/- SEM.

$kdm4aa^{-/-}$;$kdm4ab^{-/-}$ fish demonstrated significantly less activity than wildtype fish over a 24-hour period

In order to determine if the loss of Kdm4aa and Kdm4ab function leads to a change in activity, the activity of $kdm4aa^{+/+}$;$kdm4ab^{+/+}$ (*n* = 85), $kdm4aa^{+/-}$;$kdm4ab^{+/-}$ (*n* = 368), and $kdm4aa^{-/-}$;$kdm4ab^{-/-}$ (*n* = 84) zebrafish embryos were tracked over a period of 24 hours, starting from 158 hours post-fertilisation (hpf). $kdm4aa^{-/-}$;$kdm4ab^{-/-}$ fish showed consistently lower activity than other genotypes over the whole 24-hour period (Figure 7). To investigate this further, a mixed linear model analysis was performed. A significant main effect of genotype was observed (F = 4.11 (2, 162.28), p = .018, two-tailed). The main effect of Zebrabox tracking system was significant, and thus was kept in the model. There was no significant interaction between genotype and time (F = .78 (46, 6308.22), p = .86, two-tailed). Pairwise comparisons were performed to investigate the effect of genotype. We observed that $kdm4aa^{-/-}$;$kdm4ab^{-/-}$ fish were significantly less active than $kdm4aa^{+/+}$;$kdm4ab^{+/+}$ fish (p = .017, two-tailed, Bonferroni adjusted). There were no significant differences between $kdm4aa^{+/+}$;$kdm4ab^{+/+}$ fish and $kdm4aa^{+/-}$;$kdm4ab^{+/-}$ fish.

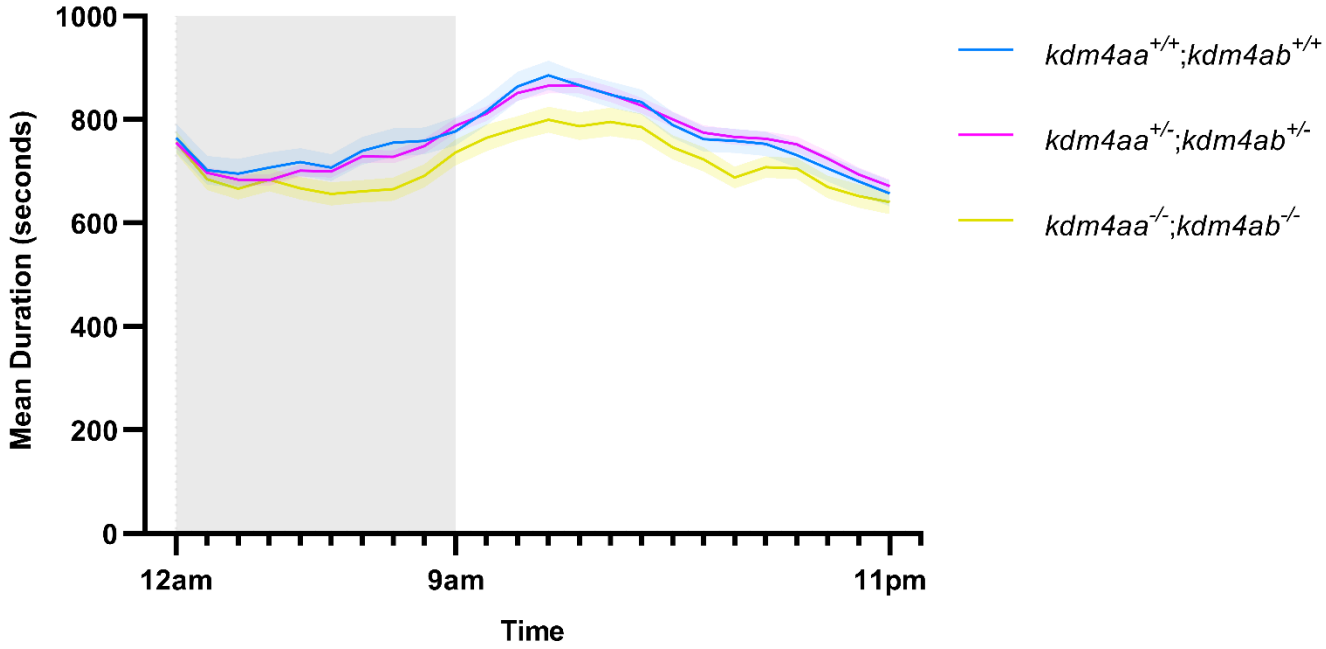

Figure 7. Activity analysis of $kdm4aa^{+/+}$;$kdm4ab^{+/+}$ (*n* = 85), $kdm4aa^{+/-}$;$kdm4ab^{+/-}$ (*n* = 368), and $kdm4aa^{-/-}$;$kdm4ab^{-/-}$ (*n* = 84) zebrafish 6 dpf embryos over a period of 24 hours. $kdm4aa^{-/-}$;$kdm4ab^{-/-}$ fish demonstrated significantly lower activity than $kdm4aa^{+/+}$;$kdm4ab^{+/+}$ fish over the whole time period. The average time spent per genotype moving in each hour time point is displayed on the Y axis. Data is from twelve biological replicates, based on power calculations performed using data from the *chmp7* 24-hour 6 dpf locomotion assay (see results section of Chapter 1, Figure 5). Error bars = +/- SEM.

Loss of Kdm4aa and Kdm4ab function doesn't alter expression levels of neural differentiation markers

Dysregulation of KDM4A has been shown to disrupt neural differentiation patterns (Cascante et al., 2014). To determine if this was the case in the *kdm4aa*;*kdm4ab* mutant line, RNA was collected from the heads of *kdm4aa*$^{+/+}$;*kdm4ab*$^{+/+}$, *kdm4aa*$^{-/-}$;*kdm4ab*$^{+/+}$, *kdm4aa*$^{+/+}$;*kdm4ab*$^{-/-}$ and *kdm4aa*$^{-/-}$;*kdm4ab*$^{-/-}$ embryos at 6 dpf. The expression of genes known to be expressed in the neuroepithelium (SRY-box transcription factor 2, *sox2*), astrocytes (glial fibrillary acidic protein, *gfap*; S100 calcium binding protein, beta (neural), *s100b*), oligodendrocytes (oligodendrocyte transcription factor 1, *olig1*; oligodendrocyte lineage transcription factor 2; *olig2*), and immature neurons (T-box brain transcription factor 1b, *tbr1b*; neuronal differentiation 1; *neuroD1*), as well as brain derived neurotropic factor (*bdnf*), was examined to determine if the prevalence of any of these cell types, or neural differentiation was altered following disruption of Kdm4aa and Kdm4ab function. Quantification using qRT-PCR showed that there were no significant differences between genotypes for any of the genes tested (Figure 8), suggesting that at 6 dpf there was no gross over or under representation of any of the examined neuronal cell types.

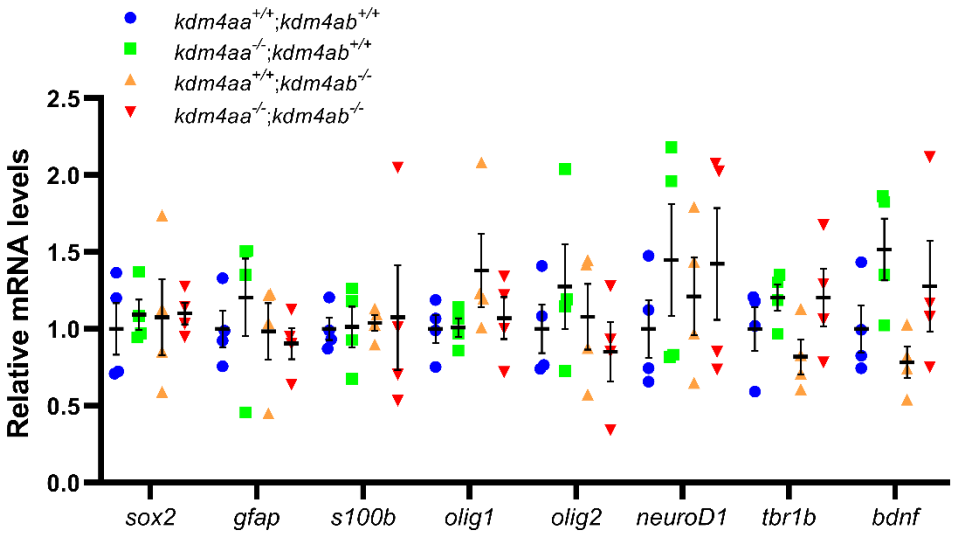

Figure 8. qRT-PCR examining mRNA levels of neural differentiation markers in *kdm4aa*$^{+/+}$;*kdm4ab*$^{+/+}$, *kdm4aa*$^{-/-}$;*kdm4ab*$^{+/+}$, *kdm4aa*$^{+/+}$;*kdm4ab*$^{-/-}$ and *kdm4aa*$^{-/-}$;*kdm4ab*$^{-/-}$ 6 dpf embryos. *eef1α1*, *mobk13*, and *lsm12b* were used as reference genes. There were no significant differences observed between genotypes for each neural differentiation marker. Data is normalised to *kdm4aa*$^{+/+}$;*kdm4ab*$^{+/+}$ values for each gene of interest, and is taken from four biological replicates. Centre lines = mean, error bars = +/- SEM.

