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Review

Zebrafish antipredatory responses: A future for translational research?

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ABSTRACT

Human neuropsychiatric conditions associated with abnormally exaggerated or misdirected fear (anxiety disorders and phobias) still represent a large unmet medical need because the biological mechanisms underlying these diseases are not well understood. Animal models have been proposed to facilitate this research. Here I review the literature with a focus on zebrafish, an upcoming laboratory organism in behavioral brain research. I argue that abnormal human fear responses are likely the result of the malfunction of neurobiological mechanisms (brain areas, circuits and/or molecular mechanisms) that originally evolved to support avoidance of predators or other harm in nature. I also argue that the understanding of the normal as well as pathological functioning of such mechanisms may be best achieved if one utilizes naturalistic experimental approaches. In case of laboratory model organisms, this may entail presenting stimuli associated with predators and measuring species-specific antipredatory responses. Although zebrafish is a relatively new subject of such inquiry, I review the recently rapidly increasing number of zebrafish studies in this area, and conclude that zebrafish is a promising research tool for the analysis of the neurobiology and genetics of vertebrate fear responses.

Contents

1. Introduction

1.1. Human anxiety, still a major unmet medical need

Throughout this review I use the term "fear" as the behavior or internal state of the subject (human or non-human animal) that

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is elicited by aversive stimuli that can potentially harm the subject and/or signal such harm or any form of danger. Here I define anxiety in a broad sense as an abnormally prolonged, exaggerated, or misdirected form of fear. When comparing human and animal responses I do not assume the presence (or the absence for that matter) of consciousness, awareness, or understanding of fear or of the stimuli that induce it.

Human anxiety is one of the most prevalent neuropsychiatric conditions. Approximately 5% of people living in westernized countries will suffer from general anxiety disorder during their life time and, for example, just in the United States as many as 10 million patients suffer from this clinically recognized disease at any given time point [101]. The numbers are likely larger for other parts of the world and certainly even more staggering if one considers other types of anxiety related disorders including panic disorders, post-traumatic stress disorders (PTSD), and specific phobias, not to speak of less severe forms of anxiety [19,36,59]. Numerous methods, including pharmacological approaches, have been developed for the treatment of such disorders but the efficacy of these methods has been limited and variable, and thus the quality of life of individuals suffering from anxiety related disorders is still significantly lower even in patients with mild forms of the disease [67].

1.2. Mechanisms of human anxiety are not well understood

One reason why better treatment has been unavailable is that the mechanisms of anxiety related disorders have not been fully understood [66]. Neuroanatomical and neuroimaging studies suggest that the amygdala and its reciprocal connections with the prefrontal cortex play a central role (for review, see ref. [66]) but other brain regions, e.g. the periaqueductal gray [71,97], have also been implicated. Numerous neurotransmitter systems and neurochemicals have been demonstrated to be involved in anxiety disorders (for review, see ref. [66]). The concentration of corticotropin-releasing factor (CRF) has been shown to be elevated in some anxiety disorders, pharmacological blockade of glucocorticoids and noradrenaline has been proposed for trauma-related anxiety, and the glutamatergic system has been implicated in other forms of anxiety (for review, see ref. [66]). The serotoninergic system has also been in focus with regard to anxiety disorders [60]. The neuropeptides substance P, neuropeptide Y, oxytocin, orexin, and galanin have also been found to be involved in anxiety (for recent review, see ref. [66]). While many of the above mechanisms represent potentially good pharmacological targets allowing the eventual development of drug therapies, the complexity of these disorders and the limited understanding of the mechanisms behind them warrants further detailed inquiries into the neurobiology of the disease.

1.3. Use of laboratory animals as tools of discovery

Laboratory animals have long been proposed for modeling numerous human neuropsychiatric disorders [33] including anxiety [54]. Most anxiety related studies have been conducted with rats. For example, a medline (PubMed) literature search with keywords “anxiety” and “rat” returns close to 8000 publications. The primary study organism of biomedical research, the mouse, is also well represented in this literature. A medline search with this species shows well over 4000 published studies. But other model organisms, including the dog (almost 500 publications) or non-human primates (62 publications) have also been utilized in the analysis of anxiety. Even the fruit fly (*Drosophila melanogaster*) has been proposed as a research organism for the understanding of the mechanisms of anxiety [56]. Clearly, there is a major effort in multiple laboratories to utilize model organisms for the analysis and/or modeling of human anxiety. Why is this so? The rationale for the use of model organisms for the analysis of human disorders is principally two fold. First, laboratory organisms represent a pragmatic compromise: they are cheaper to maintain and easier to analyze than humans and they face fewer ethical roadblocks. Second, laboratory organisms are evolutionarily related to us, which offers functional (e.g., neurobiological, physiological, biochemical and genetic) homologies that one may utilize for the understanding of the principal mechanisms of human anxiety (for examples, see ref. [24]), a point I will return shortly.

