The role of zebrafish larvae for studying anxiety-like behaviour

Muniandy, Y.

Chapter 2

The use of larval zebrafish (*Danio rerio*) model for identifying new anxiolytic drugs from herbal medicine

Yuvendran Muniandy[1,2]

[1]Animal Sciences & Health, Institute of Biology Leiden, Faculty of Mathematics and Natural Sciences, Sylviusweg 72, 2333 BE, Leiden, Netherlands.

[2]Plant Sciences & Natural Products, Institute of Biology Leiden, Faculty of Mathematics and Natural Sciences, Sylviusweg 72, 2333 BE, Leiden, Netherlands.

*Author for correspondence. y.muniandy@biology.leidenuniv.nl

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Abstract

Anxiety is a widespread psychiatric disorder. The search for a cure is still continuing since many of the synthetic drugs were inefficient in completely treating anxiety, yet caused some dangerous side effects until many of the drugs were withdrawn from the market. One promising source of new anxiolytics could be herbal medicines. The challenge is to screen plant extracts. Rodent models can be used for this purpose but are expensive. Moreover, rodent tests are costly and consume relatively large quantities of sample. For this reason, alternative animal models may be useful. Zebrafish larvae have many advantages for screening natural products. The main advantage is that they can be produced cheaply and in large numbers. Several studies have shown that zebrafish is a good model for studying drugs that affect anxiety. This review focuses on the use of animal models including zebrafish larvae, for studying anxiety and screening for herbal medicines that modulate anxiety. Finally, future prospects of the zebrafish larva as an alternative model in this field are also discussed.

Introduction

Anxiety-related disorders are the most widespread psychiatric disorders affecting humans [1]. In severe cases, anxiety disorders can lead to significant impairment of daily functioning [2]. At present, anxiety disorders are diagnosed and classified based on systems outlined in the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-V) by American Psychiatric Association or by the International Classification of Diseases, 10th revision (ICD-10) by World Health Organization [3]. DSM-V classifies anxiety disorders into (i) agoraphobia (ii) generalized anxiety disorder (GAD), (iii) social anxiety disorder (SAD), (iv) panic disorders (PD), and (v) specific phobias [4]. The etiology of anxiety is often complicated and co-morbid with other disorders such as depression[5], whereby both may occur together with anxiety predisposing depression or vice versa [6]. It is also possible that anxiety disorders may represent an external manifestation of a disrupted homeostatic balance (for example disruption of sleep and circadian rhythm) [7].

One factor that can contribute to the development of anxiety disorder and other psychiatric disorders is stress [8-10]. Stress is a hard concept to define, although the mechanisms underlying stress are highly conserved among vertebrates [11]. Almost every discussion on stress includes three prominent figures: (i) Claude Bernard, (ii) Walter Bradford Cannon, and (iii) Hans Selye. Claude Bernard was a French physiologist who introduced the idea of *milieu intérieur* – maintenance of the internal environment surrounding cells is essential for the living organism [12]. Later, in 1929, Cannon extrapolated the works done by Bernard and coined the term *'homeostasis'*, which refers to a range of values for internal variables [12, 13]. He further postulated that any threat to homeostasis might arise due to external or internal stimuli and could be physical or psychological. Hans Selye was an endocrinologist who pioneered research in stress syndrome. He used the word 'stress' in a physiological context to describe the body's non-specific response to any demand placed upon it [14].

Selye showed that acute exposure of rats to non-specific nocuous agents such as low temperatures, spinal shock, and intoxication with various drugs (atropine, morphine, adrenaline, etc.) produced characteristic and harmful syndromes [15]. Initially, Selye named this syndrome as "general adaptation syndrome", and later renamed it as "stress response" [16]. The word "stress" was used for the first time by

Selye to describe this syndrome in his first comprehensive monograph published in 1950. Though Selye's discovery was groundbreaking, he faced heavy criticism in the late 1940s and 1950s for naming both the cause and effect as stress [16]. Hence, the word "stressors" was used to reflect agents that trigger stress response [16]. Though there were many complaints from physicians and scientist regarding Selye's discovery and the confusion in the definition of stress, one physician[17] quoted the following *"Stress in addition to being itself, was also the cause of itself and the result of itself"*. The role of stress in anxiety will be further discussed in the next section.

Even after decades of intensive research using model organisms, *in vitro* studies, and clinical trials, the ability to treat anxiety effectively is still inadequate [18]. Although many drugs are available for anxiety (referred as 'anxiolytics'), individuals who suffer from anxiety and anxiety-related disorders are on the increase. Furthermore, the highly sedentary lifestyle that we are living nowadays can be an important contributing factor to the increased incidence of anxiety [19]. The reasons behind ineffective treatment for any type of neurobehavioral disorders are: (i) low bioavailability of drugs, (ii) ineffective drug-delivery method, (iii) lack of knowledge on genetic factors, (iv) lack of suitable model organism(s) for drug discovery and (v) failure to tailor the treatment program to the individual (i.e. failure to adopt the principles of personalized medicine) [20]. Therefore, these are important considerations for researchers from different fields of neuroscience in order to find new therapeutic drugs.