Discussion

This study is the first to functionally validate the association of a newly identified ADHD-GWAS hit, which maps near the *KDM4A* gene. We showed that the risk allele of a functionally predicted SNP, rs112984125 (linked to the ADHD-associated variant rs11420276), was significantly associated with higher levels of *KDM4A* mRNA from post mortem brain tissue. Further, through the use of zebrafish mutant lines, we also demonstrated that the loss of Kdm4aa and Kdm4ab function leads to a significant reduction in the activity of *kdm4aa*$^{-/-}$;*kdm4ab*$^{-/-}$ fish compared to *kdm4aa*$^{+/+}$;*kdm4ab*$^{+/+}$ fish over a 24-hour period.

The investigations in this study have provided support for the importance of *KDM4A* as a risk factor in the development of ADHD. The changes in *KDM4A* mRNA levels were significantly correlated with the ADHD risk allele, suggesting that having more KDM4A increases risk for developing the disorder (OR = 1.11 (Demontis et al., 2019)). In addition, the loss of Kdm4a proteins is linked to activity phenotypes in zebrafish. The combination of this evidence suggests that activity phenotypes can be modulated by the levels of KDM4A, with increased levels predisposing to ADHD, while reduced levels potentially have a protective effect.

Given the evidence demonstrating the importance of KDM4A in cell differentiation (Labbé et al., 2013), it is likely that developmental disruptions, or delays in the patterning of neural cell populations, are contributing to the activity defects seen in *kdm4aa*$^{-/-}$;*kdm4ab*$^{-/-}$ fish. We therefore examined the possibility that these defects were the result of obvious differences in the differentiation of neural cell populations present at 6 dpf. However, we did not observe significant differences in the mRNA levels of several neural differentiation markers, for a number of reasons. Firstly, this could be attributed to the potential changes in the differentiation of neural cells being specific to particular brain regions and, as we took cDNA from the whole head of the embryo, this method might mask the detection of subtle regional changes. Secondly, it is also possible that the regulation of differentiation is delayed in *kdm4aa*$^{-/-}$;*kdm4ab*$^{-/-}$ fish, resulting in an overall neurodevelopmental delay but no longstanding changes to the proportion of neural cell populations by 6 dpf. Lastly, some genotypes showed large variation between biological replicates depending on the gene examined. The reasons mentioned above could be contributing to this variation, as the size of neural cell populations and the rate of neurodevelopment may have differed between biological replicates, thus leading to some genes showing higher expression than others. A greater number of biological replicates could aid in determining a more accurate representation of gene expression in this case. Despite this, we have shown that gross changes in mRNA levels of neural differentiation markers in the whole head is unlikely to be contributing to the activity phenotype at 6 dpf.

There are a number of strengths and limitations with the approaches we have used in this study. The use of bioinformatic analyses to predict which SNP in LD with the ADHD-associated GWAS SNP was most likely to be functional, then the use of human post mortem brain samples to examine the impact of the predicted SNP on *KDM4A* mRNA levels lets us take an association through to a biological meaning. Following this, the use of an overexpression model, similar to results chapter 2, would have provided a way to more similarly model the directional change in expression levels we see in humans. However, the unintended impact of randomly integrating transgenes, plus our inability to know how much each individual ortholog would need to be upregulated to mimic the total *KDM4A* upregulation seen in humans, makes a double overexpression model difficult to develop. Instead, the use of a double mutant in which both genes are downregulated has allowed us to examine one more definitive answer, if a loss of *kdm4a* function leads to a decrease in activity. While protein analyses, similar to the previous chapters, would allow us to know if this is in fact due to a loss of protein, we have demonstrated that reductions in *kdm4aa* and *kdm4ab* mRNA levels is sufficient to reduce activity, similar to how a change in *KDM4A* mRNA levels in humans, is associated with ADHD.

Overall, we have demonstrated that disruptions to *kdm4a* contributes to changes in activity in zebrafish, suggesting that KDM4A may contribute to the development of ADHD-related phenotypes. Higher levels of KDM4A is associated with increased risk for developing ADHD, while decreased levels could potentially have a protective effect against a hyperactivity phenotype. This study demonstrates how the combination of human data and a zebrafish model can be used to functionally examine current and future ADHD-GWAS associations, thus furthering our knowledge of the underlying genetic contributions to the disorder.

Materials and Methods

Ethics

Fish maintenance and handling were carried out as per standard operating procedures approved by the Monash Animal Services Ethics Committee. All fish were maintained in the Fish Core facility at Monash University under breeding colony license MARP/2015/004/BC.

Prioritisation of *KDM4A* functional variants

Determination of the SNP in linkage disequilibrium with the ADHD-GWAS associated variant that showed the highest likelihood for functionality was done using the bioinformatic pipeline previously described in Tong et al., (2016).

Husbandry and genotyping of the *kdm4aa* and *kdm4ab* mutant lines

Mutant lines for *kdm4aa* and *kdm4ab* were imported from the Zebrafish International Resource Center (ZIRC, *kdm4aa*^{sa40621}, *kdm4ab*^{sa11870}), and maintained by outcrossing to wildtype (*Tübingen*, TU) fish. Imported individuals were screened for the respective mutation using allele specific KASP fluorescence assays (Geneworks), then *kdm4aa*$^{+/-}$ and *kdm4ab*$^{+/-}$ fish were crossed and the offspring raised to adulthood.

Phylogenetic tree

KDM protein sequences from human, mouse, zebrafish, and *Drosophila* were aligned using multiple sequence alignment software, ClustalX (Version 2.1 (Larkin et al., 2007))(Supplementary Table 2). The evolutionary history was inferred by using the Maximum Likelihood method based on the Jones-Taylor-Thornton (JTT) matrix-based model (Jones et al., 1992). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 58.2413)). Any positions that contained gaps or missing data were removed. The tree was calculated from the alignment using Molecular Evolutionary Genetics Analysis (MEGA) software version 6 (Tamura et al., 2013) and bootstrap values taken from 1000 repetitions using the JTT model.

Reverse transcription-PCR to examine expression of *kdm4aa* and *kdm4ab*

RNA was extracted from wildtype embryos at the: 8-somite stage, 16-somite stage, 1 day post-fertilisation (dpf), 1.5 dpf, 2 dpf, 3 dpf, 4 dpf, and 5 dpf. Total RNA was isolated using TRIzol® reagent as described by the manufacturer (Sigma), and treated with DNAse (Promega) to remove genomic DNA. One µg of total RNA from each developmental stage was reverse transcribed using the Superscript III first-strand synthesis kit (Invitrogen). PCR was performed using the following primers: *kdm4aa* forward 5'- GATGAAGAGCTGCCCAAAAG -3' and reverse 5'- GATGACGGGCTCGATGTAGT-3', *kdm4ab* forward 5'- AGGGCGAAGTGGTTCAAGTA -3' and reverse 5'- AGCTCCTCGTCCAAACTGAA-3', and actin beta 1 (*actb1*) was amplified as a positive control, using forward 5'- GCATTGCTGACCGTATGCAG-3' and reverse 5'- GATCCACATCTGCTGGAAGGTGG-3'. The PCR cycles were as follows: initial DNA denaturing step at 96°C for two minutes, 30 cycles of 96°C for 30 seconds,

57°C for 30 seconds, and 72°C for 30 seconds, followed by a final 72°C step for 5 minutes. Twenty-five µl of the PCR product was run on a 1% Tris-acetate-EDTA agarose gel for visualisation.

Quantitative RT-PCR: Zebrafish

RNA pooled from 15-20 embryos per genotype was extracted from the heads of *kdm4aa*$^{+/+}$;*kdm4ab*$^{+/+}$, *kdm4aa*$^{-/-}$;*kdm4ab*$^{+/+}$, *kdm4aa*$^{+/+}$;*kdm4ab*$^{-/-}$, and *kdm4aa*$^{-/-}$;*kdm4ab*$^{-/-}$ embryos at 6 dpf, with a constant number of fish per genotype within each biological replicate. cDNA was prepared as described in the RT-PCR section above. qRT-PCR was performed using a Lightcycler 480 (Roche) and SYBR Green Master mix (Roche). The average of the expression values from three reference genes was used, including, eukaryotic translation elongation factor 1 alpha 1 (*eef1α1*), as well as MOB family member 4, phocein (*mobk13* (Hu et al., 2016)), and Like-Sm protein 12 homolog b (*lsm12b* (Hu et al., 2016)), as these genes are considered to be stably expressed throughout the body. Primers for *kdm4aa* and *kdm4ab* were the same as those used in the RT-PCR. All reference gene primers, as well as primers for the neural differentiation analysis, can be found in Supplementary Table 1. Three technical replicates were performed for each biological replicate.

