Zebrafish Models of Attention-Deficit/ Hyperactivity Disorder (ADHD)

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Abstract Attention-deficit/hyperactivity disorder (ADHD) is a common, early onset neuropsychiatric disorder that is characterized by developmentally inappropriate inattention, hyperactivity, increased impulsivity and motivational/emotional dysregulation. However, although there is a significant genetic component to ADHD, relatively few risk genes have been identified and characterized. Furthermore, despite the effectiveness of pharmacological therapies such as methylphenidate, the long-term treatment outcome varies considerably depending on the psychosocial environment. The development of novel drug treatments has been hampered by a lack of knowledge regarding the genetics and neurobiology of ADHD. It is therefore necessary to develop animal models of ADHD in order to better understand its etiology and to improve the treatment options that are available. The aim of this chapter is to explore how we can develop zebrafish as a translational model for ADHD. We will first discuss the genetics and neurobiology of the disease. We will then consider existing animal models of ADHD and examine how the unique attributes of zebrafish can be used to extend this research. Finally, we will propose promising avenues for future research using zebrafish as an ADHD-like model.

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A.V. Kalueff (ed.), *The rights and wrongs of zebrafish: Behavioral phenotyping of zebrafish*, DOI 10.1007/978-3-319-33774-6_7

Keywords Animal model • Zebrafish • Larvae • Translational model • Psychiatric disorder • Attention-deficit/hyperactivity disorder • ADHD • Endophenotype • Hyperactivity • Impulsivity • Inattention

Abbreviations

5CSRTT	Five choice serial reaction time task
6-OHDA	6-Hydroxydopamine
ACC	Anterior cingulate cortex
ADHD	Attention-deficit/hyperactivity disorder
ATO	Atomoxetine
CDCV	Common disease-common variant hypothesis
DA	Dopamine
daMCC	Dorsal anterior midcingulate cortex
DAT-KO	*Dat* knock-out mice
DSM-III	Diagnostic and Statistical Manual of the American Psychiatric Association
fMRI	Functional magnetic resonance imaging
G×E	Gene×environment interaction
GWAS	Genome-wide association study
ITI	Inter-trial interval
LED	Light emitting diode
MPH	Methyphenidate
MRI	Magnetic resonance imaging
NA	Noradrenaline
NPD	Neuropsychiatric disorder
PCBs	Polychlorinated biphenyls
PET	Photon emission tomography
PFC	Prefrontal cortex
PT	Posterior tuberculum
SHR	Spontaneous hypertensive rat
SNP	Single nucleotide polymorphism
SPECT	Single-photon emission computed tomography
SSRI	Selective serotonin (5-HT) reuptake inhibitor
VNTR	Variable number of tandem repeats
WKHA	Wistar-Kyoto hyperactive rat
WKY	Wistar-Kyoto rat

1 Introduction to Attention-Deficit Hyperactivity Disorder

Mental illnesses, or neuropsychiatric disorders, are an extremely diverse set of diseases that affect all aspects of mental function including thinking, feeling and mood as well as the ability to relate to other people [1]. Neuropsychiatric disorders (NPD) place a massive strain on society; mental illness ranks second in the burden of diseases in established market economies [2]. Nevertheless, in spite of their prevalence, the drug therapies available to treat NPDs frequently fail to prove satisfactory long-term outcomes due to variable efficacy and intolerable side-effects. Despite the clear need for better treatments, many of the pharmacological compounds used to treat NPDs were discovered serendipitously 60 years ago and have not been significantly improved since [1]. The development of novel drugs has in part been hampered by a lack of knowledge about the underlying neurobiology of NPDs. Therefore, research into the etiopathogenesis of psychiatric disorders, led by a combination of human genetic studies and animal modeling of the identified gene variants, is mandatory in order to improve drug treatments and develop early interventions that could prevent or delay disease onset.

Attention-deficit/hyperactivity disorder (ADHD) is a common, early onset neuropsychiatric disorder that is characterised by developmentally inappropriate inattention, hyperactivity, increased impulsivity and motivational/emotional dysregulation. ADHD has similar prevalence rates across different cultural settings [3, 4], resulting in poor performance in school and impairments in multiple other domains of personal and professional life. ADHD has long been considered a childhood disorder that gradually resolves itself with maturation during adolescence. However, this view has been contested by systematic follow-up studies documenting the persistence of ADHD into adulthood [5]. The adult form of ADHD is associated with considerable risk for co-morbidity with other psychiatric disorders as well as failure of psychosocial adaptation [5]. ADHD can be further divided into different subtypes in the clinic: a predominantly inattentive subtype, a predominantly hyperactive-impulsive subtype and a combined form of both, which is the most common form of the disease [6]. The behavioral symptoms of ADHD may result from alterations to underlying cognitive and motivational/emotional processes such as behavioral/response inhibition, delayed gratification (choosing a smaller earlier reward rather than a larger later one) and executive function (higher order integrated cognitive processes that allow selective attention and the use of information in problem-solving tasks) [7, 8]. The symptoms of ADHD are thought to be—at least partially—controlled by neuronal networks in the prefrontal cortex, anterior cingulate cortex and parietal cortex, the dorsal and ventral striatum and cerebellum.