The main scope of this paper is not to review each plant species in detail in terms of its phytochemistry and pharmacotherapy as these were reviewed extensively elsewhere [21-26]. The literature is superfluous with many reviews on specific plant species (for example *Hypericum perforatum*[27, 28], *Passiflora incarnata*[29], *Valeriana officinalis[30, 31], Withania somnifera*[32]) or specific mechanistic action of herbal preparations (for example modulation of different receptors such as the gamma-aminobutyric acid (GABA receptors)[33] and serotonin receptors [34]). The main scope of this review is to discuss the perspective of using herbal anxiolytics on zebrafish larvae as a model system. Moreover, current challenges in phytochemistry and zebrafish neurobehavior were also discussed in this review.

Etiology of Anxiety

In normal situations, our body responds to threatening stimuli via different mechanisms such as defensive behaviors, autonomic reflexes, increased alertness, and catecholamine and corticosteroid secretion [35]. It is normal to feel fearful at some points in our lives when there is a perceived imminent threat to our sense of well-being [4]. Such a perceived threat could be the taking of an exam, the giving of a public talk, or the making of an important life decision. However, if there is an anticipation of a future threat, and it is either irrational or is never resolved, then a pathological state of anxiety may develop [4]. Fear and anxiety are usually emotion-based adaptive responses to stressful stimuli or threats [36]. These responses may arise due to external inputs (auditory, olfactory, visual or somatosensory stimuli); or internal inputs from the endocrine and nervous systems [36].

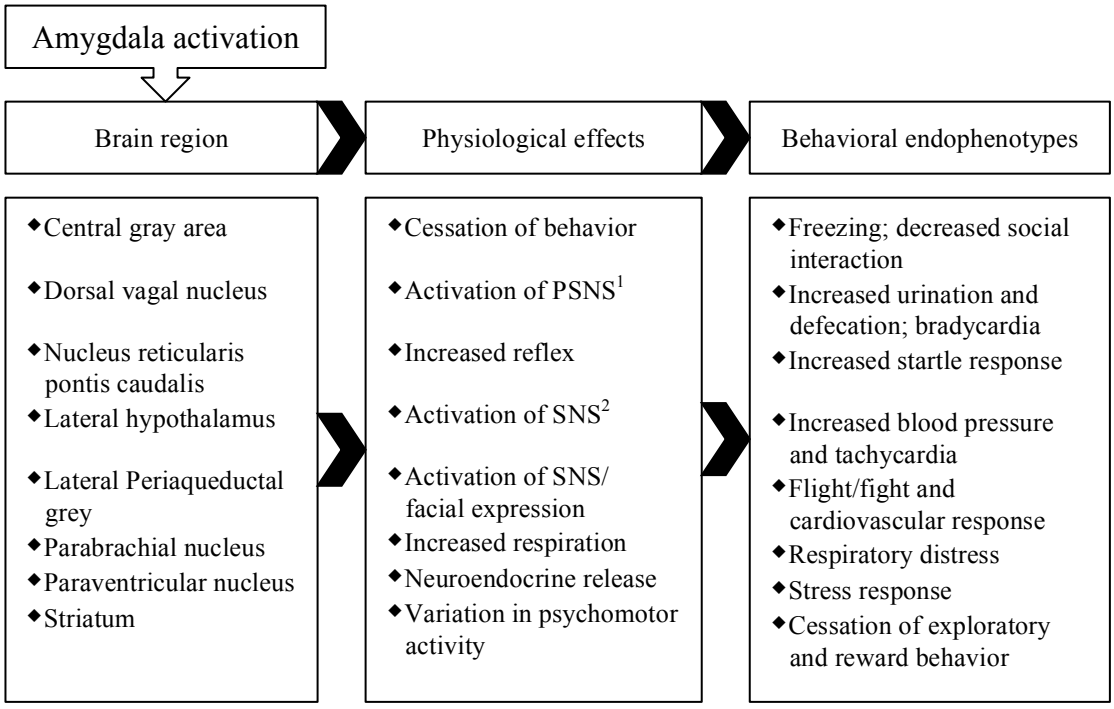
Apart from these stimuli, anxiety may also be triggered by unpleasant memories or the anticipation of stressors or threats [36]. Though anxiety and fear may represent two similar emotional conditions, they can be easily distinguished on the basis of the controllability of the threat[37] (that is, the extent to which the threat can be controlled by the individual concerned). According to Epstein's view[37], in a fear response, there is a hope of controlling the threatening situation. By contrast, anxiety appears when the attempt to control the threat (i.e. to cope) has failed, and the threat is therefore perceived to be uncontrollable or uncertain (a helpless state) [37]. Defining anxiety and fear in terms of different controllability scales has an added advantage, whereby they can relate to another psychological disorder – depression. When feelings of uncontrollability increase and last for long period, the organism enters the state of being hopeless and anxiety is replaced with depression [38, 39]. The etiology of anxiety covers different anatomical structures involving mainly the nervous and the endocrine system.