Quantitative RT-PCR: Human

qRT-PCR was performed on post-mortem brain samples from 81 unaffected Caucasian individuals obtained from the Australian Brain Bank. Seventy one percent of the samples were male, with a mean age of all subjects of 51.9 years and post-mortem interval of 28.1 years. The pH range of the brain samples was 5.75-7.02. qRT-PCR analysis was performed using tissue obtained from the inferior frontal gyrus (IFG) as it is a key node of the frontostriatal system that has been implicated in attention (Durston et al., 2006), and was shown to be dysfunctional in ADHD (Cortese, 2012). RNA was extracted from the IFG samples using TRIzol® reagent as described by manufacturers (Invitrogen/Life Technologies), treated with DNASE-1 (Qiagen) to remove any genomic DNA, and purified with the RNeasy Mini Kit (Qiagen). A standard Invitrogen/Life Technologies procedure was used to synthesize first cDNA strands of the samples. qRT-PCR was performed using a Lightcycler 480 (Roche) and SYBR Green Master mix (Roche). The average of the expression values from two reference genes was used, *β*-2-microglobulin (*β2M*), and *β* actin (*ACTB*), as these genes are considered to stably expressed throughout the body. Primers for *KDM4A* were: forward 5'- GCTGTGCTGTGCTCCTGTAG -3', reverse 5'- CTCCTCGTTGCCAGCTCTTG -3'. Primers for *β2M* were: forward 5'-GGCATTCCTGAAGCTGACAG-3', reverse 5'-TGGATGAAACCCAGACACATAG-3'. Primers for *ACTB* were: forward 5'-ACCACACCTTCTACAATGAGC-3', reverse 5'-GCGTACAGGGATAGCACAG-3'. Three technical replicates

were performed for each brain sample. Values outside a 1.5 x interquartile range were flagged as outliers and were removed.

Brightfield morphology analysis

kdm4aa$^{+/+}$;*kdm4ab*$^{+/+}$, *kdm4aa*$^{-/-}$;*kdm4ab*$^{+/+}$, *kdm4aa*$^{+/+}$;*kdm4ab*$^{-/-}$, and *kdm4aa*$^{-/-}$;*kdm4ab*$^{-/-}$ 6 dpf zebrafish were examined for gross morphological abnormalities using a brightfield microscope. At 6 dpf, embryos were anesthetised using Tricaine methanesulfonate (Sigma) at a final concentration of 0.0016% in E3 embryo medium (5 mm NaCl, 0.17 mm KCl, 0.33 mm CaCl, 0.33 mm MgSO4 in water), and set in 1% low melting agarose in clear E3 medium containing tricaine in 0.8 mm fluorinated ethylene propylene (FEP) tubing (Bola). Images were taken on an Olympus SZX16 microscope using a Ximea xiC USB 3.1 camera. DNA was then extracted from the whole embryo using 50 mM NaOH and 1 M Tris-HCl (pH 7.5) for genotyping.

24-hour locomotion assay

Embryos were collected in the morning between 9:00 am and 10:00 am and raised in petri dishes in a 14-hour day (9:00 am-11:00 pm) and 10-hour night (11:00 pm-9:00 am) cycle until 6 dpf to entrain the embryos to a day/night cycle. The tracking itself was performed in full darkness, to avoid confounding effects of light during the tracking, while still being able to record behavioural differences between day and night due to entrainment. Light intensity during the day was 300 lux ± 20 lux, while night was in full darkness. Embryos were fed 0.5 ml concentrated paramecium between 9:00 am and 10:00 am on day 5 and 6, and the water was changed between 2:00 pm and 4:00 pm each day. Between 2:00 pm and 4:00 pm on day 6, embryos were transferred to 24-well plates containing 1.5 ml of E3 embryo medium (5 mm NaCl, 0.17 mm KCl, 0.33 mm CaCl, 0.33 mm MgSO4 in water) per well to acclimatise to their new environment. Between 10:30 pm and 10:50 pm on day 6, plates were transferred to Zebraboxes (Viewpoint). At 10:50 pm the Zebraboxes were closed to allow the fish to habituate to the darkness for 10 minutes, and tracking began at 11:00 pm. The experiment ran for 24 hours and 30 minutes, after which videos of the tracking were collected for analysis, and embryos were then sacrificed and genotyped.

Video analysis

In order to analyse the movements of each fish over the experimental period, videos collected from the locomotion assays were analysed using Ethovision software (Noldus, version 14). Movement

thresholds used were: Moving, 1 mm/sec; Stopping, 0.75 mm/sec; Detection threshold, Dynamic Subtraction, Darker, 9. Voxel smoothing was used to remove small video jitters and large errors in detection, with Direct movements smaller than 0.04 mm and larger than 12 mm per frame excluded.

Locomotion assay statistical analysis

Locomotion data was processed using Microsoft Excel 2013 and analyses were performed using SPSS Statistics 26 (IBM). Data was ordered chronologically into 10-minute bins. Any time points at the end of videos that were less than 300 seconds were excluded. For each fish, activity data was summed by hour. Then, a normalised value for each hour was determined by comparing activity per hour to the average activity value of all fish (calculated as the average activity of all fish from the respective replicate, for that respective hour). Genotyping data was then assigned to individual fish, and fish with ambiguous genotypes were removed from analysis. The data was then imported into SPSS. Data points from the 30 minutes past the initial 24 hours were excluded. Data was visualised using a line graph in GraphPad Prism Version 8.

To examine differences in activity between genotypes, a mixed linear model was used. Main effects of time and genotype, and an interaction effect of time by genotype were used. To account for known differences in Zebraboxes, a main effect of Zebrabox tracking system was used. If a significant ($p < .05$) main effect of Zebrabox tracking system was observed, it was kept in the model to account for any contributing variation. Repeated measures of time (hour) were modelled using a first order autoregressive variance structure. Random effects were defined as individual animals, grouped by genotype. A natural log transformation was applied to the normalised data to meet assumptions of normality which were checked by inspection of the residuals. F tests were performed using a maximal likelihood model, with Satterthwaite estimated degrees of freedom.

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Conflict of Interest

The authors declare no conflicts of interest.

Thesis Discussion

The use of zebrafish models to examine neurodevelopmental disorders is becoming increasingly popular (Fontana et al., 2018). Their utility and versatility for exploring behavioural and morphological phenotypes make them a well-rounded tool, and should be considered when investigating the functional relevance of other ADHD-risk genes in the development of the disorder. In this thesis, I have functionally assessed the impact of three ADHD-associated variants, mapped to *CHMP7*, *DUSP6*, and *KDM4A*, on the development of activity and brain volume phenotypes. These provide the first known examples of functionally examining zebrafish lines mutant for significant ADHD-GWAS genes. I have demonstrated that variants mapped to *DUSP6* and *KDM4A* are significantly associated with changes in their respective mRNA levels in post-mortem brain tissue, similar to what has been shown in *CHMP7* (Tong et al., 2016). Further, I have demonstrated that both reduced *chmp7* mRNA, and loss of Kdm4aa;Kdm4ab protein function, are sufficient to cause increased and decreased activity phenotypes respectively, over a 24-hour period at 6 days post-fertilisation (dpf). These findings suggest that these genes play roles in the development of ADHD hyperactivity phenotypes. In addition, the hyperactive phenotype seen in *chmp7*$^{+/-}$ fish is ameliorated after application of methylphenidate, pointing to a potential role for Chmp7 in dopaminergic signalling. I have also examined loss of Dusp6 function, and no activity phenotype was observed. However, I have found evidence to suggest a synergistic increase in activity in *dusp6*$^{-/-}$ fish after application of methylphenidate (albeit non-significant), which, together with the interaction between *chmp7* and methylphenidate, highlights the variation in drug response between different ADHD genetic models. Finally, I have demonstrated that reductions in *chmp7* mRNA levels, but not the loss of Dusp6 function, result in reduced zebrafish brain volume.

One aspect of functionally characterising these genes that was unfortunately not displayed in this thesis, was the examination of the underlying mechanisms behind the phenotypes of the genes of interest. A major reason for this was the lack of appropriate antibodies for performing the experiments that were originally envisaged. For *chmp7*, examination of neurotransmitter turnover, such as the dopamine transporter, was unable to be completed due to not having an antibody specific enough to be confident in the quantification of turnover. This was similar for *dusp6*, in which quantifying the amount of stabilised Slc6a3 would have provided insight into how much the mutation induced in *dusp6* was affecting its protein function. These problems extended to not having antibodies to quantify and examine the protein produced from the mutated genes of interest, resulting in the need to rely on qrt-PCR to detect mRNA levels, and predict the effect on protein function through DNA sequencing. For each of the proteins of interest, several antibodies were tested using Western blots with varying levels of specificity, but none enough to be used for quantification. Despite this, the use

of behavioural and morphological assays provides examples of how to utilise zebrafish to do preliminary assays to determine if genes of interest can impact ADHD phenotypes in a way that is meaningful to the development of the disorder.

Using these methods, I have demonstrated that *CHMP7* and *KDM4A* are contributing to the development of activity phenotypes, while the potential role of *DUSP6* in ADHD requires further investigation. The differences between *KDM4A* and *DUSP6* highlights that significant ADHD-GWAS associations require intensive scrutiny via multiple genetic models and functional assays, to determine how, or if, their association with ADHD is contributing to the development of ADHD phenotypes. Overall, this thesis provides the first line of functional evidence that ADHD-GWAS associations play a role in the development of ADHD. I also discuss how future investigations of ADHD-associations can be optimised, to determine their functional impact on ADHD phenotypes.