1.4. Naturalistic approaches and their utility in the analysis of biological mechanisms of behavior

Some of the most successful lines of investigation into the mechanisms of a broad range of behaviors have been multidisciplinary. These studies (e.g. [47,49,65,90]) utilized behavioral, electrophysiological, neuroanatomical and molecular genetics methods to investigate the mechanisms of brain function and how such mechanisms lead to the behavioral output. An important controversy in this research, however, has been the general question of how to measure behavior [39]. While not necessarily mutually exclusive, two fundamentally distinct approaches have emerged. The classical animal psychology approach has emphasized the analysis of species invariant generalizable features that cut across multiple species and thus are expected to lead to easier translation from animal to human. On the other hand, the ethological approach has put more weight on naturalistic studies sensitive to species-specific features and the evolutionary and ecological relevance of the methods employed. I, along with others, have argued that the ethological approach is more appropriate when one is interested in the question of biological mechanisms of behavior [10,21,22,41]. The essence of this argument is twofold. First, genes that influence brain function and behavior have been under the pressure of natural selection and thus their effects observed at the behavioral level are the result of evolution, the phylogenetic argument [20]. Second, analysis of the mechanisms of phenes (i.e. phenotypical characteristics) may be best achieved when the phenes themselves are defined in a biologically meaningful manner. In case of behavior, this means methods that allow the quantification of natural, species-specific, responses that are the product of the studied organism and not of the experimenter’s subjective bias, the phenogenetic argument [20]. Last, it is also important to realize that nature-blind (usually classified as animal psychological) experiments do not necessarily translate better to our own species and may not be easier to generalize to the human clinic. As we put it in a previous publication [42], for example, “offering a sizeable financial reward to a rat and giving tasty rat chow to humans might not represent ‘rigorous laboratory control’ of motivation: ignoring species-specific characteristics can lead to less obvious, but similar, mistakes in behavioral research.”

1.5. Ethology and anxiety: studying antipredatory behavior

Naturalistic approaches thus may have an important place in research whose ultimate goal is to understand the biological mechanisms of abnormal fear responses in vertebrates including our own species (for discussion specific to fear/anxiety see [10,44,62,85]). Behavioral analysis is often deceptively simple [39] and this is especially true for anxiety paradigms [12]. It is therefore important to consider what approach or behavioral method has the highest possibility for success. Classical laboratory rodents including the rat and the mouse have been successfully employed in anxiety research using antipredatory paradigms [53]. In these tests the subject is exposed to stimuli specific to its natural predator, and the species-typical antipredatory responses of the subject (e.g. freezing) are quantified. Rosen et al. [85], for example, use

trimethylthiazoline, a chemical that is present in the fox's urine and is known to be an effective stimulus for rodents. Barros et al. [6], who studied the marmoset, used a cat (a taxidermied wild oncilla cat, the natural predator of the marmoset) as a predator stimulus. Apfelbach et al. [4] review a large variety of predator odors and their fear inducing effects in different prey species, including cat odor induced antipredatory responses in the rat. Others have utilized eye spots or eye-like structures placed on objects mimicking the appearance of predators, an approach that has been effective in a variety of species including rodents, birds and fish (e.g. [46,68] and references therein). The argument for using ethologically relevant stimuli and measuring species-specific responses in laboratory model organisms is principally based upon the notion that human anxiety disorders are likely to develop as a result of abnormal functioning of neurobiological mechanisms (brain areas, circuits and/or molecular mechanisms) that have evolved to subserve avoidance of predators or other harmful or dangerous agents in nature during our evolutionary past. Given that our species shares its evolutionary past with those of others this approach may have translational relevance. For example, Denver [24] reviews the structural and functional evolution of vertebrate neuroendocrine stress systems and explains that "Recent findings suggest that the proteins, gene structures, and signaling pathways of the HPA [hypothalamus–pituitary–adrenal] axis were present in the earliest vertebrates and have been maintained by natural selection owing to their critical adaptive roles". This author also concludes that numerous neurotransmitters and neuromodulators influencing stress-related behaviors, such as anxiety and fear, are evolutionarily conserved. Others also argue that the basic neuronal mechanisms are shared across mammalian species, and, for example, the same set of genes may regulate critical aspects of anxiety in humans and in lower species [55]. Briefly, the translational relevance of fear/anxiety paradigms is expected to be high as long as the mechanisms that evolved in the brain to subserve these behaviors are properly engaged by the experimental setup.