Functional anatomy of anxiety

Disruption in the limbic system, an important emotional center of the brain, is linked to anxiety disorders [40]. The limbic system includes the hippocampus, hypothalamus, medial prefrontal cortex, and amygdala [40-42]. Resilience towards anxiety disorders is correlated with hippocampal volume and neurogenesis [40]. By contrast, the amygdala is responsible for the formation and retrieval of fearful memories. The amygdala has many interconnections with various parts of the brain

including the hippocampus, thalamus, hypothalamus [40]. The amygdala becomes activated during the fear response and this causes various behavioral responses [36, 40, 43] (**Box 1**). Neuroimaging studies in humans verify that distinct but related brain anatomical structures motivate fear and anxiety. Fear is also known as 'phasic fear', and anxiety as 'sustained fear'. Based on experimental paradigms using rodent models, nonhuman primates, and humans, Davis and colleagues have concluded that the amygdala mediates the fear response while anxiety is mostly governed by the bed nucleus of the stria terminalis (BNST) [44].

Box 1. Schematic diagram depicting the role of the amygdala in the fear response. Upon external stimulation, the amygdala induces various physiological effects, which in turn producing some behavioral endophenotypes that can be exploited as an index for anxiety. Adapted from Fig.2 in Davis (published in *Annu. Rev. Neurosci*. with permission of the publisher, Annual Reviews©, California, USA) and with additional information from Fig. 63.1 in Charney and Drevets (published in Neuropsychopharmacology – 5th Generation of Progress with permission of the publisher, Lippincott Williams and Wilkins©, Philadelphia, USA).



[1]Parasympathetic nervous system
[2]Sympathetic nervous system

Theories of anxiety pathophysiology

There are many theories suggesting the pathophysiology of anxiety. Examples include the GABAergic theory, the stress response theory, and the monoamine theory. Different classes of biomolecules are involved in these theories such as neurotransmitters, hormones (adrenaline, noradrenaline, and cortisol), neurotrophins,

and neuropeptides. These biomolecules are involved in signaling in the brain, and between the central nervous system and peripheral tissues.

GABA-ergic theory

Gamma-aminobutyric acid (GABA) is one of the major inhibitory neurotransmitters in the central nervous system. Receptors for this neurotransmitter are localized in the brain and peripheral nervous system [45]. The role of GABA in mood disorders was first identified based on the clinical efficacy of valproic acid (a GABA agonist) in the treatment of bipolar disorder [46]. Furthermore, the gold-standard anxiolytic, diazepam (Valium), a benzodiazepine, acts via the GABA pathway. Benzodiazepines do not bind to the receptor site where the endogenous ligand GABA binds, but to a different site located between α- and γ-subunits of $GABA_A$ receptors[47, 48], which is also known as the benzodiazepine site [49].

Many preclinical and clinical studies support the role of GABA in mood disorders [50-56]. It is assumed that decreased inhibitory signaling in the GABA-ergic system could be the main reason for the pathophysiology of anxiety [57, 58]. Another important role of the GABAergic system is in the regulation of inhibition of HPA axis activity. However, the GABAergic control of the HPA axis is highly susceptible to both acute and chronic stress [59]. Although the GABAergic system is well studied, it is still not known the exact role of this system in hyperactivity of HPA axis. Scientists are still speculating whether a deficit in the GABAergic system independently causes HPA axis hyperactivity that leads to mood disorders or if the dysfunction of the GABAergic system is secondary to stress-induced HPA hyperactivity in mood disorders [60].

Stress Response Theory

The stress response is widely conserved across the vertebrate species, in order to maintain survival [61]. Nevertheless, due to our sedentary lives, this mechanism may lead to health problems [14]. In his later research, Hans Selye found that not all stress responses are bad for our health [16]. Lenard Levi's clinical and social investigations in Sweden played a prominent role in shifting Selye's mindset. According to Levi, our cerebral cortex has the ability to differentiate between adrenocorticotropic hormone (ACTH) and corticoids released under unpleasant (arguments with a spouse) and pleasant situations (pleasure of kissing a girlfriend or boyfriend) [62]. Hence, Selye

introduced the terms “eustress” and “distress” to differentiate positive and negative stress respectively [63]. Distress can be either acute (intense but of short duration) or chronic (of long duration and possibly low intensity) [64]. The concept of eustress is incomplete[65], due to the lack of clear criteria to differentiate this type of stress from distress and insufficient knowledge on the basis of eustress [66].

Perhaps the easiest way to explain the relationship between eustress, distress, and health is with the help of Yerkes-Dodson principle (depicted in **Figure 1**) [67]. According to this principle, there is a non-linear relationship between the intensity of stress levels and health. The concept of ‘*hormesis*’ could give a clearer interpretation quantitatively on the Yerkes-Dodson principle. Hormesis is a process that causes cells or organisms to exhibit a biphasic response to an increasing amount of substances or conditions [68]. In other words, lower dose exposure results in a beneficial response, while the higher dose is detrimental and toxic [68]. According to Le Fevre et al.[69], an individual’s perception and interpretation of a condition determine whether a stressor becomes eustress or distress. It could be speculated that the hump/ maximum performance (as shown in Fig. 1) is variable individually. Previous studies revealed large inter-individual variations in the stress response to psychological challenges [70-74].