Although generally accepted as being a neurodevelopmental disorder with a risk of life-long impairments and disability, the variable combination of syndromal dimensions and the diversity of comorbid disorders render diagnosis of ADHD difficult and sometimes even contentious. At one extreme the energy, exuberance

and demanding behavior of ADHD patients is said to be part of the normal spectrum and psychiatrists are accused of needlessly using medication to pacify children. The evidence for this point of view is based in part upon the increasing number of people diagnosed with ADHD each year, and the difference in prescription policies between countries despite similar prevalence rates across cultural settings. At the other extreme, ADHD is presented as a purely biological construct that is caused by the interaction of genes and the environment and is treatable with medication (reviewed in [9]). The symptoms of ADHD were first described more than 160 years ago. An early description of ADHD-like symptoms can be found in a children's book written by the pediatrician and psychiatrist Heinrich Hoffmann. In "Die Geschichte vom Zappel-Philipp" (the story of fidgety Phil), Hoffmann described a boy who "won't sit still; he wriggles and giggles and then, I declare, swings backwards and forwards and tilts up his chair" [10]. In 1902 George Frederick Still wrote an account of 43 children with poor "moral control" who were aggressive, defiant, resistant to discipline and excessively emotional [11]. By the beginning of the twentieth century, diseases with similar behavioral phenotypes were described as minimal brain damage and then minimal brain dysfunction, even though there was no evidence of brain damage in the patients studied. By the 1970s, the symptoms of ADHD were first recognized as including attention deficits [12]. The symptoms of ADHD were then formalized in the third edition of the Diagnostic and Statistical Manual of the American Psychiatric Association.

ADHD is one of the most common neurodevelopmental disorders and affects around 3–5 % of children worldwide regardless of ethnicity or cultural setting [13, 14]. Although it is predominantly considered a juvenile disease, the symptoms of ADHD also persist into adulthood in about 30–50 % of cases [15, 16]. However, it is not yet clear to what extent the genes and symptoms linked to ADHD are similar in children and adults [5]. ADHD patients generally experience significant impairment of academic, behavioral and social performance [17, 18]. ADHD can also lead to life-threatening conditions. For example, children with ADHD show an increased risk of injury in traffic accidents [19]. ADHD patients are more likely to suffer from other NPDs, including depression, anxiety and substance use disorder [20–22]. ADHD is also the most common NPD to develop following brain injury [23], giving credence to idea that ADHD can be linked to damage or dysfunction of the brain. However, in common with all NPDs, it is difficult to untangle the neurobiological root cause of the multiple symptoms that are presented by patients. For example, the comorbid symptoms of ADHD could be caused secondarily to disease pathology; poor performance at school due to inattention could in turn lead to increased anxiety and depression [24]. Finally, although ADHD is often perceived as a negative attribute it may also have a positive impact on a person's life. ADHD patients are often very creative, good at problem solving and able to focus selectively on certain captivating tasks. If channeled in the correct way, the increased drive and energy shown by ADHD patients may even be enviable.

1.1 Areas of the Brain that Are Altered in ADHD

One of the difficulties facing research into the pathophysiology of ADHD is that the neural networks controlling cognitive and emotional processes in the non-ADHD brain (including executive function, working memory and attention) are poorly understood. Nevertheless, some of the brain areas that control the symptoms of the disease have already been identified. This research has combined imaging studies with knowledge of neural network architecture such as the catecholaminergic systems in the brain [24]. Photon emission tomography (PET), single-photon emission computed tomography (SPECT), magnetic resonance imaging (MRI) and functional MRI (fMRI) have all been used to examine ADHD patients [24]. MRI is particularly suited to studies of young ADHD patients since it is non-invasive and does not require the injection of a radioactive tracer. However, there are also limitations to MRI studies. ADHD patients tend to move a lot during the scan (due to increased motor activity), the disease is etiopathogenetically very heterogeneous making standardization problematic, and it is difficult to find subjects who have not already been exposed to drug treatments that could potentially skew the results [8]. Combined data from a number of neuroimaging studies have reported a 3% reduction in white matter volume in the brain of ADHD patients [7, 25, 26]. This reduction of brain volume does not progress during adolescence suggesting that it occurs early during embryonic development [8]. The reduction of brain volume may reflect a developmental delay in the maturation of cortical circuits in ADHD patients. In agreement with this, longitudinal studies of cortical thickness have reported that the initial reduction normalizes over time in ADHD children [27]. The thickening of the cortex to normal levels with age is paralleled by a reduction of symptoms. ADHD may thus be caused by a delay rather than a disruption of brain development [27]. Studies of potential asymmetries in the brain of ADHD patients have reported differences between the left and right caudate nucleus, globus pallidus and putamen [7, 8, 28]. However, the data from these studies are inconsistent making it difficult to draw any firm conclusion about the role of brain asymmetries in ADHD.

Structural MRI studies have also identified localized reductions in the size of some ADHD brain areas. Localized reductions in the size of the prefrontal cortex (PFC) [25, 26], corpus callosum [29] and cerebellum have already been reported. These areas all contain high densities of DA neuron terminals, a neurotransmitter which is intricately linked to the symptoms of the disease. So far, the majority of studies have focused on the frontal-striatal network, made up of the PFC (including the dorsolateral- and ventrolateral prefrontal cortex) and the striatum (caudate nucleus and putamen; reviewed in [30]). The PFC is known to be important for the control of attention, working memory and executive function [31, 32], key mental processes that underlie the behavioral symptoms of ADHD. Therefore, a reduction of PFC function is in accordance with the symptoms of the disease. In agreement with this, fMRI studies have already demonstrated a hypofunction of DA in the PFC of ADHD patients. There are also some behavioral similarities between ADHD and

patients with frontal lobe injuries [33]. Finally, both methylphenidate and atomoxetine (drugs which are given to ADHD patients) ameliorate the symptoms of ADHD by increasing catecholamine levels in the PFC [34, 35]. The striatum (caudate/putamen) has an important role in controlling executive function and motor output, and is connected to the dorsal anterior midcingulate cortex (daMCC, commonly known as the anterior cingulate cortex or ACC) and dorsolateral PFC. A decrease in striatum volume is also seen in ADHD patients, a phenotype which recovers by around 16 years of age [7]. Other areas of the brain which have been linked to the symptoms of ADHD include the parietal cortex and the cerebellum (reviewed in [24]). Cerebellar areas including the vermis and inferior lobes may affect both the control of motor movements and attention via connections to the PFC [36, 37].