2. Zebrafish in the analysis of fear and anxiety

2.1. Practical simplicity and system complexity: Is zebrafish the right tool?

One commonplace in research often referred to among scientists may be summarized by a simple mathematical equation: $E \times T = C$, where E is a measure of the ease of use of a research species in the laboratory, T represents the translational relevance of this species, and C is a constant. That is, the easier a species is to use in the laboratory the less translationally relevant the results one obtains with this species will be. In other words, laboratory study species that are simple and easy enough to use often are less translationally relevant. For example, think of drosophila vs. the chimpanzee with respect to biomedical research relevance for human disorders. However, C, as defined above, may not be a constant: there may be some species for which C is higher than for others. The zebrafish appears to be one such species, at least for the purposes of behavioral brain research. I argue that while it is simple and easy to use, it is also highly relevant as a tool for the modeling and analysis of human disorders and functions. Briefly, this species represents an optimal compromise between practical simplicity and system complexity. It is a small (4 cm long) freshwater tropical fish which is easy to maintain and breed in the laboratory. Due to its highly social nature (shoaling) and its small size, a large number of zebrafish can be housed in small fish tanks. A single female may lay 2–300 eggs at every spawning and may spawn 2–3 times a week. Thus a large number of experimental subjects can be generated fast and utilized for research in a cost effective manner, which makes the zebrafish particularly appropriate for high-throughput screening applications such as those required for forward genetic mutagenesis screens or large scale drug (pharmacological compound) screens. Furthermore, the ease of use of this species is coupled with several features that make it potentially highly relevant for translational research.

Notably, zebrafish genes have been found to possess high nucleotide sequence homology (60–80%) with human genes. Perhaps even more importantly, the amino acid sequence of proteins (60–90% sequence homology), and especially at the functionally relevant catalytic or ligand binding domains of the proteins (approaching 100% sequence homology), has been found highly similar between zebrafish and human [83,84]. These findings suggest evolutionary conservation of function. Evolutionary conservation, i.e. functional and structural homologies have been demonstrated at numerous other levels of the biological organization of zebrafish, including, for example, its neurotransmitter systems [72,76,17,44] and its neuroendocrine responses to stress [2]. Conservation of function (at the gene expression level) has been found in zebrafish even in such responses as neuro-adaptation to drugs of abuse [58]. Thus the zebrafish has been deemed an appropriate model organism for the analysis of a range of human diseases [89].

2.2. Genetics: the strength of zebrafish

Another advantage of zebrafish is that, due to the past three decades of intensive developmental biology research, by now an arsenal of genetic tools have been developed for this species and the amount of information on the zebrafish genome has also become substantial. For example, a large number of genetic markers crucial for the localization and identification of randomly induced mutations have been established. These include rapid amplification of polymorphic DNA (RAPD) and amplified fragment length polymorphisms (AFLP) [25,50,98], polymorphic microsatellite markers and radiation hybrid maps with microsatellite markers and expressed sequence tags (ESTs) [37] as well as single nucleotide polymorphisms (SNPs) [96]. The latter study also utilized oligonucleotide microarrays, the gene chip technology that is rapidly spreading in zebrafish research [91]. A viral infection-based mutagenesis technique has been established for the generation of insertional mutations that could be rapidly cloned due to the presence of the viral tag in the genome [3]. An entire company was formed to use this methodology and by now a large library of mutants has been generated (see, e.g. http://www.znomics.com/; also see ref. [100]). A gene-breaking transposon-based method to generate mutations has also been developed for zebrafish [93]. In addition to forward genetic approaches, reverse genetic methods have been implemented. Morpholino antisense knockdown allows the inactivation of known genes in early embryos [73] (also see ref. [9] for a recent review). Targeted-induced local lesions in genomes (TILLING) has been successfully adapted to zebrafish [102]. Targeted gene disruption has also been achieved with the use of zinc-finger nucleases [26]. Most recently, a Gal4/Upstream Activating Sequence approach has been employed for the flexible deployment of transgenes in the analysis of expression patterns of target genes [87], and a transposon-based genetic approach has been proposed for zebrafish [74]. Importantly, all these tools and pieces of information are in the public domain (e.g., GenBank, Sanger Center website, and ZFIN, see ref. [95]). Briefly, the zebrafish has become one of the most preferred laboratory animal species of geneticists.