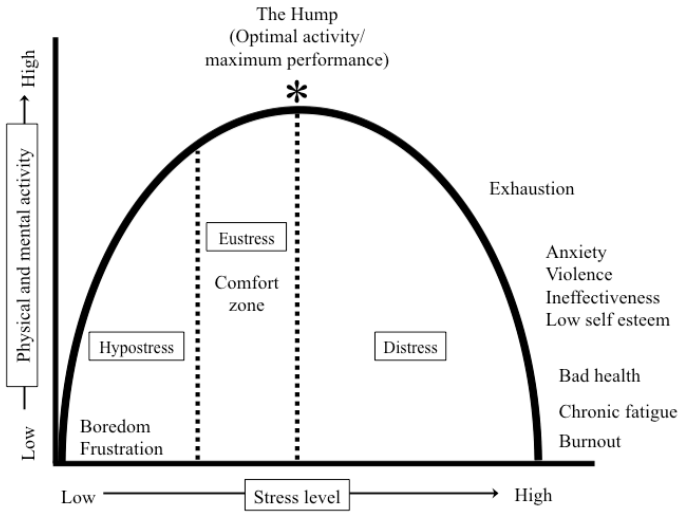


Figure 1. Yerkes-Dodson curve showing different types of stress. Stress left to the midpoint represents positive stress (eustress) while stress beyond this point is considered as negative stress (distress) that can affect our health. An extreme abundance of stress or hypostress can cause boredom and poor performance. Redrawn with modifications from Fig. 1 in Rapolienė *et al.* (published in *Adv. Prev. Med.* with permission of the publisher, Hindawi©, Cairo, Egypt) and Fig. 1.5 in Seaward (published in Managing Stress: Principles and Strategies for Health and Well-Being with permission of the publisher, Jones and Bartlett Learning©, Massachusetts, USA).

Physical and psychological stressors are capable of causing different biological response, including release of catecholamines, sympathetic arousal [also known as sympathetic-adrenal-medullary (SAM) axis], and hypothalamic-pituitary-adrenal (HPA) axis activation [75]. Acute and chronic stress are different[76], whereby the former is governed by the SAM axis, while the latter mainly involves HPA axis [75]. Acute stress-response is the immediate action of the sympathetic nervous system (SNS) that readies an organism for flight/fight response [75]. Upon activation, the SNS causes the adrenal medulla to release adrenaline and noradrenaline into the bloodstream [75]. These two hormones prepare our body for the threat by increasing the heart rate and blood pressure, dilating pupils and inhibiting gastrointestinal activity [75]. The main objective is to prepare the body for the threat by maximizing muscular output and reaction speed [14].

Although acute and chronic stress can both activate the HPA axis[77], chronic stress is thought to be the main cause of many stress-related diseases, since our body is constantly aroused for danger [64]. Different endocrine pathways govern the functioning of HPA-axis (as summarized here in **Figure 2**). Moreover, HPA axis activity is modulated by different parts of the limbic system, such as the amygdala and hippocampus. The amygdala elevates HPA axis activity while the hippocampus causes suppression of HPA axis activation [40]. Stressors trigger the short-term adaptive responses that involve short-term activation of HPA axis, whereby a negative feedback system via glucocorticoid receptors establish a homeostatic balance. Unfortunately, under excessive stress conditions, the HPA axis system becomes maladaptive. This causes a negative impact on the limbic system and increases the risk for many psychiatric disorders[78], including anxiety. Chronic stress is often characterized by hyperactivity of the HPA axis with elevated cortisol levels. A hyperactive HPA axis can be explained by two mechanisms. One mechanism suggests that impaired feedback inhibition is responsible for decreased glucocorticoid receptors activity. In the other, there is excess glucocorticoid signaling [79]. Furthermore, higher cortisol concentrations also cause toxic effects on the hippocampus through reduced brain-derived neurotrophic factor (BDNF) expression [80].