How do *CHMP7*, *DUSP6*, and *KDM4A* contribute to what we know about ADHD

The hyperactivity and brain volume reduction phenotypes observed in *chmp7*$^{+/-}$ fish confirm that previously sub-threshold ADHD-associations can be relevant to ADHD. In addition, examination of two significant ADHD-GWAS hits has demonstrated that one, *KDM4A*, is associated with an activity phenotype, while the other, *DUSP6*, does not seem to contribute to an observable activity or brain volume phenotype. While *DUSP6* requires further examination with the use of alternative genetic models, this highlights that significant ADHD-GWAS associations need to be experimentally examined in order to determine their role in the development of ADHD.

With regards to Chmp7, I have provided evidence to confirm that a reduction in *chmp7* mRNA levels can cause an ADHD phenotype, hyperactivity, in larval zebrafish. However, this effect does not persist into the juvenile or adult stages. This highlights that the lack of persistent ADHD diagnoses into adulthood could have a genetic basis. We currently know that the predominantly inattentive subtype of ADHD becomes more prevalent as ADHD groups age, moving from the combined and hyperactive subtypes (Willcutt, 2012). This has been postulated as due to the attentional demands on individuals increasing as they age, placing more emphasis on attentional phenotypes until hyperactive phenotypes are no longer detectable (Willcutt, 2012). However, the decline in hyperactive symptoms can also be attributed to neuromodulator abnormalities caused by genetic differences being resolved by adulthood. In support of this, the response of *chmp7*$^{+/-}$ 6 dpf fish to methylphenidate suggests that these fish have lowered levels of synaptic dopamine, or noradrenaline. This could lead to decreased long term potentiation (LTP) of synapses (Kerr and Wickens, 2001; Kitada et al., 2007; Tripp and Wickens, 2012), which in turn could result in a less mature neural system at 6 dpf than wildtype

counterparts. Given enough time (e.g development to adulthood), this immature neural network could undergo enough LTP to develop the proper connections that were lacking at a younger stage. This would be consistent with the developmental delay hypothesis of ADHD, and suggests that targeting genes such as, or downstream of, *CHMP7*, could help to alleviate this delay.

In the third chapter of this thesis, I have demonstrated that loss of Kdm4aa;Kdm4ab function leads to decreased activity in 6 dpf fish, thus showing the first evidence that a significant ADHD-GWAS association can have a functional impact on ADHD development. It is likely that ADHD-GWAS associations play a neurodevelopmental role (Dark et al., 2018), and in the case of KDM4A, this could be through a role in the regulatory timing of histone modifications on neural specification genes (Prajapati et al., 2019). Understanding KDM4A's role in neurodevelopment, let alone ADHD, is hardly straight forward. Demethylation via KDM4A can both activate differentiation via neural specifier genes (Prajapati et al., 2019), and promote self-renewal of embryonic stem cells (ESCs) to prevent premature differentiation (Pedersen et al., 2016). This suggests that KDM4A activity is required at a number of points in neurodevelopment.

KDM4A binding to DNA, and subsequent activation of target genes, is dependent on the transcription factor PR/SET Domain 1 (PRDM1 (Prajapati et al., 2019)). Therefore, PRDM1 and other recruitment factors may be interesting to investigate as candidate ADHD-associated genes. Examination of other members of the KDM4 family could also be of interest, as there is overlap between the demethylation targets of the KDM4 family (Labbé et al., 2013). In fact, knocking out both KDM4A and KDM4C is required to prevent self-renewal of ESCs, while knocking either out alone is not sufficient (Pedersen et al., 2016). This redundancy in demethylation targets could explain how loss of Kdm4aa;Kdm4ab function in the zebrafish does not lead to embryonic lethality.

In contrast to the results observed for the Kdm4aa;Kdm4ab mutant line, the loss of Dusp6 function did not result in a detectable ADHD phenotypes. This is suggestive that loss of dephosphorylation mediated regulation of mitogen activated protein kinase 1 and 3 (MAPK1 & 3), is either not contributing to ADHD related phenotypes, or other mechanisms are compensating for its loss. For example, it is possible that the lack of phenotype observed is due to genetic compensation from another member of the Dusp family, such as Dusp3, which is also known to dephosphorylate MAPK1 & 3 (Todd et al., 1999). Investigations into the potential roles for other DUSP members would therefore be of great interest.

More interestingly, the lack of detectable phenotypes could be due to an observation that regulation of *DUSP6* has been associated with sex-specific differences. *DUSP6* is downregulated in the ventral medial prefrontal cortex (vmPFC) of female individuals with major depressive disorder, as well as in

the vmPFC of female mice who had chronic variable stress induced depression and anxiety-like symptoms (Labonté et al., 2017). It is important to note that both depression and anxiety are frequently co-diagnosed with ADHD. Male and female mice were then exposed to enough chronic variable stress to increase their stress susceptibility, but not result in depressive symptoms. Downregulation of *DUSP6* in the vmPFC resulted in an increased susceptibility to stress in female, but not male, mice (Labonté et al., 2017). This is suggestive that regulation of Dusp6 may result in sex-dependent behavioural phenotypes. Brain volume also shows sex differences with regards to *DUSP6* genotypes. Male mice, but not females, possessing the A allele for the missense mutation, rs13480726 (mapped to *DUSP6*), show significantly decreased brain weight compared to the G allele (Bin Liu, 2008). While the combination of evidence doesn't suggest that one sex is impacted by disruptions to Dusp6 more than the other, it does highlight that an interaction between genotype and sex could be playing a role in the development of ADHD phenotypes. Therefore, it is possible that differences in behaviour and anatomy were present in the *dusp6* mutant line, but could not be identified due to the inability to determine the sex of zebrafish larvae at 6 dpf.

The role of newly discovered ADHD associations in the dopamine hypothesis

Given the propensity of previously established ADHD risk genes to play roles in neurotransmitter signalling, it is likely that a number of ADHD-associated genes, whose contributions to ADHD have not been determined, play roles is similar pathways. It is possible that both CHMP7 and DUSP6 are playing a role in dopamine signalling, either directly or by interaction with other proteins. The role of dopamine signalling in ADHD is well established (Barr and Misener, 2008). Dopamine homeostasis ensures there is an appropriate amount of dopamine in the synaptic cleft for regular activation of postsynaptic dopamine receptors, while preventing overstimulation. Too much dopamine, shown through the absence of the dopamine reuptake transporter in SLC6A3 knockout mice, leads to hyperactivity (Giros et al., 1996). On the other hand, too little dopamine in the spontaneously hypertensive rat (SHR), also demonstrates hyperactivity, potentially through increased cell surface expression of SLC6A3 (Miller et al., 2012). This evidence has led many to believe that ADHD symptoms are the result of an imbalance in dopamine signalling. This can be explained in the form of a U curve (Figure 1), in which too much or too little synaptic dopamine/dopaminergic signalling results in ADHD phenotypes.

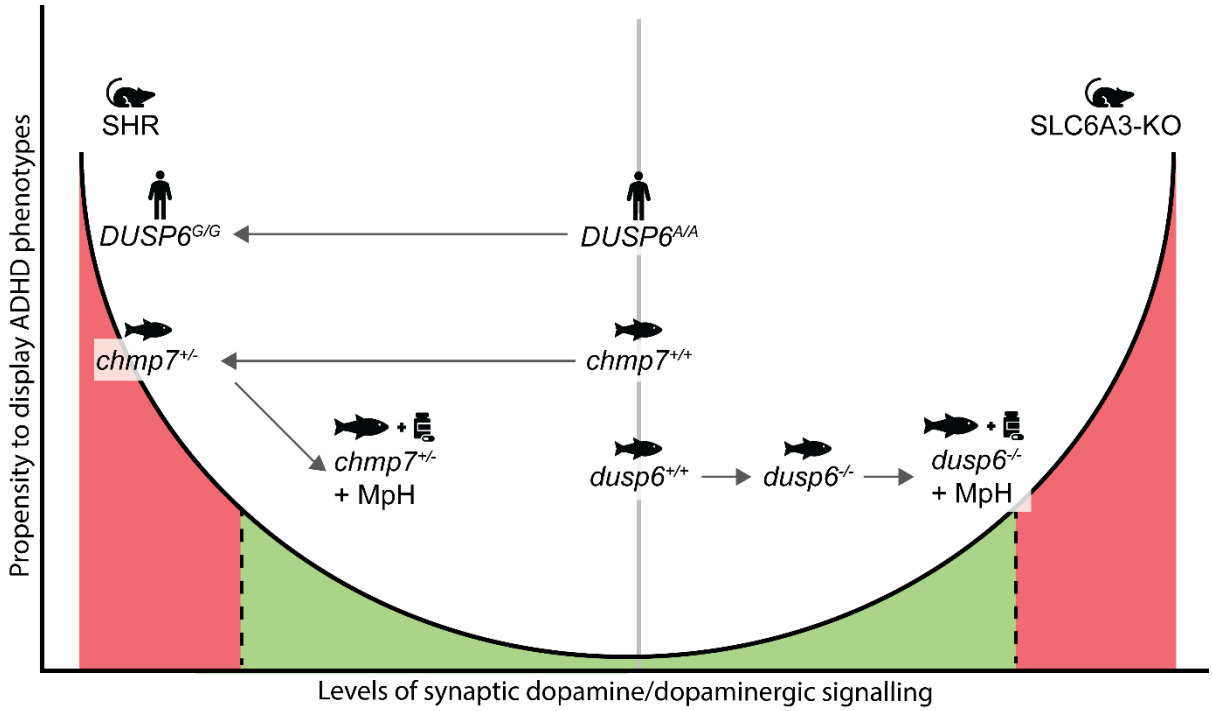

Figure 1. Representation of how *chmp7* and *dusp6* may act in the dopaminergic hypothesis for ADHD. Red zones depict the significant association with ADHD phenotypes, caused by decreased and increased synaptic dopamine/dopaminergic signalling, the levels of which are denoted by an arbitrary cut off at the dotted line. The green zone represents a "middle ground", where the levels of synaptic dopamine/dopaminergic signalling do not cause ADHD phenotypes. *DUSP6* genotypes are shown for the functionally predicted variant, rs10506971. SHR: spontaneously hypertensive rat, SLC6A3-KO: dopamine transporter knock-out, MpH: methylphenidate.