2.3. Behavior: the weakness of zebrafish

One drawback zebrafish suffers from as a laboratory study organism of brain research is that its behavior is not well char-

acterized, especially compared to other classical laboratory study species such as the rat, mouse, or the fruit fly [92]. This represents a major bottleneck for zebrafish research because behavioral phenotyping plays a central role in the characterization of brain function [38]. A crude literature search in Medline (PubMed) with the keyword "behavior" and "rat" reveals over 100,000 papers. A similar search with keywords "behavior" and "mouse" returns nearly 50,000 papers. But even for the fruit fly one finds almost 5000 publications in this area of investigation. However, a search with keywords "behavior" and "zebrafish" returns only 630 papers. Although relatively small, this number does allow for some optimism especially if one considers that a disproportionately large number of the zebrafish behavioral papers have appeared most recently. Briefly, behavioral brain research and behavior genetics appear to have discovered the utility of zebrafish. One of the relatively better studied aspects of the behavior of zebrafish is their fear responses. The rest of this review focuses on these studies with emphasis on how screening applications with zebrafish may be utilized.

2.4. The natural alarm substance of zebrafish elicits significant antipredatory responses

Zebrafish is a small freshwater tropical fish that inhabits slowly moving creeks and small lakes in India and Nepal [30]. Actual predator–prey encounters have not been documented for zebrafish in nature but being a small fish, zebrafish are likely to face a number of predators in their natural habitat. The zebrafish belongs to the Osterophisan superorder of fishes. Several species of this superorder have been shown to respond to alarm substances, natural "pheromones" first discovered by von Frisch [34,35], that are released from specialized epidermal club cells of the fish when the skin of the individual is cut or damaged [79]. The zebrafish has also been found to respond to its natural alarm substance. The first evidence for this was published more than five decades ago by Schutz [86] and subsequently confirmed by Pfeiffer [78] who later on also provided a systematic review demonstrating the alarm reaction in a large number of fish species [79]. In this latter review Pfeiffer also described the variety of behavioral reactions exhibited by fish in response to the alarm substance and concluded that these responses may significantly differ from species to species but may include: "(A) fish swimming excitedly with their heads against the bottom and with their bodies at an angle of about 60° to the floor; (B) becoming motionless and showing no movement for several minutes; (C) sinking to the bottom and spitting gas for a considerable time; (D) fleeing to the surface when they are alarmed, crowding together there and swimming hastily, frequently jumping out of the water; or (E) fleeing towards the depth where they form a dense school". Waldman [99] quantified the location of groups of six zebrafish in terms of vertical and horizontal coordinates and found that the alarm substance led to fish staying closer to each other and closer to the bottom of the large experimental tank he used. This author also anecdotally described a potential developmental trajectory of the alarm substance induced behavioral responses arguing that zebrafish may start showing the reaction approximately at their age of 50 days after hatching. Although at the time of their experiments Pfeiffer and Waldman had no access to sophisticated videorecording and videotracking equipment, it is noteworthy that currently such systems can easily and precisely track the location as well as movement pattern of fish [11,69,70] thus allowing automated quantification of behavior, a prerequisite for high-throughput screening.

Common to the early studies with the alarm substance was that the zebrafish used in the experiments were purchased from local pet-stores and thus the factors that potentially could affect the behavior and brain function of the experimental fish (e.g. age, exposure to other fish species, housing density, food, temperature and water chemistry, etc.) could not be controlled prior to arrival of the fish to the laboratory. The first study in which all these factors were controlled and the zebrafish alarm reactions were studied appeared only recently. Speedie and Gerlai [94] confirmed that under controlled laboratory conditions zebrafish, not exposed to any predatory, harmful, or aversive stimuli prior to the experiment, will still show a robust alarm reaction to the natural alarm substance, i.e. the alarm response to the substance is innate. Speedie and Gerlai [94] demonstrated a significant decrease of distance between members of the zebrafish group being tested (a shoal consisting of 5 fish at every observation session) and also found the duration and the number of episodes of erratic movement to increase. These authors also noted that freezing (complete immobility) and bottom dwell time appeared to increase, despite that the small experimental tank biased the behavior of zebrafish against such responses. Briefly, a typical alarm response repertoire was triggered by the alarm substance. It is also notable that these responses were demonstrated irrespective of whether the experimental zebrafish were or were not exposed to a live predator during the experiment, i.e. the alarm substance alone could induce the full fledged alarm reaction [94].