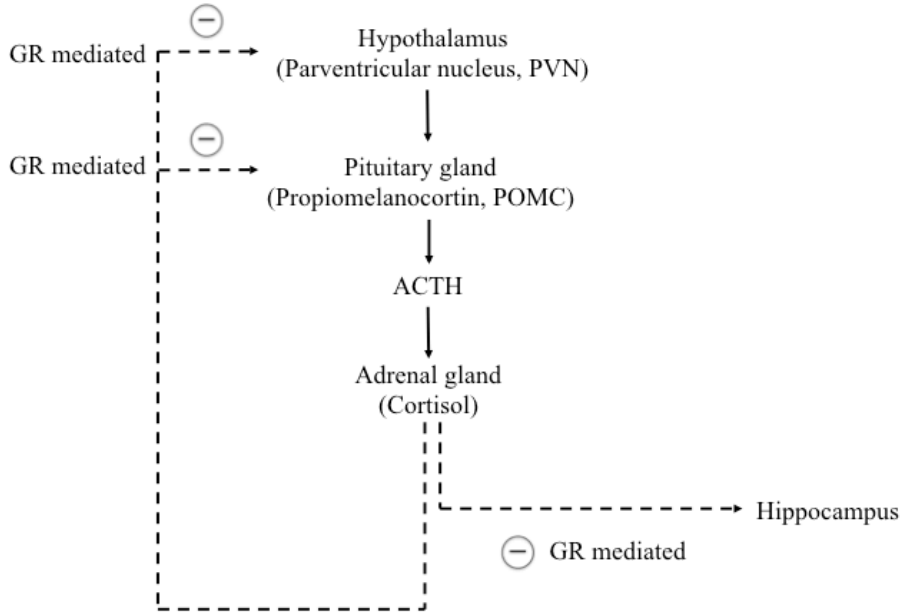


Figure 2. General organization and functioning of the HPA-axis in humans. The paraventricular nucleus (PVN) of hypothalamus induces propiomelanocortin (POMC) secreting cells in the pituitary gland to produce adrenocorticotropic hormone (ACTH). This hormone will activate adrenal glands of the kidney to release cortisol (the main stress hormone). A negative feedback system via glucocorticoid receptors (GR) establishes homeostasis of the HPA axis. Adapted from Fig. 1 in *Steenbergen et al.* (published in *Prog. Neuro-Psychopharmacol. Biol. Psychiatry* with permission of the publisher, Elseiver©, Amsterdam, Netherlands).

In 1993, McEwen and Stellar proposed a new model to give a clearer explanation of the difference between acute and chronic stress. According to them allostasis and allostatic load are the important factors that distinguish acute from chronic stress [81]. To have a better understanding of this concept, it is important to incorporate the concept of homeostasis as well. Comparatively, homeostasis is defined as physiological systems that are essential for the stability of life, while allostasis is the process that maintains these systems [82]. From a practical viewpoint, homeostasis preserves set points and various boundaries of physiological states (such as pH, temperature, etc), whereas allostasis allows for a modification of these set points in order to counter challenges [82]. Therefore, by default allostasis is positive and necessary to sustain life and it actually supports homeostasis [83]. On the contrary, the allostatic load is the body's wear and tear due to the repeated activation of the adaptive response to stress [81]. The concept of allostasis and allostatic load is reviewed in detail elsewhere[66, 81-83] and beyond the scope of this review.

One of the important features of stress response theory, especially regarding anxiety, is the coping mechanism. Coping involves physiological, psychological and

behavioral responses in order to avoid a threat or distress, and applies to both animals and humans [84, 85]. It is more apparent that susceptibility to stress-induced diseases varies between individuals and may involve a coping mechanism [86]. In the human context, this mechanism is comparable to "temperament" or "personality" traits, which are essential in maintaining an adaptive capacity under changing environments [87]. In general, coping styles among individuals can be classified into two groups: active (proactive) and passive (reactive). However, there is a possibility for large inter-individual variability within these two groups [86].

In addition to the two coping styles mentioned above, there are also two ways in which an organism may respond towards a threat or negative stimulus. One is an active strategy (involving flight-fight response)[88], whereby the main goal is to eliminate the source of threat. The other one is a passive strategy (involving conservation/ withdrawal)[89] and the main aim of this strategy is protection from the consequences of threat. The active coping strategy involves the SAM axis whereas the passive coping strategy involves mainly the HPA axis (see above). Individuals turn to a passive coping strategy whenever the flight-fight response has failed (for example arrested flight[90], entrapment[91], and defeat[90]). It is assumed that anxiety is remarkably increased when the passive coping strategy are used more frequently (**Figure 3**) [86].

Monoamine Theory

Well documented anxiolytic activity of some antidepressants such as fluoxetine (Prozac®) and amitriptyline (Elavil) suggests the involvement of the monoaminergic system in the pathophysiology of anxiety [40]. According to this theory, disruption of monoaminergic system in the synaptic cleft, specifically involving catecholamines [dopamine (DA) and noradrenaline (NA)] and the indoleamines (serotonin, 5-HT) is thought to be the main reason for anxiety [92]. One candidate gene thought to be causing a malfunction in the signal transduction of monoamines is BDNF [93]. In healthy individuals, BDNF promotes the survival of neurons in the brain. However, under a stressed condition, this gene is down-regulated. This leads to degeneration and apoptosis of neurons in the hippocampus of depleted BDNF [93].

