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Review

Behavioural assessments of neurotoxic effects and neurodegeneration in zebrafish ☆

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ABSTRACT

Altered neurological function will generally be behaviourally apparent. Many of the behavioural models pioneered in mammalian models are portable to zebrafish. Tests are available to capture alterations in basic motor function, changes associated with exteroceptive and interoceptive sensory cues, and alterations in learning and memory performance. Excepting some endpoints involving learning, behavioural tests can be carried out at 4 days post fertilization. Given larvae can be reared quickly and in large numbers, and that software solutions are readily available from multiple vendors to automatically test behavioural responses in 96 larvae simultaneously, zebrafish are a potent and rapid model for screening neurological impairments. Coupling current and emerging behavioural endpoints with molecular techniques will permit and accelerate the determination of the mechanisms behind neurotoxicity and degeneration, as well as provide numerous means to test remedial drugs and other therapies. The emphasis of this review is to highlight unexplored/underutilized behavioural assays for future studies. This article is part of a Special Issue entitled Zebrafish Models of Neurological Diseases.

1. Introduction

1.1. The importance of behavioural endpoints

In animals, and arguably all life throughout the biological kingdoms, behaviours are ways to integrate into the biotic and abiotic environment. Behaviours are the actions and reactions taken to internal and external cues that are intended to place organisms in a preferable position with respect to fitness. Conditions and situations that cause deviant or impaired behaviours therefore have clear survival implications. The inclusion of a behavioural assessment endpoint in studies of disease and neurotoxin exposure, especially those involving non-lethal impairments, will help address whether sub-organismal changes will affect survival. However, there is another reason to include behavioural endpoints—they can be used to rapidly and effectively detect changes of molecular to system level origins.

In vertebrates, all behaviours are achieved through nervous control. However, not all behavioural modifications are of neurological origin. Non-neurological alterations can also affect behaviour—some musculoskeletal issues, for example, such as those arising through injury and developmental abnormalities, can cause altered behaviours. Malformed limbs or other morphologically-based conditions may be associated with behaviours not apparent in normal individuals. There are also a suite of developmental issues that are related to neurological function, however, they are not related to neurotoxins or neurodegeneration. Cerebral palsy, for instance, can affect gross and/or fine motor control and have very apparent behavioural abnormalities [1]. Additionally, in healthy animals, internal, physiological processes can also modify neuron function and these can result in behaviour phenotypes. For the purposes of this review, only behavioural alterations arising though neurological pathways and directly involving neurotoxin exposure or progressive neurological pathologies will be given consideration.

Behavioural alterations arising from neurotoxin exposure and neurodegeneration come about through very different means, even though the behavioural "phenotype" (observable manifestation) may appear similar in some instances. Neurotoxic agents affect the functionality of "normal" neurons and may be reversible and of limited duration. In contrast, neurodegeneration collectively refers to a group of typically irreversible *processes* that work at the genetic to system level, all of which result in the loss of neurons and/or their functionality. Mechanistically, neurotoxic agents act in one of two ways: in general, non-specific ways (e.g. polar or non-polar narcosis [2]); or through affecting genetic and/or protein receptors. Either mode of action can cause changes at molecular, cellular, system and levels beyond. It must be noted that the first mode of action could include disruption of cells in addition to neurons. With neurodegeneration, mechanisms of action include protein misfolding and conformational disorders (proteopathies) and/or astrogliosis, i.e. an

Abbreviations: AChE, acetylcholinesterase; Anti-AChE, anticholinesterase; CPP, conditioned place preference; CS, conditioned stimulus; dpf, days post fertilization; OKR, optokinetic response; OMR, optomotor response; OP, organophosphorus agent; OSN, olfactory sensory neuron; PEA, phenylethyl alcohol; PTZ, pentylenetetrazole; UCS, unconditioned stimulus

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increase in astrocytes due to neuron death. For each mechanism of neuron (or neural tissue) impairment, it should be possible to isolate an individual behavioural phenotype for screening purposes.

All behaviours involve motion, and so given the appropriate methods, all are quantifiable. The evolution of software solutions in the last decade or so, including solutions from EthoVision (www.noldus.com), LocoScan (www.cleversysinc.com), Loligo (www.loligosystems.com), and Video-Track (www.viewpoint.fr) has greatly facilitated the accurate assessment of subtle and directed movements. However, unlike the movement of mammalian models, fish move in three dimensions. In studies of fish motion, movement in the vertical plane is typically ignored or minimized (i.e. by keeping the tank water shallow) [3–5], however, in at least one case, a proprietary protocol was developed to include it [6]. Nevertheless, with a model such as a zebrafish, the best solution may be to minimize water depth, to perhaps twice body length. This depth does not appear to result in overt stress and most fish settle down (acclimate) within 30 min (personal observation). Current technology permits the rapid, and potentially repeated, non-lethal testing of numerous fish (e.g. 4+ adults to 96 larvae) [7,8]. Additionally, a method has been constructed to calculate very subtle differences in the swimming mechanics of individual fish [4]. Caveats exist in behavioural assessments. Organisms are designed with systems that provide resilience and can act to retain higher level responses [9]. Also, factors that affect responses at lower organisational levels, e.g. protein level, may not necessarily have an obvious behavioural phenotype. Behavioural studies clearly need a mechanistically-based rationale for including a motion-based endpoint.