Thus the results confirmed that induction of fear responses in zebrafish is possible under controlled laboratory conditions. However, the problem with all studies employing the natural alarm substance was that the absolute concentration of the substance could not be determined. The dose response analysis in all the above-cited studies was based upon relative doses only, a dilution sequence. This is a major drawback as the appropriateness of relative concentration differences could only be ascertained within a dilution sequence but not between studies. The absolute amount of alarm substance to be extracted from the skin of zebrafish almost certainly varied from study to study no matter how precisely the extraction protocol was followed. Without knowing the chemical identity of the substance and without precisely measuring its concentration it was not possible to ascertain identical doses between studies, a major problem if one wants to conduct large-scale behavioral screens.

2.5. The synthetic alarm substance H3NO elicits significant antipredatory responses

This issue was resolved by a recent study [77] in which a synthetic alarm substance was employed. Previously, alarm substances from species of the Osteriophysan superorder of fishes were identified [79,80] and a chemical structure common to these substances was found [13,16,57]. The compound based upon this structure is hypoxanthine 3-N-oxide (H3NO), a purine derivative oxidized at the 3-position. Hypoxanthine 3-N-oxide has been shown to induce alarm responses in a number of fish species particularly those that belong to the Osteriophysan superorder [79,80,13–16]. Given that zebrafish belongs to this superorder it was hypothesized that this species too should be responsive to the synthetic alarm substance. From an evolutionary stand point being too selective about the taxonomic origin of this odor cue would be maladaptive: determining the presence of a hunting predator should not depend on what species of prey it has caught. Indeed the synthetic alarm substance was found to induce alarm reaction in zebrafish: upon administration of H3NO, erratic movements and jumps, typically observed in response to the natural alarm substance, were observed [77]. Thus, no longer were the investigators dependent upon the stochastically varying nature of the alarm substance extraction: fear responses could be induced in a precise and replicable manner.

2.6. A sympatric predator of zebrafish elicits significant fear responses

Although the above results are promising, the practical applicability of the alarm substance approach to consistent induction of fear, and thus to the analysis of the biology of fear, in zebrafish will have to be further explored. One potential issue with the alarm substance approach is that although it is ethologically relevant and now does allow one to induce fear in a controlled manner, the modality of the stimulus used makes the task practically complicated: odor cues are notoriously difficult to work with. For example, residual odor cues left behind from a prior session may influence the behavior of subsequent fish. Cleaning the test tanks is labor intensive. Furthermore, ascertaining that the odor cue used remains active also requires some attention. Last, the on-set and offset of the presentation of an odor cue is difficult to precisely control: multiple on and off periods are almost impossible to employ. Briefly, cues of other modalities, for example visual cues, may be more appropriate. Analysis of the utility of visual cues was the primary goal of study in which the effect of stimulus fish on zebrafish behavior was studied [7].