1.2 Dopamine and Noradrenaline Control the Symptoms of ADHD

Data from drug treatments and genetic analyses have suggested that alterations in dopamine (DA) and noradrenaline (NA) (and to a lesser extent 5-HT) signaling most likely underlie the symptoms of ADHD. The combination of synthesis enzymes, transporters and metabolizing enzymes that mediate neurotransmitter signalling provide many mutable targets that can potentially lead to expression of the disease. Furthermore, DA and NA act via a large number of receptors which transduce neurotransmitter signaling in discrete neural circuits. The role of DA and NA in ADHD can be understood by re-examining the function of the PFC. DA and NA neurons have a dual function in the brain, acting both tonically to maintain a basal arousal state and phasically in response to external stimuli [37]. The prefrontal cortex is very sensitive to the levels of catecholamines in the brain which act via the D1 receptor (DA) and Alpha 2 adrenoceptor (NA) [38]. Moderate levels of NA act on the PFC to increase the "signal" or response to stimulation, whereas levels of DA act to decrease background "noise" [38]. Thus, small fluctuations in DA and NA can dramatically affect the neural circuits that control attention, arousal and executive function. Furthermore, both increases and decreases of DA and NA signaling have been found to modify PFC function in an inverted U-shaped dose response [39, 40]. In summary, the symptoms of ADHD can be ultimately thought of as being triggered by fluctuations in DAergic and NAergic tone at the level of the PFC—an area of the brain that has been suggested to underlie the symptoms of many NPDs [41].

1.3 Pharmacological Therapies for ADHD

The majority of compounds used to treat ADHD to date interact with monoaminergic neurotransmitter signaling pathways. Indeed, it was the observation that methylphenidate (MPH), a dopamine (DA) pathway drug, could be used to treat ADHD

that orientated research towards monoaminergic signaling. MPH is now the most frequently prescribed ADHD treatment, and under its trade name Ritalin has even achieved a certain dubious celebrity, having been mentioned in several American cartoon series including South Park. MPH is a synthetic amphetamine derivative that causes several behavioral improvements including sustained attention, impulse control, reduction of task-irrelevant behavior and decreased disruptive behavior in ADHD patients [42–44]. MPH treatment amplifies the duration and tone of DA signaling in multiple ways, including blocking the DA transporter Slc6a3/Dat, disinhibiting DA D2 autoreceptors and activating D1 receptors on postsynaptic neurons [44]. However, MPH treatment also increases DA signaling in the nucleus accumbens, an area of the brain associated with reward behavior; thus in common with other psychostimulants MPH has the potential to be highly addictive. Drugs which target the noradrenergic (NA) system have also been successfully used to treat ADHD patients, including bupoprion and atomoxetine [42]. Atomoxetine (ATO) [45] is a selective inhibitor of the presynaptic NA transporter with minimal affinity for other neurotransmitter transporters and receptors [35]. ATO treatment increases the level of both NA and DA in the PFC (due to increased firing of anterior projections from the locus coeruleus to the PFC DA neurons) thereby improving attention and cognition [35, 46]. Although ATO does not work in all ADHD patients, it appears to be a safe and efficacious treatment for children, adolescents, and adults [42]. However, around 30 % of ADHD patients do not respond to MPH treatment, and 40 % to ATO [5], highlighting the need to improve drug therapies. Other non-stimulant medication has also been successfully used to treat ADHD patients. For example, guanfacine is a selective alpha 2A adrenoceptor agonist that stimulates prefrontal cortical networks, reducing the symptoms of the disease [47, 48]. Conversely, selective 5-HT reuptake inhibitors (SSRIs) do not reduce ADHD symptoms, questioning the importance of 5-HT in this disorder [49]. Although pharmacological treatment of ADHD (often used in combination with behavioral therapy) has been highly successful in controlling disease symptoms, the drugs which are available are only palliative and the long-term effects of stimulant medication on adolescent development are not known. There is thus a clear need to improve the drug treatments available for ADHD at every possible level, including the efficacy, number of side effects and potential for abuse.

1.4 The Genetic Basis of ADHD

The symptoms of ADHD are highly heritable suggesting that there is a genetic basis of the disease [5]. This substantial heritability, with estimates of up to 80 %, has been documented in numerous family, twin and adoption studies. However, despite this strong genetic basis, relatively few ADHD-risk genes have been identified and characterized following genome-wide approaches or candidate gene studies [20, 50–52]. ADHD has been shown to be caused by both a combination of multiple common mutations as well as polymorphisms in single neurodevelopmental genes [53–55].

The majority of ADHD-susceptibility genes examined to date have been linked to monoaminergic signaling [56–60]. For example, multiple DA signaling-related genes have been linked to ADHD. In particular, association with polymorphisms in the gene encoding the DA D4 receptor (*DRD4* [57, 59]), the DA D5 receptor (*DRD5*, [61]) and the DA transporter gene (*DAT/SLC6A3* [62]) have been reported. DAT terminates synaptic activity by reducing DA to a level at which it can no longer activate receptors. Thus, efficient DAT activity is needed in order control the strength and duration of DA neurotransmission. Most studies of *DAT* have focussed on a 40 base-pair variable number tandem repeat (VNTR) found in the 3′ untranslated region of the gene [63–65]. There is also some evidence associating the DA synthesis enzyme *Dopamine-beta hydroxylase* (*DBH*) with ADHD [66]. In the serotonin (5-HT) pathway, the 5-HT synthesis enzymes *TPH1* and *TPH2* [67, 68] and the 5-HT transporter gene (*SERT/SLC6A4* [69]) have all been linked to the disease. There is also conflicting evidence linking the receptors *HTR1B* and *HTR2A* to ADHD [70]. Recent GWAS and candidate gene studies have also identified polymorphisms in genes that are involved in cell adhesion (including *ASTN2* and *CDH13*) and synaptogenesis (S*NAP25*, *CTNNA2*, and *KLRN* [51, 55]). As well as being caused by direct modification of neurotransmitter signaling, ADHD may be triggered by more general alterations in brain formation, including cell signaling, morphogenesis and migration during development.