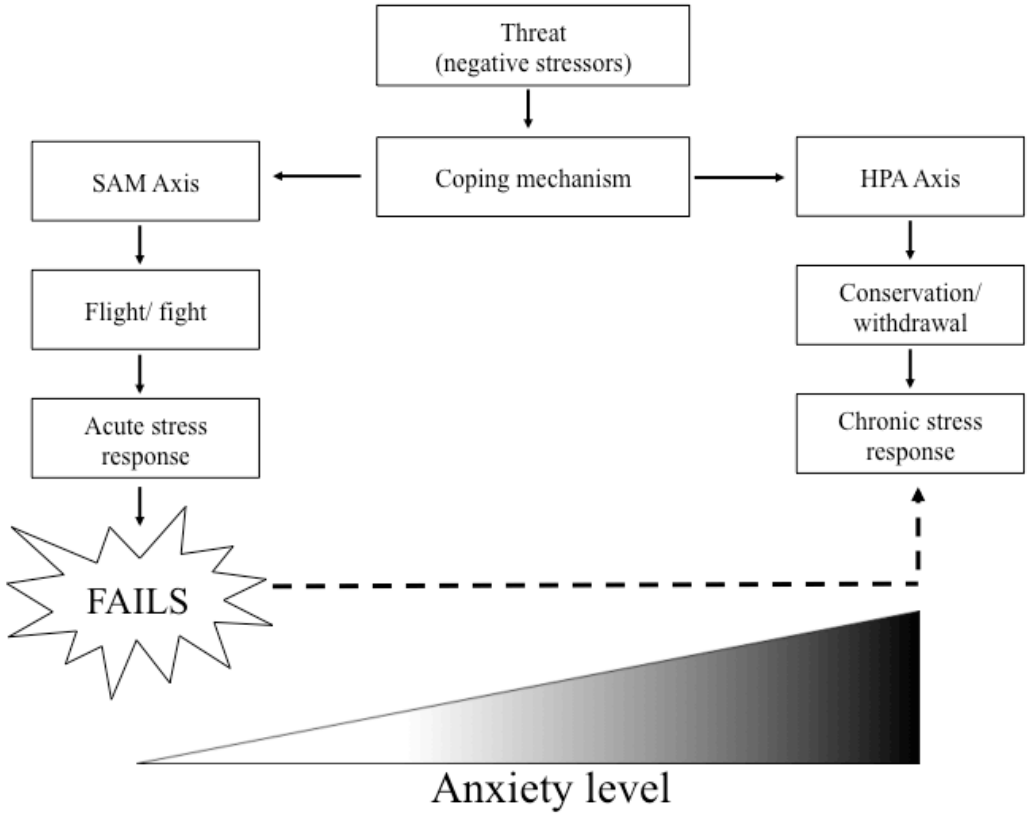


Figure 3. Coping mechanism is a response to threat (negative stressors). See text for details. Adapted from Figure 1 in Steimer (published in *Dialogues Clin. Neurosci.* with permission of the publisher, Les Laboratoires Servier©, Suresnes, France).

Model Organisms for Studying Human Psychological Disorders

The purposes of using animal models to study anxiety are to (i) understand the basic mechanisms (pathophysiology) of anxiety and (ii) develop new therapies [94, 95]. McKinney and Bunney have suggested that a model organism should have the following minimum requirements in comparison with humans: (i) similar pathophysiology (face validity), (ii) comparable etiology (construct validity), (iii) common treatment (predictive validity), (iv) causes behavioral changes that can be monitored accurately, and (v) most importantly reproducible between investigators [96].

Theoretically, a model organism should reproduce all features of a human disease or disorder under investigation. However, this is rarely achieved since psychiatric disorders (including anxiety) are characterised by several clusters of symptoms [97].

Therefore, no model organism can reflect the whole range of symptoms associated with anxiety [98]. Despite this limiting factor, animal models are still useful in research concerning anxiety, since psychiatric disorders are characterized by endophenotypes [99, 100]. The term endophenotype indicates a series of behavioral characteristics that are associated with altered processes involved in particular illnesses [101]. Therefore, instead of replicating the whole syndrome of a specific psychological disorder, an animal model is more suited to replicate particular cluster of symptoms involved in anxiety [98]. Using endophenotypes to design animal models for psychological disorders offers many advantages. For instance, Bakshi and Kalin have highlighted that endophenotypes offer higher chances for construct and predictive validity in the model [98].

Behavioral Models for Studying Anxiety

So far, the rodent model has been the most extensively and successfully-used laboratory animal in anxiety research. The rodent behavioral models used to study endophenotypes of anxiety can be broadly classified (see **Table 1**) into two categories: (i) unconditioned responses (measuring the organism's innate exploratory behaviour[98]) and (ii) conditioned responses (which often involve training, and interfere with memory and motivational processes [102]). The startle response is a common endophenotype in both conditioned and unconditioned responses. The startle response involves reflex movements upon a stimulus [103]. There are three types of startle responses observed in rodent models: (i) a general startle response (measured while the animal is not subjected to any stimuli), (ii) fear-potentiated startle (observed after the animal is exposed to a stimulus or stimuli), and (iii) context-potentiated startle (promoted by the uncertainty of whether the threat is present or not [103]).

According to Montgomery, animals exposed to a novel environment may respond either by showing an exploratory tendency (driven by curiosity) or withdrawal cues (driven by fear) [104]. There are many factors that affect these behaviors, such as the degree of novelty, the complexity of the situation, and the internal state of the animal [104-108]. Moreover, in a novel environment, animals with low anxiety levels will tend to explore the new environment, while the anxious ones will hesitate to take risks. Therefore, evaluating locomotion and exploratory tendency as the main parameters gives information on the degree of anxiety. The above-mentioned two parameters are useful for evaluating potential new anxiolytic drugs, whereby a

decreased value for these two parameters could indicate anxiolytic activity [109]. However, it is important to consider that a decline in these two parameters might not be purely anxiolytic if they cause additional effects such as locomotion inhibition, toxicity or sedation [109].

Table 1. Rodent models of anxiety and the various tests that they include. Adapted from Table 1 in Steimer (published in *Dialogues Clin. Neurosci.* with permission of the publisher, Les Laboratoires Servier©, Suresnes, France).