1.2. The relevance of zebrafish models

Ten years ago, zebrafish were identified as an up and coming model for genetic disorders and developmental biology in humans [10]. Since this time, the zebrafish genome has been fully sequenced and many genes of high mammalian homology have been identified. Five years ago, zebrafish were proposed as an untapped behaviour-genetic model organism [11]. There are now high-throughput behavioural zebrafish-based screens able to detect specific neurological alterations/pathologies, some of which take advantage of protocols similar in function and purpose to those used on rodents (e.g. olfactory-based endpoints). Furthermore, zebrafish are more amenable to manipulation and behavioural testing during early development. Their transparent larvae facilitate localization of proteins and the use of morpholino manipulation. For these reasons, and given that rodents are more expensive and require greater growth periods than zebrafish, the adoption of zebrafish models continues in typically rodent dominated fields. This review is intended to communicate the current use of behavioural endpoints in zebrafish, and how protocols used with other animals may be adapted. Numerous behavioural assessment avenues remain unexplored.

2. Types of behavioural endpoints

As with most animals, fishes exhibit intricate behaviours that depend on locomotion and may be the result of complex decisions [12]. Behavioural responses must be viewed in a three-part hierarchical manner: (1) basic motor responses, which underlie (2) sensorimotor responses, that facilitate and/or integrate with (3) learning and memory (Fig. 1). Although the first "behavioural level," arguably does not involve true behaviour since it does not necessarily include sensory responses, many studies have included locomotory (activity) changes as a behavioural endpoint [13–15]. While this endpoint may appear simplistic, it does not mean it is not meaningful, in fact, quite the opposite: all behaviours are predicated on the ability to move. Furthermore, in tests of higher-level behaviours, such as those involving learning, it may be difficult to rule out potential neurological issues of the lower founding "levels." For example, if feeding response decreased in fish exposed to a dissolved neurotoxin such as an organophosphorus (OP) insecticide, the effect could be the result of impaired locomotory

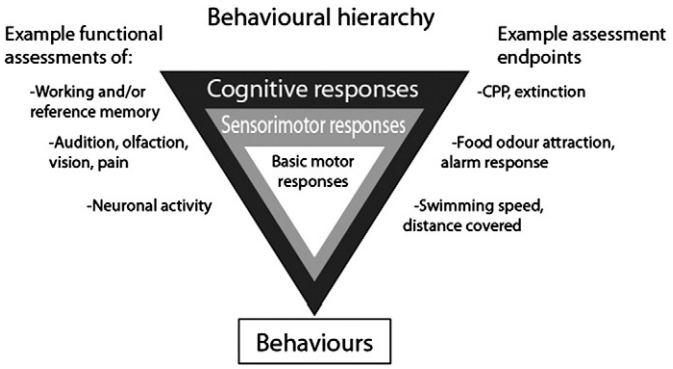

Fig. 1. Behavioural responses can be evoked by internal and external signals and/or be altered by impaired neurological condition. With zebrafish, assessment methodologies exist to test neurological performance in absence of signals through to the ability of the brain to retain and integrate sensory signals of diverse origins, which makes zebrafish a powerful model for testing compounds that affect neurons over brief to life-spanning timeframes. CPP = conditioned place preference.

ability, and/or impaired olfactory sensory neuron (OSN) function, and/or cognitive ability.

Implicit in neurodegeneration and neurotoxin exposure is that organism condition, and so likely behavioural responses, will change with time. With neurotoxins, there are four temporal phases to describe their effects: direct, secondary, tertiary and quaternary effects. The first two phases deal with the presence and actions of an agent within the organism, and the latter two, without. Direct effects are those local or systemic effects that occur over the short run and would typically be the "intended" effects, such as the actions of an anticholinesterase (anti-AChE) drug. Secondary effects consist of adaptations made over longer periods to restore equilibrium from drug actions, such as upregulation of acetylcholinesterase (AChE) expression. Tertiary effects are those that follow from the discontinuation of administration of an agent that has become physically depended, i.e. involve withdrawal and stress. Over extended periods, tertiary effects may give way to quaternary effects, such as malnutrition. The majority of behavioural assays focus on direct and secondary effects, however, there are studies of tertiary effects, e.g. withdrawal from cocaine [16].

In the following, a diverse array of endpoints using apparatus from simple, one chambered tests, to complex multi-chamber, decision-based challenges are discussed (Table 1). The majority of the behavioural endpoints can be conducted on zebrafish 3–4 days post fertilization [17,18], excepting learning/memory-based endpoints.

2.1. Basic motor response endpoints

Compounds that modulate neuron firing rate have potential to affect locomotory activity. In fishes, locomotory activity endpoints include swimming speed [19], distance covered [14], and turning rate (angular velocity) [20]. Changes in zebrafish activity have been noted in studies investigating the mechanisms of addiction to amphetamines [21], cocaine [16], ethanol [11,22], and nicotine [23], as well as the effects of pesticides [24,25].