Environmental factors also play a significant role in the risk of suffering from ADHD. Environmental factors that have been linked to expression of the disease include exposure to nicotine, alcohol or psychosocial adversity (including child abuse, single-parenthood, marital discord or parental psychiatric disorders) [71–73]. For example, interaction of *DAT* and nicotine [73–75], alcohol [71] and psychosocial adversity [76] have been found to increase ADHD susceptibility. The gene-by-environment (G x E) interactions that increase ADHD susceptibility are still relatively poorly understudied, perhaps reflecting the difficulty inherent in conducting this research. It is also important to remember that the environmental factors that increase the risk of ADHD may also be under genetic control; the propensity of a mother to drink or smoke could be caused by genetic influences related to maternal ADHD [7, 76]. Thus environmental influences could ultimately reflect the interaction of the parent's and child's genome in controlling the expression of mental illness.

2 Translational Models of Human Diseases

Although recent studies have uncovered some of the genes linked to PDs, only few of these have been validated experimentally. It is therefore essential to use animal models in order to investigate whether a loss- or gain-of-function leads to disease pathology in each case. The complicated genetic basis of NPD makes it difficult to fully recreate them in animal models. One way to simplify this problem is to measure endophenotypes, neuropsychological or biological markers that correlate to a

disease-gene's activity [77, 78]. An ideal endophenotype should be controlled by a single gene, be associated with expression of the disease in the population and be both heritable and state independent (meaning that it is expressed even when the illness is not active; [79]). Although endophenotypes for NPDs have rarely fulfilled all of these criteria, successful markers have been developed for mood disorders [80, 81], Alzheimer's disease [82, 83] and ADHD [7, 84]. Endophenotypes may help to translate information from animal models to human patients. Furthermore, the division of NPDs according to endophenotypes may help redefine psychiatric diseases. Diseases could thus be reclassified on the basis of their molecular pathology instead of behavioral symptoms, both simplifying their diagnosis and providing an explanation for co-morbidity with other diseases [77].

Despite the difficulty of modeling NPDs, studies in animals still have the potential to give insights into the etiology of mental illness making them critically important. The first animal models were established on the basis of behavioral phenotyping. Behavioral changes that appeared to mimic some aspect of the human disease were rescued with specific treatment drugs, thus validating the model. However, the advent of tools to modify genes or their expression has now allowed the creation of animal models that are firmly based upon the genetic pathways underlying a disease. A perfect animal model should have three main attributes: construct validity (meaning that it conforms to the underlying rationale of the disease), face validity (mimicking some of the characteristics of the disease) and predictive validity (allowing the prediction of novel disease symptoms, or identification of disease treatments [85–87]). The animal model should also combine genetic tractability, tools to visualize and manipulate neurons in vivo, and the ability to translate findings to patients based upon conserved neurobiology.

2.1 Modeling Psychiatric Disorders in Zebrafish

Although rats, mice and fruit flies have been extensively used to model human diseases, recent work has demonstrated that zebrafish are poised to become a valuable translational model [88]. Zebrafish have already been established as one of the premiere organisms to study vertebrate development. In parallel, a battery of tests for behavioral analysis of both larval and adult zebrafish has also been developed [89, 90]. Zebrafish develop rapidly outside of the mother, making it easy to collect and manipulate embryos. By 6 days, larval fish swim continuously, search for food and are able to escape from predators thus demonstrating a range of innate behaviors. Zebrafish are optically transparent until larval stages allowing the study and manipulation of neural circuits at the cellular level in the intact brain [91]. The large number of identified mutant lines, genetic tools (such as TALENs and Zinc-Finger nucleases to knock-out genes [92–94], genetic ablation [95] and optogenetics [96, 97]) and techniques to monitor neural activity (including calcium indicators and electrophysiology [98]) make zebrafish an ideal model for neuroscience. Although the formation, position and function of neurotransmitter signaling pathways

sometimes differ between zebrafish and other vertebrates, comparative studies are beginning to precisely map these differences, allowing the transfer of information gained in zebrafish to other species [99]. These attributes have already been used to investigate the genetic basis of complex behaviors including reward and prepulse inhibition as an endophenotype for schizophrenia [100–102].

In spite of the experimental advantages of zebrafish it does not yet rival rat or mouse as a translational model for human disease. Thus, the challenge faced by zebrafish researchers is to design studies that harness the strengths of fish as a model system including live imaging, optogenetic interrogation of neural circuits and high-throughput screening of novel compounds. Indeed, although zebrafish are often touted as an excellent high-throughput system, this potential has been relatively under used (but see [103, 104]). Another challenge facing the field of translational research (in zebrafish as well as other animals) is to develop models for other, less-well characterized, diseases. In the rest of this chapter we will concentrate on one such NPD—attention-deficit/hyperactivity disorder.

3 Modeling ADHD in Zebrafish

Although the existing animal models for ADHD have provided novel insights into the genetics and neurobiology of the disease, there is clearly still room for development of new models. Despite the numerous advantages of zebrafish for developmental biology and neuroscience, there are currently very few studies that have reported an ADHD-like model in zebrafish [105, 106].

Since we do not know a priori which of the innate behaviors shown by fish could constitute an ADHD endophenotype we have to start by using a candidate gene approach. Starting with data from genome-wide screening approaches of ADHD patients we can identify and clone the homologous gene in zebrafish. We can characterize the expression of the ADHD-linked gene during neural development and then abrogate gene activity by either injecting morpholino oligonucleotides [107] or creating a novel mutant line. We can then assay the behavioral changes that are manifested by morphant (gene-specific morpholino injected) or mutant zebrafish in an attempt to identify novel ADHD-linked endophenotypes. The ensuing behavioral changes can be measured with- and without application of an ADHD treatment drug, thus providing face validity for the zebrafish model. Finally, the morphants can be used to investigate alterations to neurotransmitter signaling triggered by loss of gene function, as well as to screen for novel therapeutic compounds. In this approach we use endophenotype in the loosest sense of the word—a measurable behavioral phenotype that corresponds to the activity of a disease gene and represents a subset of the symptoms of the disease.