It is possible that both *CHMP7* and *DUSP6* are relevant to the dopaminergic hypothesis of ADHD, and disruptions to either gene could result in an imbalance of dopaminergic signalling. It is known that DUSP6 plays a role in stabilising SLC6A3 at the plasma membrane, which leads to increased dopamine reuptake (Mortensen et al., 2008). The higher expression of *DUSP6* mRNA in humans possessing the ADHD risk allele suggests that these individuals may have increased SLC6A3 at the plasma membrane and thus, increased reuptake of dopamine, resulting in ADHD symptoms (Figure 1). In this thesis, *dusp6*$^{-/-}$ fish were used to test the hypothesis that loss of Dusp6 function would lead to hyperactivity, expected following destabilisation of Slc6a3 and increased synaptic dopamine. However, loss of Dusp6 did not result in a detectable hyperactivity phenotype. Further, the application of methylphenidate to *dusp6*$^{-/-}$ fish was expected to increase activity, via a synergistic effect of increasing the levels of synaptic dopamine. Despite a positive trend, methylphenidate did not result in a significant activity

increase. This suggested that either the levels of synaptic dopamine were not sufficient to lead to an activity phenotype, or the loss of Dusp6 function did not sufficiently destabilise Slc6a3 to result in a substantial increase in synaptic dopamine. It would still be of interest to see if increased Dusp6, leading to reduced synaptic dopamine as previously demonstrated (Mortensen et al., 2008), could display a hyperactive phenotype.

In contrast to *dusp6*, *chmp7*$^{+/-}$ fish demonstrated significantly increased activity levels compared to wildtypes. While there was no prior evidence linking *CHMP7* to dopamine signalling, my finding that methylphenidate could rescue the hyperactivity phenotype of *chmp7*$^{+/-}$ fish suggests an increase of synaptic dopamine is enough to reduce hyperactive phenotypes in these fish (Figure 1). It is important to note that this could also be the result of increased synaptic noradrenaline, however, for the purpose of this discussion, I will focus on *CHMP7*'s potential role in dopaminergic signalling.

CHMP7 is known to interact with ESCRT-III, and overexpression of GFP-tagged CHMP7 leads to dominant negative effects in the form of decreased breakdown of endocytosed epidermal growth factor, intended for degradation by the lysosome (Horii et al., 2006). In addition, disruption to ESCRT-III subunits results in the accumulation of NMDA receptor subunit 1, suggesting reduced degradation (Lee et al., 2011). It is possible that reductions in CHMP7 could lead to disrupted endosomal recycling pathways, potentially involving neurotransmitter receptors as seen in Lee et al., (2011), which could extend to the recycling of dopamine receptors. Endosomal recycling of dopamine receptor D2 (DRD2) is known to be important for maintaining levels of the receptor at the plasma membrane (Li et al., 2012). While DRD2 has not been consistently associated with ADHD, dopamine receptors D4 and D5 (DRD4, DRD5), have (Gizer et al., 2009). Impaired endosomal recycling could result in reduced turnover of postsynaptic dopamine receptors such as DRD4 and DRD5. This would result in less dopamine receptors at the postsynaptic membrane, and thus decreased dopaminergic signalling. Experimental examination of CHMP7's role in the turnover of dopamine receptors could test this, and potentially provide a mechanistic basis for its contribution to ADHD phenotypes.

Currently, it is not known how, or if, the loss of KDM4A function contributes to dopamine signalling. However, the same was true for CHMP7 prior to the investigation with methylphenidate treatment. Not every ADHD-associated variant will contribute to the dopaminergic theory of ADHD. However, given the well-defined role of this pathway in this disorder, examining the impacts of new variants on dopamine homeostasis and signalling, would be a recommended starting point to determine the molecular mechanism underlying their contributions to the development of ADHD.

The benefits of testing ADHD drug treatments in zebrafish ADHD models

The use of commonly prescribed ADHD drugs to understand the molecular mechanism of the disorder can also provide pharmacogenetic value by predicting the efficacy of the drug prescribed for individuals possessing the ADHD risk alleles. It is known that variability in individual response to drug treatment is a problem when prescribing drugs for ADHD (Contini et al., 2013; Gilbert et al., 2006; Polanczyk et al., 2010). Therefore, examining the effectiveness of commonly prescribed ADHD drugs using ADHD-associated mutant lines could help establish a pharmocogenomic approach for ADHD. For example, while *chmp7*$^{+/-}$ fish responded to methylphenidate treatment by significantly reducing activity levels (results chapter 1, Figure 6), *dusp6*$^{-/-}$ fish did not, instead trending towards increased activity, although not significant (results chapter 2, Figure 7). This highlights how ADHD-associated variants mapped to *CHMP7* and *DUSP6* could be contributing to the variability we see in drug response. Examination of drug responses in fish mutant for ADHD risk genes could help to determine the most appropriate treatment for each variant. The combination of all of an individual's ADHD-associated alleles could then be used to design tailored drug treatment plans.

Testing drug treatments can also give preliminary indications of what neuromodulator systems are underlying the phenotypes seen in mutant lines. SLC6A3 knock-out mice show hyperactive phenotypes which can be attenuated through application of selective serotonin reuptake inhibitors, but not specific noradrenaline reuptake inhibitors (Gainetdinov et al., 1999). This suggests that serotoninergic, but not noradrenergic, transmission plays a role in reducing the hyperactivity phenotype. In *chmp7*'s case, the effectiveness of methylphenidate in *chmp7*$^{+/-}$ fish shows that the hyperactive phenotype is reduced, potentially by increasing the levels of dopamine in the synaptic cleft (Figure 1).

Overall, it should be noted that there are still some limitations regarding drug treatments in zebrafish, as drug absorption rates are not always as expected (Rubinstein, 2006), and pharmacokinetics studies are still needed for many drugs (Van Wijk et al., 2019). However, a large number of studies have examined neuropsychological drug treatments in zebrafish to great effect (Khan et al., 2017), including the effects of methylphenidate (Das et al., 2020; Kung et al., 2015; Lange et al., 2012; Parker and Brennan, 2016). Zebrafish, therefore, show great potential for ADHD pharmacological studies.

Examining ADHD in animal models: What are we really looking at?

In this thesis I have demonstrated how zebrafish can be used as a model to investigate ADHD-associated DNA variants. However, the first question that comes to mind, and is often asked, is can zebrafish get ADHD? The answer to this, in my opinion, is surprisingly simple: humans can have ADHD,

zebrafish can have ADHD-related phenotypes. This applies to any animal model, as ADHD is a disorder of human behaviour and it is important that we do not anthropomorphise the animal models we use. Instead, we utilise animal models to examine phenotypes that underpin what it means to have ADHD. However, the type of phenotype that we wish to examine can dictate what animal model is most appropriate, and vice versa. Having previous knowledge of the ADHD subtype that a variant is associated with can help to determine which phenotypes to examine. In the absence of this however, common ADHD phenotypes such as hyperactivity, inattention, and reduced brain volume can be analysed. In this thesis I have examined both the hyperactivity and reduced brain volumes commonly seen in individuals with ADHD.

I have utilised a 24-hour locomotion assay to measure fish activity, which is superior to short, 5-10-minute locomotion assays, for examining ADHD-related activity phenotypes. By their nature, behavioural phenotypes are highly variable (Raftery et al., 2014; Renart and Machens, 2014), and basing the sole measurement of this phenotype on one 10-minute time point is not sufficient to demonstrate a typical baseline level of activity. In the approach I took, movement was tracked every 10 minutes, and activity was highly variable between each time point for individual fish, and between fish at each time point. I summed the activity for each fish over each hour-long period, which gave a more stable indication of activity levels. However, the assay demonstrated how variable this behavioural phenotype can be for a single 10-minute time point. Furthermore, my results suggest that a 10-minute habituation period before analysis may not be sufficient time and there may still be prolonged exploration of the novel environment. An assay that assesses locomotion over a longer period of time avoids this question altogether, as it provides much longer for any prolonged habituation effects to wear off. Therefore, it is recommended that when investigating behavioural phenotypes such as activity, examination periods of up to 24 hours are better suited for capturing differences in baseline levels of activity. The use of zebrafish for these assays allows a high number of fish that can be examined in one experiment, despite requiring a tracking system for 24 hours at a time. This significantly increases the power to detect subtle differences between genotypes, while keeping the total number of experiments to a minimum.