Whether any given drug/neurotoxin increases or decreases activity is related to concentration/dose and time. For example, exposure to a low concentrations of D-amphetamine caused hyperactivity while higher concentrations caused hypoactivity [21]. Cholinesterase inhibiting drugs/pesticides are well known to have similar effects [26–29]. Clearly, agonizing stimulatory receptors or inhibiting neurotransmitter degradation will potentially lead to activity increases in the short run that are not sustainable in the long. In fact, persistent stimulation may result in excitotoxicity and neuron apoptosis. Behavioural manifestations of neurotoxin exposure may not appear until later in life. Additionally, some activity changes

Table 1
Behavioural test endpoints and their associated stimuli available to fishes, including zebrafish. Some endpoints that are underutilized or hold promise in toxicity or degenerative studies are in bold.

Apparatus	Stimulus	Stim. type	Type of test	References
Open arena	None	None	Activity level[A]	[14,16,20–22,24,25,30]
	Space	Vis	Searching behaviour[A]	[22,23,70,90]
	Food, dead	Vis; Olf	Appetitive behaviour[A]	[37]
	Food, alive	Vis; Olf	Prey capture[A]	[40,42,43]
	Tap	Aud	**Startle response**[A]	[7]
	Glass bead	Aud; Vis	Startle response[A]	[58]
	Predator	Vis	Alarm response[A]	[39,40]
	Predator scent	Olf	Alarm response[B]	[68–70]
	Simulated conspecific	Social	Aggressiveness[B]	[11,22]
Confined tube, or flume	Water flow	Motion	***U*crit**[B]	[83,84]
	Social	Vis; Motion	**Schooling**[B]	[58,59]
Moving background (striped drum)	Simulated object	Vis	**OMR**[A]	[47,49,50]
		Vis	OKR[A]	[51,52]
Counter-current olfactometer	Odours*	Vis; Olf	**Attraction; avoidance**[B]	[15,74,75]
Y-maze	Odours*	Olf	Attraction[B]	[76–79]
T-maze	Preferred location	Vis	CPP[B]	[93]
	Food	Chemo	CPP[B]	[97]
	Location	Drug	CPP[B]	[97,98]
Two-chamber tank	Shade	Vis	**CPP**[B]	[22]
	Electric field	Pain	**Aversive CPA**[B]	[16]
	Colour	Vis	**CPP**[B]	[99]
Three-chamber, gated	Confinement	Vis; Stress	**CPP, CPA**[B]	[100]
Three-chamber, separated	Social	Vis	**Schooling**[B]	[22]

Abbreviations: Aud, auditory; CPA, conditioned place aversion; CPP, conditioned place preference; OKR, optokinetic response; Olf, olfactory; OMR, optomotor response; *U*crit, critical swimming speed; Vis, visual. Note: Zebrafish age may affect whether specific endpoints are appropriate, as the endpoints may depend on the development of swimming ability, sensory responses, and memory. In general, assays requiring swimming or sensory-evoked swimming are available as soon as 48 post hatch (~4 dpf; endpoints marked [A]) [37,106]. Swimming intensive and memory based endpoints have mostly been conducted on adults (approx. ≥3 months), however many of these assays could likely be conducted on juveniles (endpoints marked [B]). An exception: OMR relies on immobilizing fish, and so fish cannot be>7 dpf [51]. A caveat: olfactory-based alarm responses may be most robust and least variable at 50 dpf [73].

* Odours include food, conspecific, pheromones, predator, and synthetic compounds.

may only be evident after the removal of the drug, i.e. during withdrawal [16].

Zebrafish activity has proven a viable endpoint for detecting neurological impairments received during development. For example, embryonic zebrafish exposed to chlorpyrifos, an organophosphorus insecticide and ubiquitous environmental pollutant, had reduced swimming activity 6+ days later [24]. If persisting neurological impairment or neurodegeneration are not readily apparent, a drug can be used to evoke activity. Altered neurology will be apparent in a lower response threshold to the drug. The convulsant (seizure inducing drug) pentylenetetrazole (PTZ) has been used in recent studies in zebrafish [30], including high throughput 24 and 96 well plate assays [3,31,32], for just such a purpose. Specifically, zebrafish embryos injected with domoic acid (DA), a neurotoxin of diatom origin, reduced the concentration of PTZ required to evoke stage I and II seizure activity [33]. Activity endpoints can also be used to detect neurodegeneration. Specifically, to model the effects of Parkinson disease, neurotoxic drugs can be injected directly into specific brain regions (using microinjection), although this has historically not carried out on fish [34]. A recent study demonstrated that unilateral injection of the drugs 1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA), two neurotoxins with specific targets, reduced the speed and distance zebrafish travelled [14]. Microinjection techniques make the zebrafish model portable to a variety of neural ablation studies.

A consideration for activity-based sensory endpoints is that they may not actually be stimulus-free. When a fish or other animal is introduced into a new environment, such as a test tank, they will likely begin searching and forming a cognitive map of their new surroundings [35]. This process will draw upon one or more senses and involve synaptic plasticity (discussed below). Even with elimination of searching behaviour, perhaps accomplished through tank acclimation, activity changes may be based on input from the interoceptive senses, i.e. the perception of internal movement and/or pain (aspects of the somatosensory system). Activity-based endpoints clearly have application, but they may be more of an umbrella for other neurological impairments isolatable through sense-specific tests.