Some fish species have been found to be particularly responsive to their sympatric predators. For example, paradise fish (*Macropodus opercularis*) responded both to the sight and smell of snakehead fish (Chana) without any prior exposure to this predatory fish, i.e. without any learning, suggesting an innate predator avoidance reaction [40]. Paradise fish were also shown to be able to flexibly learn to associate previously innocuous visual stimuli with aversive stimuli (pain or predators), a response that was dependent upon genetic factors (ref. [68] and references therein), suggesting that innate predator recognition and plastic learning-based antipredatory behavior are not mutually exclusive features of a species. Zebrafish have also been shown to be able to associate stimuli of different modality (both visual and olfactory) with predatory threat [51] but what was previously not known was whether zebrafish could respond to its sympatric predator without prior learning. Most recently, however, such a response has been demonstrated. Bass and Gerlai [7] showed that zebrafish exhibited elevated number of jumps in response to the Indian leaf fish (*Nandus nandus*), a sympatric predator that is found in the same geographical region and microhabitats where zebrafish live. This antipredatory response was induced by the Indian leaf fish the first time the experimental zebrafish saw this species but such a response was not found when the zebrafish were exposed to an allopatric predator or to non-predatory fish species. It is important to emphasize that in Bass and Gerlai [7] study the zebrafish were physically isolated from the stimulus fish and thus the induction of antipredatory response was based solely upon visual cues. Briefly, although predator–prey interaction between the leaf fish and zebrafish has not been observed in nature [30], the above suggests that the zebrafish may be evolutionarily "prepared" and is particularly sensitive to the visual cues that characterize at least one of its sympatric predators. Thus visual cues can be used to induce fear responses in zebrafish and given that such cues are easier to control than olfactory cues, the above finding has important practical implications for high throughput behavioral screening. The problem, however, with the use of live stimulus fish such as the Indian leaf fish is that their behavior may be variable and thus the visual stimulus they provide may be inconsistent across sessions, which then leads to experimental error variation. Better experimental control is required if one wants to conduct large scale screening studies. A possible way to achieve consistent stimulus delivery is to present computer controlled visual cues, but could such visual cues induce fear responses?

2.7. Animated image of the sympatric predator of zebrafish elicits significant fear responses in zebrafish

To answer the above question, Gerlai et al. [44] presented animated (moving) images of the Indian leaf fish to zebrafish and found that this stimulus also was able to elicit erratic movement and jumping from zebrafish, behaviors that were previously found in response to the alarm substance or to the live Indian leaf fish. What aspect of the moving image was effective, i.e. what makes a good predatory stimulus for zebrafish, has not been analyzed. It is also not known whether other predatory fish sympatric with zebrafish could induce fear responses. The Indian leaf fish has a particular color and pattern (brown patches and markings on a silver background), size (about 10–12 cm long), body proportions (relatively large head and mouth), and movement pattern (slow or stationary ambush predator). Any one of these features or perhaps any combination of these features may be responsible for the induction of fear responses in zebrafish. It is also possible that some key features of a sympatric predator may need to be exaggerated to induce a maximal fear response in zebrafish. Perhaps other parameters of the fear paradigm may also need to be optimized. The dimensions and other physical features of the experimental tank may be changed. For example, larger tanks may allow zebrafish to exhibit a wider range of fear responses including increased bottom dwell time if the tank is deeper and increased freezing if the tank provides some physical shelter or hiding place (e.g. artificial plants). Irrespective of these potential improvements, however, the Gerlai et al. [44] study has already demonstrated that precisely controlled computer images can be employed in the induction, and thus in the future biological analysis, of fear responses in zebrafish.

2.8. The antipredatory response of zebrafish can be quantified using automated methods

Perhaps the most sophisticated pattern detection device is the human brain: the experimenter can identify complex motor and posture patterns as he/she watches the fish [43]. This classical ethological method has been employed in countless behavioral studies on numerous species successfully. However, an important drawback of this method is that it is slow and extremely time consuming. Employing observation-based methods makes sense at the early phase of characterization of behavioral responses as it allows one to obtain highly detailed information about the observed animal's behavior. However, once the experimenter established how the animal responds in the given behavioral paradigm it makes sense to try to develop automated behavior quantification methods. But could such fear responses as erratic movement and/or jumping be measured automatically?

Currently, a number of computerized systems are available from videotracking [11] to force-transducer-based methods [32] that allow automated quantification of behavior. For aquatic organisms force transducer-based detection is clearly inappropriate. Although possibly appropriate, video-image or videotracking analysis systems have not really been developed for the detection of particular movement patterns in fish. This technology is being developed for the mouse (for review, see ref. [38]). Nevertheless, in a recent study videotracking-based automated quantification of fear responses of zebrafish did reveal significant effects of the animated image of the Indian leaf fish [44]. The results showed, for example, reduced swimming speed, increased variability of swimming speed, increased turn angle and increased variability of turn angle upon presentation of the predator image. It is important to note that variability in the above responses represents the temporal variability of the behavior of a subject, i.e. within individual variability and not between individual variability. Although the above videotracking parameters were not designed to define erratic

movement and jumping, it is noteworthy that these motor patterns are expected to manifest exactly as increased variability of speed (due to occasional bouts of very fast swim episodes), increased variability of turning (straight swimming interrupted by fast turning episodes) and increased turning (more frequent and sharper turns). Briefly, quantification of fear responses was possible using the automated videotracking method. If one considers that the induction of fear responses was also computerized, one can argue that now a high-throughput fear paradigm is available for zebrafish [44].