High-throughput imaging of brain volumes using confocal microscopy is also possible in zebrafish, and is perhaps an even better ADHD-related phenotype to measure than activity. This is because changes in anatomical features will be less variable than behavioural phenotypes. The procedures for measuring brain volumes in this thesis (outlined by Gupta et al., (2018)), allow for the dissection of volumes from different regions of the brain, giving detailed analysis of where in the zebrafish brain is affected. In spite of this, examining changes in brain volume and structure may be better suited to rodent models, as rodent brains are more comparable to human brains than fish are. However, the

mechanisms that underlie neurodevelopment are highly conserved in zebrafish (Kalueff et al., 2014; Tropepe and Sive, 2003), despite the morphological differences to humans. This, in addition to the combination of high power and rapid brain development of zebrafish models, allows us to gather preliminary brain volume information on a large number of ADHD-associated variants in a short time frame, making the zebrafish more suitable for initial analysis of variants.

One ADHD phenotype that is not as straight forward to measure in zebrafish, is attention. However, attention as a construct has been well modelled in rodents (Bushnell, 1998; Bushnell and Strupp, 2009). In addition, there are well established tasks that can be used to examine sustained attention, such as the five choice serial reaction time task (5-CSRTT (Carli et al., 1983)), and the five choice continuous performance test (5-CCPT (Young et al., 2009)). The 5-CSRTT involves placing the mouse/rat in a chamber with five identical potential light sources on one side, and an automatic food dispenser on the opposite side. One of the light sources will randomly flash and if the animal correctly identifies the source of light by nose-poking the light source, it is rewarded with food. Multiple variables can be adjusted, such as the length of light flash, the brightness of the light, length of time between flashes, and the presence of distractors such as noises (Bari et al., 2008). The accuracy to which the correct source of light is detected, and then selected, is used as a measure of attention. The 5-CCPT utilises the same set up as the 5-CSRTT, however, there is also a condition where all five lights flash at once, which requires no response, adding a layer of response inhibition (Young et al., 2009). This is thought to have increased similarity to CPTs performed in humans, which are widely validated for examining sustained attention (Shalev et al., 2011).

Despite the extensive work on attention in rodents, a gold standard measure of attention in zebrafish has not yet been established. A zebrafish version of the 5-CSRTT has been developed for examining impulse control (Parker et al., 2013), however, its ability to measure attention is currently limited. A major criticism of attention assays in zebrafish is that it is difficult to prove that they are direct indicators of attention itself, rather than a correlative consequence of a learned or classically conditioned stimulus (Choo and Shaikh, 2018). A number of tasks have been adopted from rodent models (Echevarria et al., 2011), yet none truly measure attention, rather they infer attention phenotypes from correlates of attention, such as learning, and the ability to make choices based on visual and spatial cues (Bilotta et al., 2005; Colwill et al., 2005; Gerlai et al., 2009; Lau et al., 2006; Levin et al., 2003; Parker et al., 2012; Sison and Gerlai, 2010). These assays struggle to assess sustained attention, which requires the build-up of a cognitive load on the individual, thus assessing if attention can be maintained through periods of higher cognitive strain. A recently developed task, called the virtual object recognition test (VORT (Braida et al., 2014)), has shown promising results for examining attention. This modified version of a novel object recognition test, utilises 2D geometrical shapes on

two digital screens either side of the test tank to test the animal's visual attention to novel stimuli. The shapes stay the same for 10 minutes, then the fish is moved back to its home tank for 5 minutes, then is returned to the test tank for a further 10 minutes, with a novel shape replacing one of the old shapes. The fish's ability to discriminate as to which shape is new is measured through the time spent exploring the new shape compared to the old. Thus, more time spent exploring a novel shape suggests the fish is paying more attention to the fact that the shape has changed. Different factors such as how difficult it was for fish to discriminate between certain shapes, and making the shapes move in the same or different directions can also be used to change the cognitive load. Interestingly, adding movement to the shapes significantly improved the ability of fish to discriminate between shapes they previously could not discriminate between, suggesting movement plays a large part in how zebrafish pay attention to stimuli. In addition, injection of nicotine, which has been demonstrated to improve cognitive performance in attention tasks (Young et al., 2013), also improved discrimination between previously poorly discriminated stimuli. This task validation with nicotine suggests that the task is in fact examining attention in zebrafish, making the VORT very promising for examining ADHD inattention phenotypes. However, it is important to note that the assays that have been developed so far only examine these phenotypes in adults, which highlights a need to further develop these tasks for use at larval stages. Until then, the use of adolescent mice models for examining attention deficits in young animals will be a more viable option (Ciampoli et al., 2017; Remmelink et al., 2017), although the use of the VORT for examining attention deficits in adult zebrafish will at the least be able to identify if zebrafish ADHD models have attention phenotypes that persist into adulthood.

It is therefore possible to examine different ADHD phenotypes effectively in animal models. Knowledge of the ADHD subtype associated with a risk allele, such as from ADHD symptom scores, can help guide our assessment of what phenotypic assays to use, and thus what animal model would be most appropriate.

Examining non-coding ADHD variants in zebrafish

Uncovering the genetic background of ADHD through examination of singular variants, and how they impact gene function, is definitely not straight forward. While the immediate impact of coding sequence variants on protein structure is relatively easy to predict, the functional impacts of non-coding variants are much more difficult to discern. In addition, ADHD-GWAS variants are common in the general population (allele frequency > 5%), making them unlikely to be highly deleterious. Given that the vast majority of ADHD variants map to non-coding regions, understanding how these non-coding variants are functionally contributing to the development of the disorder is a challenge. As

highlighted throughout this thesis, mapping a significant non-coding association to a specific gene allows us to then examine the function of that gene in ADHD as a whole. However, it does not tell us if it is the functionally predicted variant, or another variant in strong linkage disequilibrium (LD) with it, that is impacting gene function. In addition, we can't even be sure that it is a single variant that is contributing to the phenotypic changes we see. Even if a variant is predicted to be more likely to be functional than the original association, it could be the combination of both, or even a whole haplotype of variants in strong LD, that affect gene function.

The uncertainty of which variants are functionally important leaves one wondering, what is the point of assessing single variants at all? In the cases shown in this thesis, once the genetic marker was mapped to a specific gene, the gene was functionally examined as a whole. To instead determine the functional consequence of a single variant, evidence that it was directly affecting transcription or translation would be necessary. Even then, that wouldn't exclude the contributions of other variants in LD, and testing each of those variants for causation would be very time-consuming. In fact, what is arguably more important than determining the influence of each variant individually, is understanding how a haplotype that is associated with ADHD influences the function of a gene as a whole. This would be a more natural representation of how a combination of variants works together to result in an ADHD phenotype.

But how could we examine this? Finding a conserved non-coding GWAS variant between humans and zebrafish is very rare (Madelaine et al., 2018), let alone a full haplotype. Rodents models have higher DNA sequence homology to humans than zebrafish, but it is incredibly unlikely that the full haplotype will match. In addition, the effect size predicted by a group of linked variants will be just as low as the original detected association, making it difficult to detect subtle differences without very large sample sizes. It is possible to produce transgenic models in the zebrafish that utilise human promoter sequences (Hou et al., 2006), however, this will not recapitulate the effects of the full haplotype of non-coding variants mapped to the gene. Locus specific genome editing using homologous recombination to replace endogenous promoters, UTRs, and enhancers with human sequences would allow the examination of the effect of the human variants on the endogenous zebrafish gene, but this is an inefficient and labour-intensive process. Until the availability of methods that allow easier examination of multiple DNA variants at once, the simplest solution is to examine how these haplotypes impact gene regulation, such as changes in expression that are associated with the ADHD-associated variant. This way, regardless of how each of the individual variants in the haplotype contribute to a particular change in expression, the end product of the model is the same.

A framework to examining non-coding ADHD associated variants could be summarized as follows. Initially, utilise bioinformatic pathways, such as that employed by Tong et al., (2016), to determine which non-coding variants are strongly linked to the associated marker. These can then be prioritised based on their likelihood for functionality and mapped to their respective genes. Following this bioinformatic approach, we can utilise brain cDNA libraries to examine whether or not there are changes in gene expression associated with the prioritised variant, and thus the linked haplotype. From this, we can gain insight into which genetic manipulation method is best suited for modelling the expression changes seen in humans.