Unconditioned response models	Conditioned response models
1. Exploratory behavior	*1. Conflict test*
• Elevated plus maze (EPM)	• Geller-Seitfer test
• Elevated T maze	• Vogel test
• Open field test (OFT)	
• Hole-board test	
2. Light/dark preference test (LDPT)	*2. Avoidance test*
• Light/dark box	• Active avoidance
• Light/dark open field	• Passive avoidance
3. Social behavior	• Fear-potentiated startle
• Social interaction test (SIT)	
• Stress-induced vocalization	
4. Others	
• Baseline startle response	
• Stress-induced hyperthermia (SIH)	
• Predator based model	

In conditioned response anxiety models, an animal's ability to predict aversive events (fear conditioning) is exploited. In this model, an aversive stimulus (unconditioned stimulus, US), such as mild electric shock, is often paired with a neutral stimulus (conditioned stimulus, CS), such as smell, light or sound [110]. Usually, after several pairings of neutral and aversive stimuli, the animal learns that the neutral stimulus is associated with a negative experience [110]. This will eventually elicit fear responses whenever the animal is presented with a neutral stimulus only. One of the important fear responses shown by animals in this model is freezing behavior (characterized by complete cessation of movement, except respiration) [111].

An important behavioral endophenotype assessed in the open field test (OFT) is thigmotaxis. Thigmotaxis is characterized by a preference for an environment adjacent to the periphery, rather than the centre of an arena. This endophenotype has been used as a key index to measure anxiety in mammals was reported in rat[112] and mice [113]. Light dark preference test (LDPT) is another experimental model largely

based on rodents' innate aversion of brightly lit environments [114]. Behavior in LDPT reflects a conflict in animals between the preference for protected areas (for e.g., dark compartment) and innate motivation to explore a novel environment [115]. In both OFT and LDPT, the key parameters are 'percentage time' and 'percentage distance' spent in both 'safe' and 'unsafe' zones of an arena [109]. Moreover, total distance moved in two zones also included since this can give valuable information on the side effects of the drugs. The starting point for all three tests are very important as the animals may show freezing behavior when placed in an 'unsafe' zone [109].

Besides the above-mentioned experimental models, there are other models to study anxiety: one example would be by inducing chronic stress. In the field of stress research concerning anxiety, the main objective is to have a long-lasting stressor that can impair homeostatic state in order to resemble a state of being anxious [109]. There are many ways to accomplish this in the laboratory. Some examples include the following: (i) prenatal stress, (ii) olfactory bulbectomy stress, (iii) repeated restraint stress, (iv) repeated unpredictable stress, (v) repeated social defeat stress [109]. Chronic stress in mice was reported to be inducing anxious-like behavior[116] with elevated levels of DA, NA, and 5-HT levels in the cerebral cortex [117].

Obstacle in Using Animal Models for Anxiety

Though anxiety can be modeled in the laboratory as explained above, there are several problems that need to be addressed by behavioral scientists when developing animal models of anxiety. Perhaps the first question that arises is whether anxiety is exchangeable with fear, stress, panic, or sensitivity towards an aversive situation?[118] In the animal kingdom, fear has evolved as an adaptive response to provide protection from possible dangerous environments[118], whereas anxiety is fear produced in an anticipated manner towards an imprecise threat. Despite, both fear and anxiety cannot be interchanged, but they can be modulated by the same factors such as environmental and genetic factors [118]. Therefore, it is a normal practice to use fear-related behaviors in a rodent model to investigate anxiety disorders [119]. However, in the field of neuroscience, fear and anxiety appear as two strictly different yet related paradigms [118].

Another important criterion in neurobehavioral research that is often overlooked is the ability to make implicit assumptions when designing animal models of anxiety. In

most animal models of anxiety, a random population of animals is used to study anti-anxiety drugs [120]. However, in the human population, only a small group severely affected by anxiety seek medical attention [121]. Furthermore, it is also necessary to distinguish between 'trait-anxiety' and 'state-anxiety'. According to Lister "State-anxiety is an anticipated fear one experiences at a particular moment and often increased by the presence of an anxiogenic stimulus. Conversely, trait-anxiety is a continuing attribute in an individual with no variation from time to time"[121] The difference between trait-anxiety and state-anxiety can be explained by the following example. Individuals with ophidiophobia may have trait-anxiety at a normal level and low baseline level of state-anxiety under most circumstances. However, the introduction of snake in their environment may increase the state-anxiety. Often, most behavioral studies focus on therapeutics for 'state-anxiety', whereby an animal is exposed to an anxiogenic stimulus before the effect of candidate drug is assessed. Although this approach is easy, fast, and logical, it oversees important factors that contribute to high trait-anxiety, which might not be beneficial to a chronically anxious individual [121].