2.2. Sensorimotor endpoints

Fishes have a suite of exteroceptive senses analogous to those of mammals. These include vision (photoreceptor-based), audition (mechanoreceptor-), olfaction, gustatory (both chemosensor-), balance and somatosensory, which includes touch (mechanoreceptor-), pressure, temperature (thermoreceptor-) and pain (nociceptor-). Fishes also posses senses that do not have mammalian analogs, e.g. magnetoreception. Furthermore, some of the analogous senses, e.g. gustation, are morphologically dissimilar—fishes, including zebrafish, have solitary chemosensory cells (SCCs), which are externally-located taste buds [36,37]. And unlike mammals, sound and motion are not only perceived by the ear and vestibular (inertial detection) system, but by an additional system, the lateral line. In this system, neuromast cells structurally similar to inner ear mechanoreceptor "hair" cells, detect changes in water pressure and motion relative to themselves. These cells can also detect sounds and provide information regarding acceleration and velocity [38]. In the below, known and potential sensorimotor endpoints will be discussed.

The senses enable reflexive, innate responses to "unconditioned stimuli" (UCS), as well as learned responses to "conditioned stimuli" (CS). Both UCS and CS can be used to test sensorimotor responses, and can be positive (attractive) or aversive. UCS evoked behavioural responses stimuli have been included in this section, but their modification is included in the proceeding section on learning and memory. Positive UCS or CS stimuli may arrive via various senses but uniformly enable the organism to place itself in an actual or perceived improved fitness position that otherwise offers some benefit or reward; example stimuli include food, cover, mate call or odour. Aversive stimuli do the opposite; examples stimuli include electric shocks, abrupt and severe changes in lighting, temperature, sound,

(unpleasant) odours, or other sensory assaults causing discomfort. Stimuli can also be neutral, i.e. perceptible but without an associated behaviour [8]. For behavioural endpoints, both CS and UCS stimuli are routinely used in behavioural studies in fishes, including those with and without conditioning.

Changes in sensory responses can be due to impaired sensory perception or due to impaired motor performance. Separating perceptual vs. motor impairment in sensory studies may involve using an additional endpoint, such as cell or tissue level assessment of neuron function. Another challenge is that most behaviours are multisensory, and isolating specific impairments may be difficult or impossible. Nevertheless, behavioural endpoints exist to evoke responses through each of the senses.

2.2.1. Visual endpoints

Unless experiments are conducted under darkness or with visually occluded fish, all behavioural endpoints arguably have some visual component. In fish, vision specific endpoints include predator (simulated or actual) avoidance, prey capture, optomotor and optokinetic responses. All of these methods have clearly defined endpoints and involve coordination, except the last, which uses immobilized fish.

One of the most basic assays draws upon phototaxis, i.e. light seeking behaviour. Zebrafish are active during the day, and so should spend time in light unless they perceive a risky (predation-prone) condition. When given a choice between a lighted or shaded area in an open arena, zebrafish did indeed spend less time under shade (~40%) [22]. Ethanol exposure caused a concentration-dependent decline in the proportion of time spent under shade. With exposures to ethanol, zebrafish sheltering was reduced to ~10–15%, i.e. exposure evoked risky behaviour. Such a robust and easily quantifiable behavioural contrast could be exploited to test other neurological impairments.

Alarm (antipredator) behaviours are often used in fish research, although they are usually investigated using olfactory cues. Nevertheless, visual cues can be used to evoke alarm [39,40]. Specifically, presenting fish with a large shape modelled after a predator or an actual predatory fish, such as in a neighbouring tank, can evoke dashing, freezing and shelter seeking. Fast start responses (s-starts) have been shown to increase in response to the presentation of a simulated predator (painted falcon tube), and be sensitive to the effects of ethanol exposure [22]. A recent paper suggested that alarm response could be a very valuable tool for mechanistically understanding the roles of signalling molecules, specific receptors and neurons in neural circuitry [41]. One of the advantages to aversive responses is that they are easy to score either manually or through video analysis. However, in some stocks of lab reared fish, innate responses may have been lost. In this case, conditioning would need to be carried out.

Visually guided prey capture (as opposed to chemosensory-guided) has been used in a variety of fish species, although not typically on such a small one. Nevertheless, visually guided appetitive behaviours can be carried out even in 7-day-old zebrafish using paramecia [42]. Other prey items such as daphnia, hyalella and brine shrimp have been used in other species, and there is no reason why they cannot also be used with zebrafish. Assay endpoints include time to initiate attack (latency), number of attacked or captured prey, capture efficiency, and time to cessation [40,42,43]. For example, a recent study on striped bass noted that time to prey capture was increased 6 days following exposure to an anti-AChE pesticide, the OP diazinon [44]. Another used zebrafish larvae with laser ablated premotor neurons in the retinotectum and reticulospinal neurons to isolate neural circuitry associated with visually directed prey capture [42]. Tracking software currently uses contrast (i.e. a dark object, the animal, against a light background) to track as many as ten objects in one open arena (EthoVision; www.noldus.com). However, prey may not be easily discernable, and their removal from an arena may not yet be easily quantifiable. A high throughput version of this assays remains for development.