Morpholinos are an excellent tool to transiently knock-down gene activity, but they cannot be used to mimic gain-of-function mutations or to assay the impact of SNPs on gene function. One method that could be used to address this issue (but which has not yet been used in zebrafish ADHD studies) is to combine morpholino

knock-down with co-injection of mRNA encoding either a "humanized" form of the zebrafish ortholog or the human disease gene itself (reviewed in [108]). Thus the human gene can replace the function of the zebrafish paralog during development and alterations to neuroanatomy and behavior can be assessed. In this way, the importance of SNP polymorphisms that have been identified in human genes to disease progression can be studied. This method constitutes a promising avenue for linking novel SNP polymorphisms to the formation of ADHD and should be further explored in the future.

Of the three major symptoms of ADHD—inattention, hyperactivity and increased impulsivity—it is easiest to devise tests to measure hyperactivity in zebrafish. Although hyperactivity can be readily measured in both adult [109, 110] and larval fish [90, 111–113], for the purposes of this chapter we will concentrate on larvae since they are more amenable to high-throughput analysis and live-imaging. Conversely, it is perhaps better to measure impulsivity and inattention in adult fish since we do not know at which age the larval brain is mature enough to mediate these behaviors. The small number of protocols available to measure impulsivity and inattention perhaps reflects the difficulty of designing tests to measure them (see [114]), as well as the general under-appreciation of adult zebrafish as a behavioral model [115]. Behavioral analysis of adult zebrafish may require the use of stable zebrafish mutant lines, since morpholino knock-down is transient and gene activity will recover by 3–4 days of development [107]. Thus morpholino-mediated knock-down may not be suitable for studies of impulsivity or inattention. Regardless of this drawback however, injection of morpholinos can still alter the expression of ADHD-linked behaviors in adult fish. For example, reduction of *nr4a2* (an ADHD-linked dopaminergic orphan nuclear receptor; [116]) activity during development leads to permanent hyperactivity, indicating that a critical developmental process was affected that does not appear to recover over time [117]. Correlated permanent changes to the neuroanatomy of adult *nr4a2* morphants have not yet been studied.

3.1 Zebrafish ADHD-Like Endophenotypes: Hyperactivity and Motor Impulsivity

Zebrafish larvae hatch from their chorion at around 4–5 days post fertilization at which point frequent bouts of swimming occur [118–120]. Changes in the speed of locomotion are easy to measure and can even be quantified without using sophisticated equipment. For example, the number of times that larvae cross gridlines drawn on a Petri dish within a defined time-window could be counted. Nevertheless, we prefer to use videotracking software to measure the locomotory behavior of larval fish (such as Zebralab from Viewpoint Life Sciences, or Daniovision from Noldus) [90] (Fig. 1). Videotracking allows the automated tracking of multiple animals at the same time, reduces both observer bias and inter-observer variability and permits the simultaneous measurement of multiple parameters (including speed and distance swum, turning angle, time spent at the side or middle of an arena and the total

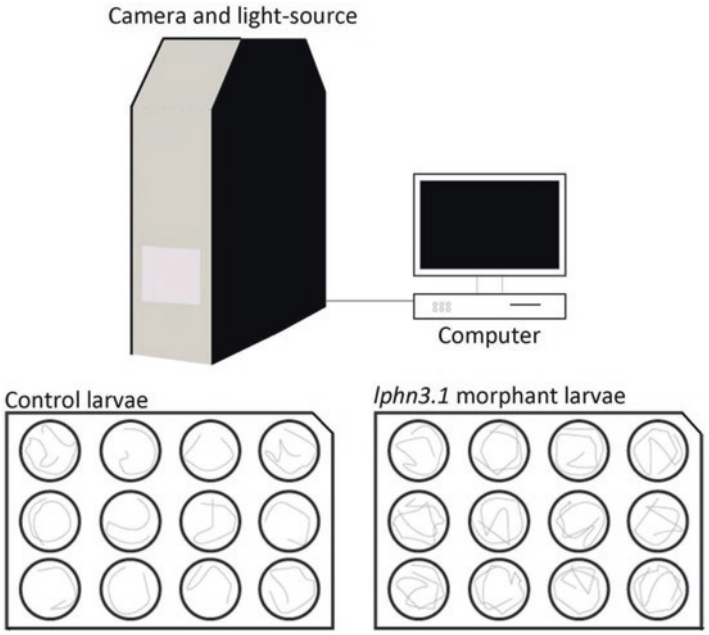

Fig. 1 Equipment used to measure larval locomotion. Cartoon representation of setup used to measure locomotion in *lphn3.1* and control morphants. Larvae are placed into twelve-well plates which are then mounted in a box containing a light-source and camera. A computer running specialized software tracks the position of the larvae during a 5-min experiment, allowing the distance swum and speed of locomotion to be calculated

time spent resting during the experiment). The hyperactivity shown by ADHD patients can be remarkably stable over time and may also be maintained at night [121–123]. Computer-automated setups use infra-red light to detect motion, thus allowing the activity of larvae to be recorded in the dark. As well as the total increase in the distance swum in a defined time-window, the pattern of larval swimming can be measured by looking at the bursts of acceleration that 6-day old larvae use to propel themselves. ADHD-associated increases in impulsivity can be subdivided into both motor and cognitive components [124, 125]. The locomotion curves of hyperactive larvae may show sharper peaks of acceleration than animals with normal activity levels, a pattern interpreted as motor impulsivity.