Methods such as morpholino oligonucleotide gene knockdown and CRISPR genome editing can be used to reduce protein levels, while transgenic overexpression models are beneficial for investigating increased protein levels. However, there are advantages and disadvantages to each model. Morpholinos can block translation to allow the examination of a relative reduction of protein levels, based on the relative amount of morpholino injected (Stainier et al., 2017), and they don't trigger genetic compensation. However, they show variation in phenotypes due to off target effects (Joris et al., 2017), difficulty in maintaining a stable level of injected morpholino (Stainier et al., 2015), and the dilution of morpholino effects 4-5 days post-injection (Czopka and Lyons, 2011). CRISPR genome editing allows both loss of function (through inducing mutations in the DNA sequence), and knock-in (through homologous recombination) models at the endogenous gene locus. The direct targeting of the genomic sequence is a distinct advantage over morpholino and transgenic models. However, the efficiency of certain techniques, such as targeting non-coding regions, and knock-in models, is relatively low, and genetic mutants have been shown to trigger genetic compensation by other genes to make up for the loss of the mutant protein (Sztal et al., 2018). Transgenic overexpression models, driven by the endogenous promoter of the gene of interest, increases gene expression in regions where the gene is naturally expressed. Further, the addition of enhancer sites, and 3'UTR modifications that increase stability of the mRNA transcript, allows the degree of overexpression to be more accurately controlled. The random integration into the genome, however, can result in variation in the transgene's expression due to the position of which the gene has inserted, such as into other genes, or non-coding regulatory elements. Overall, the methods available for manipulating gene function and expression allow us to mimic the consequence of human mRNA level changes as closely as possible. However, while each method presents particular advantages, they also come with challenges that can influence the practicality of achieving a true representative model. Therefore, careful consideration is needed when selecting the most appropriate model for the future examination of ADHD-associations.

Exploring the need for multigenic ADHD animal models

Examining single gene models is useful for determining the function of ADHD-associated genes, and how they contribute to ADHD in isolation. Unfortunately, ADHD is not a monogenic disorder. A multitude of variants, all contributing small effect sizes, is the hypothesis behind the majority of studies searching for ADHD-associations, similar to other complex diseases and disorders (Hawi et al., 2015; Maher, 2008; Manolio et al., 2009). Therefore, animal models that better recapitulate the genetic background of ADHD would require genetic manipulation of several genes at once. In this vein, multifactorial animal models could be utilised to examine disorder relevant gene interactions. This would provide valuable information as to how the combination of ADHD-associated variants leads to a final ADHD phenotype, potentially in a synergistic way, or the phenotypic effects of two gene models could cancel each other out. For example, we would expect *chmp7*$^{+/-}$;*kdm4aa*$^{-/-}$;*kdm4ab*$^{-/-}$ fish to cancel out their respective phenotypes, while an overexpression model of *dusp6* or *kdm4aa*;*kdm4ab* crossed to *chmp7*$^{+/-}$ fish could result in an even stronger hyperactivity phenotype than is seen in *chmp7*$^{+/-}$ fish alone.

Multigenic animal model approaches have already been demonstrated in models of other diseases. For example, in mice models of prostate cancer, mice with either inactivated retinoblastoma (Rb) or p53 proteins led to the development of lesions on the luminal epithelium by 20 months of age (Zhou et al., 2006). However, crossing both lines quickens the development of lesions to around 8 months, and double mutant lesions are actually more representative of human prostate carcinomas than single mutants (Zhou et al., 2006). Looking at a zebrafish model, this time of Parkinson's disease, knockdown of Parkinson protein 7 (*park7*) leads to greater dopaminergic neuron loss following treatment with hydrogen peroxide, compared to wildtypes (Bretaud et al., 2007). However, when the negative regulator of p53, *mdm2*, is also knocked down alongside *park7*, fish undergo loss of dopaminergic neurons without the previously required oxidative stress of hydrogen peroxide (Bretaud et al., 2007). This demonstrates how multigenic models can provide greater insight for the accurate modelling of complex multigenic disorders.

Multigenic animal models can be designed in the same way suggested for single genes earlier. The main limitation here, is that depending on which techniques are used to mimic these expression changes, there quickly becomes a limit to the number of genes that can be practically examined within one organism. Gene knockout models and transgenics follow mendelian inheritance, meaning examining even two genes at once vastly increases the number of genotypes involved, making acquiring the number of subjects required for experiments more difficult to achieve, as in the cases for *kdm4aa*;*kdm4ab* double mutants (see results chapter 3). However, in combination with transgenic overexpression to mimic increases in mRNA, injecting multiple morpholinos to reduce function, can

circumvent this problem. Although this increases the risk of off-target effects and effects are limited to early developmental stages.

Despite the challenges in generating multigenic models of ADHD, the potential of synergistic amplifications of phenotypic effect size could mean that the number of animals required in multigenic models is much less than anticipated. As mentioned earlier, the knockdown of two prostate cancer causing genes caused an exacerbated phenotype, greater than each individual knockdown (Zhou et al., 2006). If individually examined mutations led to the same phenotype, then combining these mutations, and increasing the effect size, would reduce the number of animals required. Overall, the closer we get to modelling the multigenic elements of ADHD, the better understanding we will have of how different combinations of ADHD-associated variants lead to the phenotype in humans. This will eventually lead to more detailed, accurate diagnosis, and a greater potential to tailor treatment to suit the individuals' neurochemistry and genetic makeup.

Final conclusions

So, what is the endgame for investigations into the genetic background of ADHD? For starters, being able to genotype a child at birth for all known functionally validated ADHD-associated variants or haplotypes would provide a polygenic risk score (PGRS) for the disorder. The PGRS would give parents the overall likelihood of their child developing ADHD. It would also inform as to what ADHD subtypes the child is likely to exhibit, and whether they are likely to have a persisting diagnosis of ADHD into adulthood. This can help prepare families for the onset of ADHD symptoms, improving their understanding of how best to manage the progression of ADHD phenotypes seen in the child.

Knowledge of the full complement of ADHD risk alleles in an individual could also provide insight into what pharmacological treatments would be most appropriate, preventing the need to test different medications until an effective treatment is found. In the future, it may even be possible to use this information to predict which neurodevelopmental processes will be impacted in an individual. It may then be possible to rescue neurodevelopmental phenotypes through pharmacological treatment, potentially removing the need for lifelong symptom management. Overall, the earlier we can predict a potential ADHD diagnosis, the better chance we have of preventing the negative impacts associated with the disorder. While the scenario of using personal polygenic risk scores to tailor diagnoses and treatment plans is a long way off, the work in this thesis paves the way for future examination of newly discovered ADHD-associations. Hopefully, this will develop our understanding of this disorder, and one day result in improved patient outcomes for affected individuals.

Supplementary Material

Supplementary Material: Results Chapter 1

Supplementary Table 1. Primers used in the generation and genotyping of the *chmp7* mutant line

Primer	**Sequence**
chmp7 exon 2 gRNA sequence	GCCTCTGAAATGGACCCTGT
chmp7 exon 2 STOP cassette	CCGGCCTCTGAAATGGACCCGTCATGGCGTTTAAACCTTAATTAAGCTGTTGTAGTGTCGGCTCTGCTGGGCAGT
chmp7 exon 2 gRNA genotyping forward	TGTGGATTGAGCGTGTTTTC
chmp7 exon 2 gRNA genotyping reverse	GGGCGAACAATTTTGACTTC

Supplementary Table 2. Genes and sequences used for CHMP phylogenetic analysis

Organism	Gene	Sequence
Human	CHMP1A	ENSP00000380998.3
	CHMP1B	ENSP00000432279.1
	CHMP2A	ENSP00000310440.1
	CHMP2B	ENSP00000263780.4
	CHMP3	ENSP00000263856.4
	CHMP4A	ENSP00000324205.9
	CHMP4B	ENSP00000217402.2
	CHMP4C	ENSP00000297265.4
	CHMP5	ENSP00000223500.7
	CHMP6	ENSP00000317468.5
	CHMP7	ENSP00000324491.7
Mouse	CHMP1A	ENSMUSP00000000759.8
	CHMP1B	ENSMUSP00000147285.1
	CHMP2A	ENSMUSP00000005711.4
	CHMP2B	ENSMUSP00000004965.6
	CHMP3	ENSMUSP00000109815.3
	CHMP4B	ENSMUSP00000036206.9
	CHMP4C	ENSMUSP00000029049.5
	CHMP5	ENSMUSP00000030128.5
	CHMP6	ENSMUSP00000026434.6
	CHMP7	ENSMUSP00000047700.8
Zebrafish	Chmp1a	ENSDARP00000141533.1
	Chmp1b	ENSDARP00000141620.1
	Chmp2ba	ENSDARP00000055865.6
	Chmp2bb	ENSDARP00000008354.7
	Chmp3	ENSDARP00000055486.5
	Chmp4ba	ENSDARP00000017897.7
	Chmp4bb	ENSDARP00000023938.6
	Chmp4c	ENSDARP00000014221.6
	Chmp5a	ENSDARP00000115597.2
	Chmp5b	ENSDARP00000138817.1
	Chmp6a	ENSDARP00000127696.1
	Chmp6b	ENSDARP00000130680.1
	Chmp7	ENSDARP00000060627.4
Drosophila	Chmp1	FBpp0074859
	Chmp2b	FBpp0076869

Supplementary Material: Results Chapter 2

Supplementary Table 1: Primers used in the generation and genotyping of the *dusp6* mutant line

Primer	**Sequence**
dusp6 exon 3 gRNA sequence	GAGGCCCGTGGACTGAAGTG
dusp6 exon 3 STOP cassette	GATGAGGCCCGTGGACTGAAGTCATGGCGTTTAAACCTTAATTAA GCTGTTGTAGTGTGGCGTGCTTGTTCACT
dusp6 exon 3 gRNA genotyping forward	CAGTCATGCACTAGAAATCCCA
dusp6 exon 3 gRNA genotyping reverse	ATGTCATAAGCATCGTTCATGG