A test of optomotor response (OMR) was pioneered over 80 years ago (see [45]) and has been frequently used on a variety of animals since, including mice [46] and zebrafish [47]. The test is one of tracking: animals reflexively keep pace with a rotating a striped or "grated" drum. At a high enough rotation speed, the lines will blur and fish will cease to follow. The parameter of interest is the point at which the animal ceases to track. Excitatory and inhibitory drugs should increase and decrease this point, respectively, and neurodegeneration will obviously affect it. Another way to conduct the test is to hold the drum speed constant but vary the illumination [48]. This method was capable of resolving optomotor differences between strains. A different version of the test replaces the uniform stripes with one large stripe. The large stripe represents a threatening object, and so fish will swim away from it. This test proved capable of detecting retinal degeneration in a "night blind" mutant [49].

A variation of the OMR assay includes using competing visual grating [50]. Specifically, a background reference grating is kept at a constant speed in one direction while a test grating of variable speed is moved in the other. Fish will move in the direction of the more strongly perceived cue. Three scenarios are possible: the fish will track the reference cue when the test cue is of lower contrast, or they will track the test cue when it is of higher contrast, or when there is insufficient contrast between the two, they will stop moving. The null motion point provides an index of visual guided response, with acuity inversely related to contrast. This test identified performance differences from a mutation in a vesicular glutamate transporter.

The OMR has been adapted to isolate eye tracking ability in static larval zebrafish [51,52]. In this "optokinetic response" (OKR) assay, larvae are immobilized in methylcellulose gel, placed in the same visual scenario as adults (a circular arena with a rotating striped perimeter), and smooth tracking and rapid saccades with stripe sweep are recorded. Impaired altered visual performance will be evident in decreased saccadic movement [52–54]. The output of eye velocity in degrees/s can be determined using computer software (a protocol is available [55]). A consideration is that fish cannot be >7 dpf because gel immobilization becomes problematic [51]. The OKR test is functionally equivalent to the finger tracking-based field sobriety test, where the degree at which eye motion switches from smooth tracking to saccadic movement is indicative of intoxication.

Zebrafish are a schooling species that exhibits territoriality [56,57]. Although schooling and aggressive behaviour are not solely driven by vision–they can rely on sensory input from the octavolateralis (sound/motion), etc.–they are predicated on it. The propensity of a small group of zebrafish that was physically but not visually isolated from a larger school to swim near the larger group, i.e. their "social preference," was inhibited in a concentration-specific manner by ethanol [22]. School cohesion, apparent in "nearest neighbour distance" (NND), may also be used as a sensitive indicator of intoxication [58] and genetic-based differences [59]. Aggressive behaviour (agonistic behaviour) consists of alternating and/or coincident fin displays and attack behaviours. Displays consist of erection of dorsal, pectoral or anal fins, and/or slapping of the caudal fin; attack behaviours include biting motions and directed swimming at the attacker [22]. An inclined mirror can be used to simulate a conspecific, and assess aggression [11,22]. In an "aggressiveness test," ethanol exposure was correlated with increased time near the mirror and aggressive displays [22]. Automatic video analysis of fin motions obviously poses a challenge, but changes in speed and distance near the mirror would be easy to determine.

2.2.2. Olfactory endpoints

In fish, olfaction is so important to so many behaviours that it cannot be done without (reviewed in [60]). This is the reason numerous studies have focused on its impairment through exposure to a variety of neurotoxic contaminants (reviewed in [61]). In humans, olfaction is not so indispensable, however, its loss does correlate with neurodegenerative diseases such as Parkinson's and Alzheimer's [62]. The use of

zebrafish olfactory-based behavioural endpoints of neurodegeneration remains largely unexplored. A recent publication suggests olfactory endpoints may also be viable in prion research [63].

In fishes, odorants and odorant classes are associated with specific behavioural responses, which facilitate isolating specific neuronal impairments. Odorants include amino acids, bile salts and pheromones, which evoke feeding and antipredator, social (assumed), alarm and mating-based responses, respectively [60]. Amino acids associated with food, such as L-alanine [64], evoked appetitive behaviours such attraction (directed movement up a concentration gradient) [65]. A mixture of ten amino acids, including L-alanine, evoked increases in turning rate and activity level at 4 dpf [37], suggesting the possibility of using amino acid responses in high throughput assays. Bile salts remain untested as behavioural modulators in zebrafish, but given their efficacy at evoking OSN responses [66], they may be of use in olfactory-based assays. Pheromone-based behavioural endpoints of mating have been restricted to other species (e.g. F-type prostaglandins evoke substrate probing in salmonids [67]), however, the behaviours evoked by alarm pheromone are well studied.