3.2 Zebrafish ADHD-Like Endophenotypes: Inattention

The ability to pay attention is a complex behavior that includes a number of hypothetical cognitive processes. Indeed, the measurement of attention in animals remains a controversial subject among neuroscientists [126]. However, despite the

difficulty of measuring attention in animals, its importance in a number of NPDs makes it necessary to design experiments to probe this issue. In a recent review of attention studies in animals, Bushnell proposed that attention can be divided into five types of cognitive process: orienting, expectancy, stimulus differentiation (selecting between two stimuli), sustained attention, and parallel processing [126]. Therefore, each of these processes could be used as a basis to develop endophenotypes for attention in animals. To date there is no single behavioral test that can directly measure attention (or inattention) in zebrafish. However, it might be possible to infer information about attention from the results of other behavioral tests [127]. For example, behavioral paradigms include visual discrimination in a T-maze or plus maze [128, 129], or appetitive instrumental conditioning in a choice assay [130, 131] could be used. Indeed, the appetitive conditioning tests are fairly similar to the 5 choice serial reaction time task (5CSRTT) described below, without a variation in the inter-trial interval [185,186]. Nevertheless, whilst these tests demonstrate a certain amount of cognitive ability in zebrafish, it is still not clear to what extent attention is being measured, or whether there is any link to ADHD. It is clear that inattention tasks for adult zebrafish require more development before they can be proposed as endophenotypes for ADHD.

3.3 Zebrafish ADHD-Like Endophenotypes: Cognitive Impulsivity

There are very few studies that have reported measurements of cognitive impulsivity in zebrafish. In rodents a five choice serial reaction time task (5CSRTT) has been established, in which impulsivity is defined as a premature response during an inter-trial interval (ITI)—the animal is unable to wait for a stimulus presentation before performing a behavioral response (usually a nose-poke [132]). Brennan and colleagues have developed a 5 choice serial reaction time task (5CSRTT) that can be used to measure impulsivity [114, 133, 134]. The 5CSRTT is measured in a tank that has a green LED on one side and five yellow LEDs in separate compartments on the other. Following illumination of the green LED, adult zebrafish are taught to only enter the compartment where the yellow LED is switched on. The correct execution of this behavior is reinforced with a food reward. Following a training period, in which the fish learns to associate the yellow light with a reward, the 5CSRTT can begin. The green stimulus LED is first activated and is then followed by a 10 s ITI. Following this pause, one of the yellow LEDs is lit, and the fish is rewarded with food upon entering the correct compartment. However, entry into any compartment before the end of the ITI, perhaps indicative of impulsivity, will result in a punishment (a 10 s time-out with no food). Entry into an incorrect compartment on the other side (i.e. one in which the yellow LED is not illuminated) will also trigger the punishment. Interestingly, ATO treatment reduces- and MPH increases anticipatory responses in this test [134], providing a possible link to ADHD-like behavior. The 5CSRTT is an impressive test for adult zebrafish, and appears to be a promising

paradigm to measure ADHD-linked cognitive impulsivity. Nevertheless, it has only been tested on wild-type animals and so needs to be applied to zebrafish lacking the function of an ADHD-linked gene before we can decide whether or not it constitutes an ADHD-related endophenotype.

3.4 Reduction of Lphn3.1 Activity During Development Triggers ADHD-Linked Alterations in Larval Zebrafish Behavior

As an example of how zebrafish can be used to analyze the function of ADHD-linked genes, we have recently conducted an analysis of *latrophilin 3.1* (*lphn3.1*) during zebrafish development [105]. *LPHN3* is an orphan adhesion-G protein-coupled receptor whose gene contains a variation that conveys a risk haplotype for ADHD. *LPHN3* was identified by linkage analysis of a genetically isolated European population in Columbia (that originated from Spain), followed by fine-mapping in several North American and European populations [135]. Replication of the finding in a cohort of Spanish ADHD patients suggests a role for LPHN3 in the adult form of the disease [136]. *LPHN3* was also identified as one of 86 risk genes in a genome-wide association study of patients with substance abuse disorders, suggesting that ADHD and substance dependence share a high degree of comorbidity [137]. LPHN3 has the capacity to moderate cell-cell interactions. It can act as one of the receptors for α-latrotoxin, a component of black widow spider venom, causing exocytosis of neurotransmitter-containing presynaptic vesicles. The connection between Latrophilin activity and synaptic signaling has been strengthened by the recent identification of two families of endogenous ligands for Latrophilins, the Teneurins and the FLRTs (Fibronectin leucine-rich repeat transmembrane proteins; [138, 139]). For example, FLRT3 appears to specifically interact with LPHN3, is expressed in restricted areas of the developing mouse brain and may control the number of Glutamatergic synapses which are formed [138]. Thus, although the normal physiological function of LPHN3 is not well understood, its function in relation to the formation of synapses during brain development is a particularly promising area for future research.

latrophilin3.1 is one of two zebrafish homologues of human *LPHN3*, both of which are expressed in differentiated neurons throughout the brain up to 6 days post fertilization. We reduced *lphn3.1* function during zebrafish development by injecting one of two gene-specific morpholinos. We then measured larval behavior at 6 days and found an increase in the distance swum by the morphants, a hyperactive phenotype (Fig. 2). The hyperactivity of *lphn3.1* was maintained during the night, suggesting a permanent increase in locomotion compared to control-injected animals (data not shown). *lphn3.1* morphants also show an increase in the number of bursts of acceleration while swimming, indicative of motor impulsivity (Fig. 3). In order to probe the link between changes in *lphn3.1* morphant behavior and ADHD, we rescued the hyperactivity and motor impulsivity by applying the ADHD treat-