2.9. Novelty and darkness: other methods of fear induction in zebrafish

Novelty has long been known to induce fear responses in animals and humans. One of the most well studied behavioral paradigms still frequently employed in the laboratory is the open field task in which rodents [82,21] or other animals including fish [22,28] are exposed to a novel environment. Behavioral responses in the open field have been shown to represent a compromise (and a conflict) between active exploration (believed to be adaptive as it may lead to finding food, mates and escape routes, for example) and passive fear responses (believed to be adaptive as it reduces predation risk) [21]. Quantitative genetic analyses of the behavior of mice in the open field, for example, demonstrated that an ambidirectional selection force (disfavoring either extreme) operated during the evolutionary past of the mouse [21], a finding that extends to other vertebrates including fish [48]. It is likely that zebrafish has also been under a similar selection pressure and thus exposing this fish to a novel environment will induce moderate levels of fear reactions. It is also noteworthy that all behavioral experimentation involves handling of animals by humans, and handling itself is expected to have a significant effect and induce fear. Novelty induced fear responses were analyzed by Levin et al. [61] who showed that exploratory activity of zebrafish was initially low in a novel open tank and slowly increased in the course of 5 min. Zebrafish in this task also spent more time on or near the bottom of the tank initially, a response that diminished over the course of the 5-min test. Similar findings were obtained most recently by Egan et al. [28]. Levin et al. [61] also analyzed the effect of nicotine and confirmed that this substance alleviated novelty induced fear responses. This was a significant finding because zebrafish have rarely been employed for pharmacological validation and nicotine is known to have anxiolytic properties. Before further discussing the few but already promising pharmacological studies with zebrafish, however, let us consider another simple fear paradigm that may have utility in high throughput screening for fear related phenotypes in zebrafish.

The light–dark choice task has long been employed with rodents [52,8]. In this task the subject is expected to prefer (hide in) the dark compartment and avoid the well illuminated (exposed) compartment. The evolutionary significance of this response has been argued to be the reduction of exposure to predators: in a dark hiding place the nocturnal rodent may be safer. Interestingly, the diurnal zebrafish has also been shown to prefer areas of the test environment with a dark background [88]. Given the simplicity of this task and the fact that recording of the location of the fish can be accomplished easily using automated detection methods (e.g. videotracking), the task has relevance for high throughput screening. However, some controversy does exist regarding the set up. Although Serra et al. [88] reported dark preference, Gerlai et al. [46] found that zebrafish avoided the dark compartment of a shuttle box. The difference between these two studies is simple: in the former, the authors used a dark background but the compartment was well illuminated, in the latter task, the dark compartment was truly dark, it was covered on all sides (including the top), except the one that allowed entry into it. This seemingly trivial difference between the two tasks was likely the reason for the opposite preference response exhibited by zebrafish: while a dark background may allow zebrafish to bland in (zebrafish has a dark olive-brown back) and thus avoid danger, a dark cave may harbor predators that remain difficult to detect for the diurnal zebrafish that uses vision as one of its primary senses.

2.10. The pharmacology of zebrafish fear

Despite the infancy of zebrafish behavioral research, the above demonstrates that by now numerous promising tests paradigms exist with which fear responses may be properly induced and quantified in this species. But have these tests been pharmacologically validated? Pharmacological validation is an important point when one develops a novel behavioral test method. The question is whether the task can detect the efficacy of known anxiolytic drugs. This question is often referred to as "predictive validity" of the test because the issue at stake is whether the new test will be able to find novel compounds with similar behavioral pharmacology profiles to those that have been proven efficacious in the human clinic or in other experimental model animals. Predictive validity is an important question for the use of novel model organisms too. Here, the argument fundamentally rests upon the evolutionary notion of homology, i.e. conservation of biological function across the previously utilized and the novel laboratory study species, in this case zebrafish. Zebrafish has rarely been utilized in psychopharmacological analyses but the few studies that have been completed gives room for optimism.