Alarm pheromone is anxiogenic and makes for a very reliable endpoint since it is an innate reflex. Alarm responses involve a set of stereotypical behaviours, which include dashing, freezing, jumping and erratic movements [68–70]. All the actions are "fight or flight" in nature, and are associated with increases in stress hormone concentrations [70]. Many fish studies have included alarm response as an endpoint of olfactory neuron function [61]. Numerous neurotoxic agents, including anti-AChE pesticides, alter alarm response, possibly through impairing both peripheral (OSNs) and central neurons [61,71]. Alarm behaviour endpoints are highly sensitive, with some OPs impairing responses with exposures to just 1 ppb [28,71]. Studies wishing to test the functionality of the hypothalamic-pituitary-interrenal (HPI) axis (analogous to the hypothalamic-pituitary-adrenal (HPA) axis in mammals) may benefit from the inclusion of an alarm assay [41].

A recent study found that hypoxanthine 3-N-oxide (H3NO) evoked alarm response in zebrafish [72]. Whether this compound is alarm pheromone or a component thereof, this discovery has great potential to improve the standardization of alarm response testing, since a molecularly based behavioural concentration-response curve can now be constructed. The previous alarm response testing methods involved the use of a skin extract or filtrate derived from the skin of conspecifics. These methods suffer by not being able to standardize the unknown(s) pheromone, however some did endeavour to do so by measuring total protein [6]. In zebrafish, the alarm response may be most robust and least variable 50 dpf [73].

One of the simplest tests for olfactory-evoked motion involves the use of an avoidance trough or "counter current olfactometer." In this device, water/odour sources flow from either end of rectangular trough and exit in the center without mixing appreciably. In this way, there are effectively two odour zones in the tank, and fish can choose to spend time in either one. As long as a fish is swimming back and forth prior to odorant introduction (i.e. has not preferred an end before stimulus introduction), both attractive and aversive responses can be determined [15,74,75]. This test is not unlike a Y-maze, where odours flow down two arms to a null or mixing zone. Y-maze can be problematic in some cases because fish may elect to make no decision, i.e. not explore the arms. Nevertheless, both Y-mazes and avoidance troughs have been useful in fish studies of the attractive or aversive qualities of amino acids, pheromones and neurotoxic contaminants [76–79].

2.2.3. Auditory endpoints

Even though the anatomy of the zebrafish ear is dissimilar to that of mammals (i.e. no cochlea), the neuron hair cell structure is highly conserved. Additionally, the lateral line cells are structurally and functionally the same as ear hair cells. Both ear and later line cells can be disturbed by compounds of human origin (i.e. "ototoxic" agents). For example, the antibiotic streptomycin can negatively affect fish behaviour [80]. For these reasons, hair cell alteration has mammalian parallel (reviewed in [17]).

An auditory-evoked startle response has recently been adapted from rodents to zebrafish [7]. In the study, adult zebrafish were placed individually in an eight tank array and video was recorded from overhead. The stimulus consisted of an automated, mechanical "tap" to the tank bottom. Fish swimming distance was determined over the following 5 s, and the taps were repeated every minute for a total of ten times. This session was then subsequently repeated. This design is particularly interesting because its neurological assessment is two-dimensional: it tests an innate, behavioural reflex (which includes integration of sound and space, and depends on physiological condition), as well as the ability to adapt (habituate) rapidly to a stimulus. In their study, they found that both endpoints could show changes due to larval exposure to chlorpyrifos. However, it was arguably the ability to adapt that highlighted the biggest neurological alteration. The difference in startle response magnitude was greater and more significant with repetition. The purpose of this high throughput auditory-based test was to assess the persisting effects of chlorpyrifos exposure, however there is no reason this assay could not be used to screen/assess a variety of neurological conditions/pathologies. Furthermore, given their findings regarding habituation, studies including other stimuli (such as odorants), could benefit from including such a parameter.

2.2.4. Interoceptive sensory endpoints

Fish rely on balance and experience pain. However, unlike mammals, equilibrioception (balance) is accomplished through the octavolateralis system, which includes neurons that perceive sound and motion. Additionally, fish do not rely upon their "limbs" (i.e. fins) for balance. In fact, removing two or more fins has frequently been used as a non-invasive, low impact means of identifying fish [81,82]. Nevertheless, neurotoxins affecting the vestibular portion (i.e. inertial sense) of the inner ear, or neurodegeneration in general, may conceivably affect balance, and so may impair rheotactic ability. Performance deficiencies could be gauged through measuring the time to current orientation.

Decades of research on fish swimming performance has relied on mild electrical shock to evoke swimming [83,84]. Detecting altered neurological performance through nociceptor-based response is not common in fish, but it does show promise, particularly with agents/conditions which modify anxiety. A surprising result of a recent study was that cocaine did not alter a behavioural avoidance of a mild electrical field [16]. Given that establishing a local electric field in an open arena is not difficult, and that fish will instinctively avoid it, this method has promise for screening neurotoxins with anaesthetic effects.