Supplementary Table 2. Genes and sequences used for DUSP phylogenetic analysis

Organism	**Gene**	**Sequence**
Human	DUSP1	ENSP00000239223.3
	DUSP2	ENSP00000288943.4
	DUSP3	ENSP00000226004.2
	DUSP4	ENSP00000240100.2
	DUSP5	ENSP00000358596.3
	DUSP6	ENSP00000279488.6
	DUSP7	ENSP00000418566.1
	DUSP8	ENSP00000329539.4
	DUSP9	ENSP00000345853.3
	DUSP10	ENSP00000355866.3
	DUSP11	ENSP00000272444.3
	DUSP12	ENSP00000356920.4
	DUSP13	ENSP00000361785.2
	DUSP14	ENSP00000478406.1
	DUSP15	ENSP00000278979.3
	DUSP16	ENSP00000228862.3
	DUSP18	ENSP00000333917.3
	DUSP19	ENSP00000343905.6
	DUSP21	ENSP00000343244.4
	DUSP22	ENSP00000345281.5
	DUSP23	ENSP00000357087.1
	DUSP26	ENSP00000256261.4
	DUSP27	ENSP00000271385.5
	DUSP28	ENSP00000344235.2
Mouse	DUSP1	ENSMUSP00000025025.6
	DUSP2	ENSMUSP00000028846.6
	DUSP3	ENSMUSP00000003612.6
	DUSP4	ENSMUSP00000033930.4
	DUSP5	ENSMUSP00000047900.6

	DUSP6	ENSMUSP00000020118.4
	DUSP7	ENSMUSP00000126984.2
	DUSP8	ENSMUSP00000049414.3
	DUSP9	ENSMUSP00000019701.8
	DUSP10	ENSMUSP00000045838.7
	DUSP11	ENSMUSP00000032071.9
	DUSP12	ENSMUSP00000027970.7
	DUSP13	ENSMUSP00000074553.2
	DUSP14	ENSMUSP00000018792.5
	DUSP15	ENSMUSP00000045815.5
	DUSP16	ENSMUSP00000098419.3
	DUSP18	ENSMUSP00000057346.4
	DUSP19	ENSMUSP00000028384.4
	DUSP21	ENSMUSP00000026018.2
	DUSP22	ENSMUSP00000089260.6
	DUSP23	ENSMUSP00000027826.5
	DUSP24	ENSMUSP00000051216.4
	DUSP26	ENSMUSP00000046794.7
	DUSP27	ENSMUSP00000083155.2
	DUSP28	ENSMUSP00000057690.6
Zebrafish	Dusp1	ENSDARP00000137487.1
	Dusp2	ENSDARP00000133300.1
	Dusp3a	ENSDARP00000081638.4
	Dusp3b	ENSDARP00000078867.5
	Dusp4	ENSDARP00000065663.4
	Dusp5	ENSDARP00000005408.7
	Dusp6	ENSDARP00000095269.3
	Dusp7	ENSDARP00000130880.1
	Dusp8a	ENSDARP00000022233.7
	Dusp8b	ENSDARP00000057317.5
	Dusp10	ENSDARP00000068814.3
	Dusp11	ENSDARP00000090031.4
	Dusp12	ENSDARP00000102008.2
	Dusp13	ENSDARP00000106948.1
	Dusp14	ENSDARP00000074788.3
	Dusp16	ENSDARP00000141033.1
	Dusp18/21	ENSDARP00000098678.2
	Dusp19a	ENSDARP00000059260.4
	Dusp19b	ENSDARP00000066449.4
	Dusp22b	ENSDARP00000058288.6
	Dusp23a	ENSDARP00000011527.7
	Dusp23b	ENSDARP00000111211.2
	Dusp26	ENSDARP00000131844.1
	Dusp27	ENSDARP00000141748.1
	Dusp28	ENSDARP00000077695.4
Drosophila	Dusp6	FBpp0074803

	Dusp10	FBpp0081288
	Dusp11	FBpp0087183
	Dusp12	FBpp0074510
	Dusp13	FBpp0074420
	Dusp15	FBpp0075564
	Dusp19	FBpp0110401
	Dusp22	FBpp0075564
	Dusp23	FBpp0079115
	Dusp26	FBpp0074420
	Dusp28	FBpp0292332

Supplementary Material: Results Chapter 3

Supplementary Table 1. Primers used in the zebrafish qRT-PCR experiments

Primer	**Sequence (5'-3')**
ef1α forward	CTGGAGGCCAGCTCAAACAT
ef1α reverse	ATCAAGAAGAGTAGTACCGCTAGCATTAC
mobk13 forward	AGCATTAAGGAATCATCTGTGGC
mobk13 reverse	CGAAACGGGTGAAGCGATG
lsm12b forward	CGTCGTAATCTCACCACCGT
lsm12b reverse	TCCTTCTGTGTTTGCTGTGC
gfap forward	GCAGACAGGTGGATGGACTCA
gfap reverse	CCGCTTCATCCACATCTTGT
neuroD1 forward	ATACCACGAAGGGCATGAAA
neuroD1 reverse	GGTCTTGTCCACGTCTCGTT
bdnf forward	TAGTTGCGCGGAGGTCTTAT
bdnf reverse	GCAGCTCTCATGCAACTGAA
olig1 forward	GGAGTTTGCGGACTGAAAGT
olig1 reverse	CCCTGGAGACTCCCAACAT
olig2 forward	TCAATTCTGCAAAGCCACAC
olig2 reverse	GAAACCCACGGACTTCTTGA
tbr1b forward	CAAAGCGCAGGTTTACCTCT
tbr1b reverse	TCAGCAAGAATCACGTCCAC
s100B forward	AACTCAAGGAGCTGCTCACG
s100B reverse	TCGAAAAACTCATGGCAACA
sox2 forward	CAGACTGCACATGTCCCAAC
sox2 reverse	TTTCCCTCCCCAAAAGAAGT

Supplementary Table 2. Genes and sequences used for KDM phylogenetic analysis

Organism	Gene	Sequence
Human	KDM1A	ENSP00000383042.4
	KDM1B	ENSP00000297792.5
	KDM2A	ENSP00000432786.1
	KDM2B	ENSP00000366271.3
	KDM3A	ENSP00000386660.1
	KDM3B	ENSP00000326563.5
	KDM4A	ENSP00000361473.3
	KDM4B	ENSP00000159111.3
	KDM4C	ENSP00000370710.3
	KDM4D	ENSP00000334181.5
	KDM4E	ENSP00000397239.2
	KDM4F	ENSP00000491279.1
	KDM5A	ENSP00000382688.2
	KDM5B	ENSP00000356234.3
	KDM5C	ENSP00000445176.1
	KDM5D	ENSP00000444293.1
	KDM6A	ENSP00000367203.4
	KDM6B	ENSP00000254846.5
	KDM7A	ENSP00000380692.2
	KDM8	ENSP00000286096.4
Mouse	KDM1A	ENSMUSP00000101473.1
	KDM1B	ENSMUSP00000038373.8
	KDM2A	ENSMUSP00000047683.7
	KDM2B	ENSMUSP00000038229.9
	KDM3A	ENSMUSP00000128789.1
	KDM3B	ENSMUSP00000037628.7
	KDM4A	ENSMUSP00000102014.2
	KDM4B	ENSMUSP00000025036.4
	KDM4C	ENSMUSP00000030102.5
	KDM4D	ENSMUSP00000061632.6
	KDM5A	ENSMUSP00000005108.7
	KDM5B	ENSMUSP00000038138.7
	KDM5C	ENSMUSP00000108207.2
	KDM5D	ENSMUSP00000061095.7
	KDM6A	ENSMUSP00000061539.8
	KDM6B	ENSMUSP00000091620.4
	KDM7A	ENSMUSP00000002305.8
	KDM8	ENSMUSP00000033010.2
Zebrafish	Kdm1a	ENSDARP00000150698.1
	Kdm2aa	ENSDARP00000134050.1
	Kdm2ab	ENSDARP00000099910.3
	Kdm2ba	ENSDARP00000152592.1
	Kdm2bb	ENSDARP00000133053.2

	Kdm3b	ENSDARP00000138150.3
	Kdm4aa	ENSDARP00000126239.1
	Kdm4ab	ENSDARP00000009763.7
	Kdm4b	ENSDARP00000156548.1
	Kdm4c	ENSDARP00000082162.4
	Kdm5a	ENSDARP00000143887.1
	Kdm5ba	ENSDARP00000156841.1
	Kdm5bb	ENSDARP00000023794.9
	Kdm5c	ENSDARP00000110667.2
	Kdm6a	ENSDARP00000116325.2
	Kdm6al	ENSDARP00000077934.4
	Kdm6ba	ENSDARP00000142796.1
	Kdm6bb	ENSDARP00000120451.2
	Kdm7aa	ENSDARP00000147148.1
	Kdm7ab	ENSDARP00000050378.6
	Kdm8	ENSDARP00000133489.1
Drosophila	Kdm2	FBpp0307736
	Kdm4a	FBpp0087961
	Kdm4b	FBpp0302636

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