Perhaps one of the better studied drugs with regard to its behavioral effects on zebrafish has been alcohol (ethanol, ethyl alcohol). For example, strain dependent effects of developmental alcohol exposure were demonstrated [64], significant behavioral effects of early embryonic alcohol exposure were found in the adult [31], adaptation (tolerance) after chronic alcohol exposure and significant effects of withdrawal from alcohol after chronic alcohol exposure have been shown [44,45], and acute effects of alcohol have been also thoroughly investigated [46]. The significance of these studies from the perspective of the current review is that alcohol has both anxiolytic properties (for the effects of lower doses of alcohol in zebrafish see [46], also see [28]) and also anxiogenic effects (for the effects of prolonged exposure to alcohol and during withdrawal in zebrafish see [44], also see [28]). The effects of other drugs of abuse have also been demonstrated in zebrafish. For example, the rewarding properties of cocaine have been demonstrated and behavioral screening for cocaine sensitivity in mutagenized zebrafish has been performed successfully [23], also the reinforcing properties of drugs of abuse have been analyzed [75]. Drugs of abuse, just like alcohol, can have significant anxiolytic and anxiogenic properties depending on concentration and dosing regimen employed. For example, Lopez-Patino et al. [63] report on a cocaine withdrawal induced anxiety response in zebrafish. Classical anxiolytic drugs have also been shown to be efficacious in zebrafish. As already mentioned, nicotine has been found to reduce novelty induced fear responses [61], α-fluromethylhistidine also exhibited an anxiolytic profile [81], diazepam has been shown to reverse cocaine withdrawal induced anxiety, and the benzodiazepine inverse agonist FG-7142 has been found to induce anxiety in zebrafish [63]. Furthermore, acute administration of caffeine, which has been known to induce anxiety in humans [18] and rodents [29], also showed an anxiogenic behavioral profile, eliciting reduced frequency of visits to the upper half of the experimental tank and increased erratic movements in zebrafish [28].

Analysis of physiological responses to stress and anxiety, for example the levels of stress hormones, has also been success-

fully conducted using zebrafish [1,2]. The similarities between zebrafish and human stress responses at the hormonal level further strengthen the translational relevance of zebrafish in fear and anxiety research. For example, visual access to a predator has been shown to induce elevated cortisol levels in zebrafish [5]. Interestingly, this stress response hormone (cortisol) is also the primary stress hormone of the HPA axis in human but not in rodents, where corticosterone plays a more important role. Correlation between behavior and cortisol responses has also been documented in zebrafish. After chronic fluoxetine treatment (a selective serotonin reuptake inhibitor also known as Prozac in the human clinic) zebrafish exhibited several signs of reduced fear including more time spent in the top portion of a novel tank and dramatic reduction of the number of erratic movements as well as reduced whole-body cortisol levels [28], responses that parallel those seen in rodents [27].

2.11. The future of zebrafish in anxiety research: concluding remarks

Although it is too early to be certain about the utility of zebrafish in the biological analysis of vertebrate fear responses and how such analysis may translate to human and whether this translation will lead to betterment of human health, i.e. therapeutic applications that can reduce some or all forms of anxiety, the necessary components for future success are present for zebrafish. The main point of using zebrafish for this research purpose is simply a question of numbers. The complexity of the mechanisms of anxiety related problems may be tackled, at least initially, using large scale screening approaches. These big "fishing" experiments, albeit often not hypothesis driven, have the potential to identify numerous novel compounds (pharmacological screens) and/or a web of novel biochemical pathways (forward genetic screens) without having to have a particular focus, and thus bias, as to what mechanism to look for. It is important to note here though that I am not advocating this approach as the only possible or only potentially fruitful one. Clearly, more directed and hypothesis driven in depth analyses have their important role in research too. But at the early phase of data accumulation the screening approach may have utility. Given the growing power of genetics and of bioinformatics that allow making better sense of intricate biological information as to how large number of genes may contribute in concert to the phenotype, and given the increasingly large number of compounds pharmaceutical companies and academic laboratories generate, it is likely that screening applications will significantly add to our understanding of complex diseases such as human anxiety. And this is exactly where zebrafish has a potentially sizeable advantage over other laboratory organisms. The practical aspects of this species make zebrafish ideal for high throughput screening. The pioneering studies, some of which I have reviewed here, suggest significant homologies at virtually every level so far investigated, demonstrating the translational relevance of zebrafish. These studies also suggest that numerous zebrafish responses to fear eliciting stimuli may be induced and measured easily and in an automated manner. Is this a recipe for guaranteed success? At this point it is hard to forecast: time will tell whether this small fish will create big enough waves.

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