2.3. Learning and memory based endpoints

In mammals, the hippocampus is involved with cognitive plasticity. Zebrafish lack a hippocampus, but they do have a developmentally similar region, the lateral pallium, that appears functionally equivalent [85]. Reference memory (i.e. acquired memories) and working memory (i.e. the attentional component of short-term memory), and their interplay (i.e. cognitive plasticity) can be tested in a variety of ways, including both classical (Pavlovian) and operant (instrumental) conditioning. Both conditioning methods evoke response(s) using a pre-existing behaviour (evoked by either CS or UCS), but for *classical conditioning, a neutral stimulus is converted into a positive CS* (through their repeated pairing), and in *operant conditioning consequences are used to reinforce or extinguish a CS*. In the below, endpoints are discussed for (1) attention, for (2) searching (map building) behaviour, for (3) the acquisition of responses to neutral stimuli (classical conditioning), as well as for (4) the

reinforcement or extinguishment of positive and negative stimuli (operant conditioning).

2.3.1. Attentional endpoints

Behaviours generally result from the integration of multiple signals. What is important is the ability to discern important signals amidst background noise. Neurological filtering in vertebrates has been referred to as the "cocktail party effect," coined to describe the ability of a person to hear a signal such as their name spoken in a room full of chatting people [86]. Implicit in "selective attention" is that distracting signals be ignored. One assessment methodology used in non-human primates involves coupling a positive stimulus, such as food, with the occurrence of another stimulus, such as a particular visual signal, and varying delay between the two [87]. Distractability can be assessed though inserting irrelevant stimuli in the delay period. Since filtering draws upon reference memory and working memory, it is a useful correlate of neurological function. While numerous paradigms exist to test selective attention-based tests in rodents and primates [87,88], no obvious correlate currently exist for zebrafish. However, a functionally similar test might be carried out by challenging zebrafish with competing cues of fitness relevance. For example, fish could be exposed to a food odorant, which would initiate appetitive behavioural response, then interrupted with an alarm cue, which should evoke anti-predator behaviour. The degree and rate at which the appetitive behaviour is replaced and anti-predator responses exhibited (i.e. evokes attention) could prove meaningful endpoints of sensorimotor function and cognition. To explicitly test memory function, the UCS food and/or predator cues could be replaced with CS cues, such as behaviourally neutral amino acids or PEA. An attentional endpoint could be especially useful in testing cholinergic system functionality, since working memory is dependent on it [89], and since many drugs agonize or antagonize cholinergic receptors and neurodegenerative diseases such as Alzheimer's impair it.

2.3.2. Searching (spatial) endpoints

Introduction to new surroundings will stimulate searching behaviour and map building [35]. The formation of a new cognitive map is another form of neural plasticity and therefore a possible performance metric for neurological impairment. Numerous studies have already validated that searching behaviour in an open arena is a meaningful endpoint in zebrafish [22,70,90]. Searching behaviour was recently used in combination with two anxiogenic factors, alarm pheromone or caffeine, and two axiolytic factors, ethanol and fluoxetine (i.e. the SSRI Prozac), as an endpoint in "a novel tank test" [70]. Release into a new tank evoked a bottom seeking in control fish, which was followed by subsequent upper tank exploration. With alarm pheromone and caffeine exposure, the number of upper tank explorations was reduced, and the time taken to explore (latency) increased. With exposure to ethanol or fluoxetine, the reverse pattern was noted: transitions to the upper tank increased, and the latency to upper tank searching was greatly diminished. Nicotine had the opposite effect, fish were not as inclined to dive to the tank bottom [23].

Spatial memory is mediated through the lateral pallium. Studies have found that lesioning the functionally equivalent region in rats (hippocampus), abolished searching behaviour [91]. Given the ability to focally lesion larval zebrafish brain tissue and neurons using laser ablation [42], studies of searching hold promise for cognitive endpoints. A tried and true test of spatial memory in rats is the Morris water navigation task, which is essentially map building under stress and with visual cue reference. The rate of learning of the platform location can be gauged by the latency to platform discovery and distance covered in successive trials. Learning under stress in fish could be accomplished in a variety of means, such as by the ability to find an escape portal (to another potion of a test tank), under the threat of predation, evoked by visual or olfactory stimuli. One zebrafish study using a net as a freight stimulus showed that the latency to discover an escape portal decreased significantly over six training sessions [92].

2.3.3. Classical conditioning endpoints

Zebrafish studies have demonstrated that both visual [93] and olfactory [8] cues unassociated with behavioural response(s) can be converted to behavioural response cues. For example, over a few (<10) trials, zebrafish were able to visibly discriminate and select coloured arms of a T-maze where they would receive a food reward [93]. As for odorant-based learning, a recent study demonstrated that by pairing PEA (a neutral stimulus) with food odours (L-alanine and L-valine) over a number of training sessions, appetitive swimming behaviour (turning rate) could be evoked by the addition of PEA alone (i.e. it was converted to a CS) [8]. Classical conditioning endpoints may be increasingly adopted since conditioning can be achieved rapidly. For example, just one pairing of a neutral stimulus (red light) with alarm pheromone (UCS) evoked significant conditioning in a related species (fathead minnow) [94]. These data suggest that the strength of the innate response determines the rate of learning.