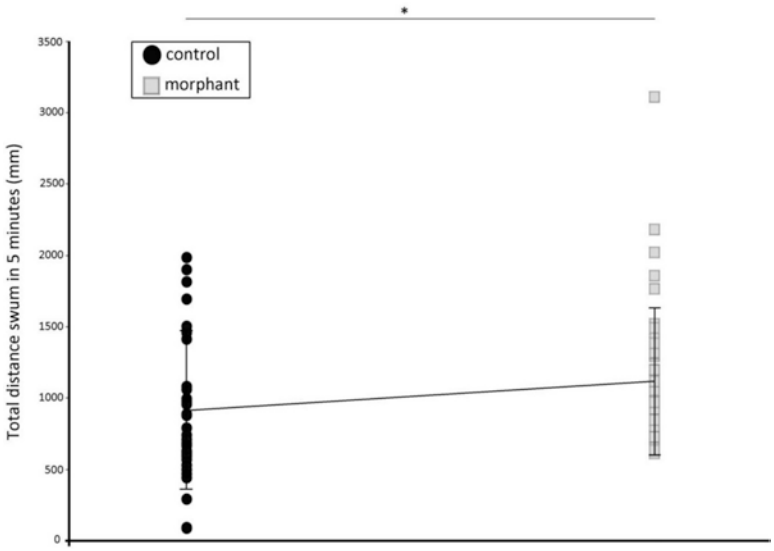

Fig. 2 *lphn3.1* morphant larvae are hyperactive. Mean distance swum in a 5-min time interval by 6 dpf larvae injected with either a control morpholino or *lphn3.1*-specific morpholino. Control larvae n = 39 and *lphn3.1* morphant larvae n = 44. *t*-test reveals a significant difference between the two groups, $*p < 0.03$

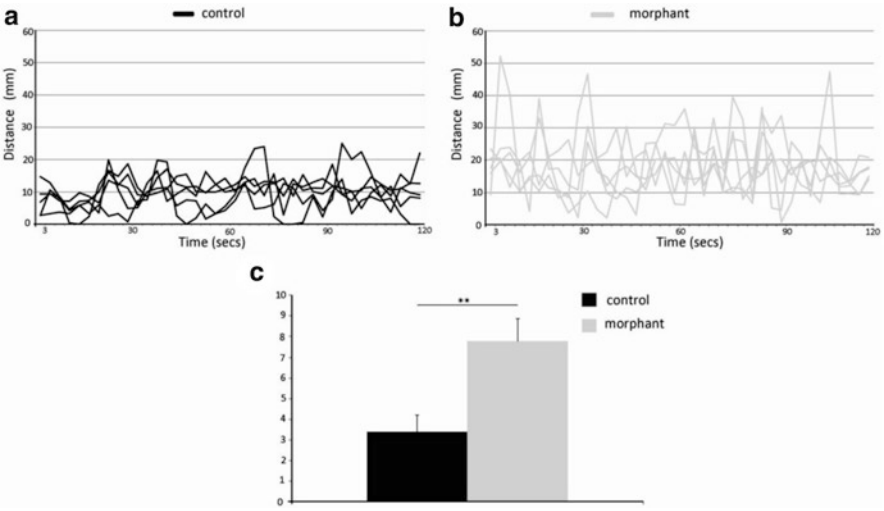

Fig. 3 *lphn3.1* morphant larvae show motor impulsivity. (**a**, **b**) Hyperactive *lphn3.1* larvae display motor impulsivity, revealed by the sharp peaks of locomotion in each of separate morphant locomotion curves (**b**) compared to those of control animals (**a**). (**c**) Lphn3-MO1 morphant larvae exhibit more activity peaks compared to the Lphn3-CO. The number of activity peaks for the two populations (control larvae and *lphn3.1* morpholino-injected larvae) is significantly different during a 120-s experiment. A peak is defined by 5 mm acceleration in at least 12 s. n = 6 for each group. *t*-test, $**p < 0.01$ for number of peaks

ment drugs MPH and ATO. Acute treatment of either drug had no effect on control-injected larval behavior at the doses used (10 μM MPH or 1 μM ATO for 1 h), but rescued morphant behavior, bringing locomotion back to control levels (Fig. 4). *lphn3.1* morphants also display a parallel reduction of dopaminergic cells in the posterior tuberculum (PT), a prominent group of dopaminergic neurons in the ventral diencephalon. The PT acts as a locomotory centre in the larval brain [117, 140,

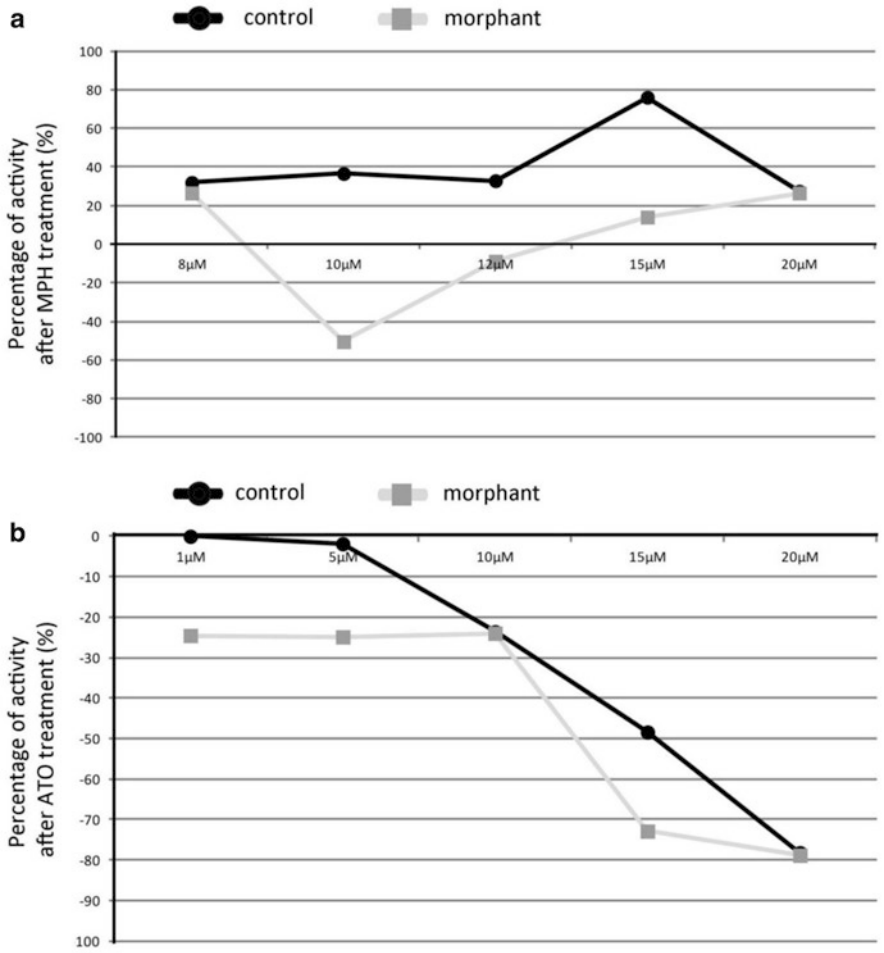

Fig. 4 Application of ADHD treatment drugs rescues *lphn3.1* morphant hyperactivity. (**a**) Dose response curve showing locomotion following methylphenidate (MPH) treatment. Values depict the percentage of change in the distance swum following a 1-h MPH treatment (8, 10, 12, 15 or 20 μM). Control larvae n = 12 and *lphn3.1* morphant larvae n = 12, for each drug concentration. (**b**) Dose response curve showing locomotion following atomoxetine (ATO) treatment. Values depict the percentage of change in the distance swum following a 1-h ATO treatment (1, 5, 10, 15, or 20 μM). Control larvae n = 12 and *lphn3.1* morphant larvae n = 12, for each drug concentration

141] and sends projections both anteriorly to the telencephalon and posteriorly to motorneurons of the spinal cord [142]. In parallel, immunohistochemical, in situ hybridisation and high pressure liquid chromatography (HPLC) studies of other neurotransmitter systems suggest that NA, 5-HT, GABA and glutamate are not affected by loss of *lphn3.1* function [105].

Our study of *lphn3.1* morphant larvae provides several pieces of information regarding the use of zebrafish as an ADHD-like model. Firstly, we have identified ADHD-like endophenotypes in larval zebrafish including hyperactivity (both during the day and night) and motor impulsivity. Secondly, we have provided novel insights into the expression of *lphn3.1* during embryonic development and identified a critical role in controlling the development of dopaminergic neurons. Finally we have provided some of the first concrete evidence that zebrafish may constitute a valid model organism to study ADHD. *lphn3.1* morphant larvae are an excellent tool to begin tease apart the genetics and neurobiology of ADHD. Nevertheless, future work will be required in order to understand how a gene that is expressed in a seemingly wide-spread pattern can lead to such a restricted loss of a few dopaminergic neurons. The possible maintenance of the phenotype into adulthood also needs to be analysed, since morpholino knock-down is only transient. *lphn3.1* morphants provide the ideal tool to search for novel ADHD-like endophenotypes in zebrafish. If the hyperactivity is maintained into adulthood (meaning that *lphn3.1*-mediated changes to embryonic development are sufficient to trigger permanent alterations to behavior) then it would be fascinating to use the 5CSRTT to measure impulsivity.

3.5 period1b Mutant Zebrafish

In an interesting recent study, Huang and colleagues have studied *period1b* (*per1b*) mutant zebrafish in connection with ADHD [106]. A key symptom of ADHD is hyperactivity that can result in sleep deprivation [143]. Furthermore, GWAS studies of ADHD patients have identified circadian clock genes [144], and mice with lacking *Clock* gene function exhibit hyperactivity and reduced sleep as well as other behavioural changes [145]. Zebrafish *per1b* mutants are hyperactive at both larval and adult stages and spend more time attacking a mirror, behaviors that can be rescued with the ADHD drugs MPH and deprenyl [106]. They also need more time to learn in an active avoidance test and are more impulsive in a reaction-time task similar to the 5CSRTT. These behavioral phenotypes are correlated with a reduction- and misplacement of DA neurons in the posterior tuberculum (similar to *lphn3.1* morphant animals) and global alterations to DA and NA turnover. Importantly, *Per1b* knock-out mice show a similar phenotype (hyperactivity, learning impairment and reduced DA levels in the brain) demonstrating a conserved function for this gene across species [106]. The possible connection between ADHD and circadian biology is fascinating and should form the basis for further research in the future.

3.6 Future Directions in Zebrafish ADHD Research

The ease of generating zebrafish morphants in large numbers makes our *lphn3.1* larvae an ideal platform with which to identify novel potential ADHD treatment drugs. Zebrafish are the perfect model system for pharmacological studies, since compounds can be directly diluted in small volumes of embryo medium and embryos, thus reducing the amount (and cost) of the compounds used. Therefore, an automated screening setup could be developed that would allow the comparison of hundreds of chemical compounds under standardized conditions. One area of research that has been explored by several groups is the use of zebrafish to look at the effect of ADHD-linked environmental toxins on development. For instance, both lead and bisphenol exposure during embryonic development have been linked to increased susceptibility for ADHD [146], and these compounds have already been applied to zebrafish during development [111–113]. The behavioral effect of MPH during zebrafish development has also been reported by Levin and colleagues [147]. Acute MPH application during the first 5 days of development leads to an increase in DA, NA and 5-HT in the 6-day old larval brain, as well as behavioral changes in adult fish. Drug-treated zebrafish show a reduction of anxiety (measured by a tank-diving assay) and decreased learning in a choice assay compared to mock-treated controls [147].

However, despite the promise shown by zebrafish as an ADHD-like model, there is still a clear need to expand the number of endophenotypes that can be measured, in particular to include those that quantify impulsivity and attention. Such work will be mandatory in order to demonstrate that we are specifically modeling ADHD rather than general NPD-related changes in behavior. The large number of groups that are now beginning to develop and validate protocols to measure adult zebrafish behavior suggest that the search for endophenotypes of NPDs may well be fruitful. The future of zebrafish as a translational model for NPDs looks bright.

Acknowledgements We are grateful to all members of the Bally-Cuif laboratory for their helpful discussions related to this work.

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