In many species of vertebrates, shaping is used to evoke a desired behaviour. This method consists of reinforcing successive steps towards a target behaviour. This method of reference memory creation may be unreasonable for fish; however autoshaping may be a viable option. Autoshaping was developed in pigeons and consisted of the pairing of a visual cue (light) with food. Initially the pigeons will respond (peck) to the UCS, but over time will respond to the CS alone [95]. There is no reason why this model would not also work in fish. Zebrafish will swim into odour sources and sometimes "bite" at water inflows including food odour (personal observation). With repeated trials, they could be expected to bite at a water flow source alone.

2.3.4. Operant conditioning endpoints

A variety of methods using open arena to multi-chambered arenas exist to test stimulus reinforcement or extinguishment. Open arenas have been used for tests involving food odour and predator scent. In the first, an appetitive response to food flake was enhanced by pairing it with amino acids food odours [8]. In the second, a study of a closely related species (fathead minnow) found that it took 6 to 8 days and two to four days to visually and olfactorily acquire a predator, presumably by the pairing of a UCS (alarm pheromone) with visual cues and scent, respectively [39]. Visual and odorant cues clearly have potential for assessing cognition, and the differential time course could provide a valuable diagnostic tool.

A common method of operant conditioning involves presenting an animal with one or more choices between arena arms or areas, and based on the decision, either rewarding the animal with food or punishing it with pain or stress. A popular paradigm is "conditioned place preference" (CPP) or sometimes just "place preference" (PP), in which animals are rewarded with a drug after selecting a particular location [96]. The reinforcing stimulus needs to be a positive psychoactive stimulant, such as cocaine [97] or amphetamine [98]. A version of CPP is "conditioned place avoidance" (CPA), which is where the conditioned area was initially avoided [99]. For example, by "addicting" zebrafish to D-amphetamine, preference for a side of the tank with two large freight inducing black dots could be evoked [99]. In general, CPP and CPA are good models for testing the neurological bases for addiction, but they remain largely unadopted but available in toxicity or degenerative studies.

At least two methods exist to test vision-based learning in fishes, the T-maze and the three chambered arena. Both methods share some similarities to the radial arm maze (RAM), frequently used in mammalian models. In the T-maze, fish are introduced into the long arm (base of the T) and they will generally swim to one or both of the sort arms. However, as many as 5% fish may not move from the base

arm unless provoked [97]. The intended outcome is for a fish to favour one arm based on the desirability of the settings ("spatial preference") [97], or a reward coupled to a visual cue [93]. In tests using the three chambered arena, fish are introduced to a center area that is flanked on either side by gated chambers [100]. To generate avoidance behaviour of one of the side chamber, fish are penalized (using confinement) for making repeated selections of one tank side (in general, to condition an animal to avoid an area based on a visual cue, nociceptor and stress based methods are used [101]). The three chamber confinement test demonstrated that cholinergic agonization can improve the number of correct decisions [100].

In all operant models discussed above, a conditioned CS will lose its behavioural stimulatory efficacy as its repeated presentation goes unpaired with the initial pre-existing, positive stimulus [8]. As with acquisitional endpoints, the rate of change in the reinforced decision can provide a correlate of neurological function [102].

3. Problems associated with behavioural endpoints

Variation in responses/traits/parameters increases with organisational level, and so behaviours are obviously the most variable of organismal responses. Additionally, in fish, as other animals, their state will affect memory recall and motivation, and they are not without personality. Furthermore, with aquatic species, special consideration must be made for the water source. Municipal water is often rife with neurotoxic contaminants, which may vary in concentration considerably even over brief time periods. Water needs to be carbon filtered and tested to remove/account for any confounding chemical effects.

An appreciable amount of individual variation can be accounted for by classifying behavioural phenotypes, such as low or high activity subgroups. Doing so will increase the power of the test; however, not all traits that appear obviously related to behaviour may be so. In juvenile brook trout, for example, swimming performance ability was not associated with actual (conative) swimming activity [103]. Some temporal issues can be exploited; food responsiveness and willingness to take risks, for example, are enhanced with brief periods of food deprivation [65,103]. Social status can also affect stress level which will in turn modulate activity and stimuli responses [103]. If social endpoint was to be used, accounting for dominance behaviour in fish may present a challenge. Careful observation and selective removal of individuals of stronger resource utilization (e.g. food consumption) could help resolve variation.

4. Conclusions

Vertebrates have been proposed as the best models for elucidating mechanisms of neurodegeneration [104]. Among vertebrate models, zebrafish are finding ever increasing application. In studies of drugs, zebrafish make for excellent models, since delivery can be achieved in many cases by simple addition to the tank water. Furthermore, microinjection techniques are now available to deliver agents directly to specific cells/tissues, even within larval fish [105]. This review has highlighted behavioural endpoints available to zebrafish that probe basic neurological function, that test behaviours predicated on the function of diverse types of sensory neurons, and that investigate cognitive performance. Some behavioural endpoints test multiple neurologically-based abilities; this review is intended to provide a template by which to link and/or apportion specific physiological, perceptual and cognitive impairments. Most methodologies described herein have very tight parallel with mammalian models. Furthermore, many behavioural tests, especially those using high throughput screens, remain in evolution. The future holds promise for the development of a suite of standardized behavioural assays that can be used to determine the mechanisms by which neurotoxic effects and neurodegenerative diseases develop and progress.

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