As mentioned earlier, a good experimental model must have good predictive validity. This is hampered by the ambiguous psychological and pharmacological theories of anxiety. For instance, pharmacologically it is validated that there are standard anxiolytic drugs. Despite this fact, it is undeniable that there is a dispute on which drugs should be used as standards [121]. According to Lister pharmacological validity alone cannot make a good model for anxiety. This is because of many drugs used for anxiety cause various side effects including ataxia, anterograde amnesia, and sedation [121]. Another important remark by Lister is that if an anxiolytic drug is functional in a particular experimental paradigm, it is not necessary for that particular paradigm to be assessing anxiolysis [121]. This is even harder with drugs that have multiple behavioral effects. For example, anxiolytic drugs often function as anticonvulsants as well [122]. Some studies have reported the ability of drugs to antagonize convulsive actions of PTZ as anxiolytic agents. Lister argued that such experimental models must be classified as a correlation model[123] and not as an anxiety model.

Pharmaceuticals for Anxiety

Anxiety disorders are very heterogeneous in nature; therefore not all anxiety patients are the same, clinically [98]. As mentioned earlier, the pathophysiology of anxiety overlaps with other psychological disorders, such as depression. Initially in the 1960's the treatment for anxiety and depression were distinctly different, whereby the diagnostic notions were clearly dichotomized into major depressive disorder (MAD) and generalized anxiety disorder (GAD), while other anxiety disorder subtypes were clustered together [93]. However, starting from the 70s and 80s, antidepressants overlapped with anxiolytics used in treating anxiety disorder subtypes [93]. At present, the pharmacological treatment for anxiety includes benzodiazepines, 'non-benzodiazepine anxiolytics', tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs), and selective serotonin reuptake inhibitors (SSRIs) [124].

Benzodiazepines affect the GABA system[23] via different mechanisms such as induction of ionic channel transmission, alteration of membrane structures [125], inhibition of GABA transaminase or glutamic acid decarboxylase [33], or just simply by binding to the benzodiazepine site of GABA receptor. Increased GABA neurotransmission produces a damping effect on stimulatory pathways, which eventually provides a relaxing effect and thus alleviating anxiety [126].

TCAs include amitriptyline, clomipramine, doxepin, and trimipramine. This class of drugs targets the norepinephrine and serotonin reuptake mechanism located on the presynaptic membrane of the noradrenergic and serotonergic neurons. By doing so, TCAs increase the accessibility of noradrenaline and serotonin to their corresponding postsynaptic receptors and thus allow enhanced neurotransmission [127].

Additionally, monoamine oxidases (bound to the outer membrane of mitochondria[128]) regulate monoamine levels by breaking down endogenous monoamines (NA, 5-HT, and DA) released in the neuronal cytoplasm in order to avoid excessive build-up and lethal interactions [129]. MAOIs prevent the catabolism of monoamines by blocking the actions of this enzyme. This results in an escalated concentration of monoamines at the synaptic cleft and at postsynaptic receptors [130].

Motivation to use Natural Products for the Treatment of Anxiety

Many medicines of plant origin have been used for centuries to calm the mind. In most Asian countries traditional herbal medicines have a long history of usage in disease prevention and treatment. Herbal medicines also appear to be popular in the

West. For example, European countries spent $4.96 billion on over-the-counter herbal medicines in 2003 [131]. In the same year, Ginkgo and St. John's Worst were the most commonly reimbursed herbal medicines among German health insurance providers [131]. More information on the prevalence and the type of herbal medicines used by adults who experience anxiety and anxiety disorders is reviewed elsewhere [132]. In Asia, the two main traditional medicinal systems that exploit plant-based treatment are Ayurvedic medicine and traditional Chinese medicine (TCM). Some examples of the plant species used as anxiolytics in those systems include ashwagandha (*Withania somnifera*), passionflower (*Passiflora incarnata*), St. John's Wort (*Hypericum perforatum*), and valerian root (*Valeriana officinalis*). It is also important to note that in Western Europe there was a centuries-long tradition of using plants as anxiolytics and these plants were listed in the official Pharmacopoeias. For example, the British Pharmacopoeia of 1885 lists valerian root among numerous other plant remedies [133].

Most synthetic drugs act according to a 'single-disease/single-target/single-drug' strategy [134]. However, herbal medicines exert their therapeutic actions via interactions of multiple active compounds (known as a synergistic effect). This synergistic effect can be defined as a collective effect produced by a combination of compounds rather than from an individual contribution alone [135]. The concept of synergism is common in traditional medicinal systems [136]. Moreover, there is a possibility for conventional modern (allopathic) medicine to overlook complex mechanisms underlying anxiety. Therefore, the reductionist approach seen in treatments using synthetic compounds could be one of the reasons for the ineffective treatment of anxiety and other psychological disorders. Herbal extracts are speculated not to be directly involved in pathophysiological processes, but instead alter the absorption, distribution, metabolism, and excretion (ADME) of bioactive compounds, or even reduce their side effects [137].

Often, herbal medicines are reported to have 'adaptogenic' effects and are referred to as 'adaptogens' [21]. In 1947, Dr. Nikolai Lazarev introduced the term 'adaptogen' [22]. Adaptogens are substances that are suggested to produce a state of raised resistance, enabling an organism to manage different kinds of stressors [138]. According to Breckhman and Dardymov [139], an adaptogen must have the following: (i) produce a nonspecific response, (ii) have a normalizing effect on the

body, and (iii) do not influence normal body functions. However, The European Medicine Agencies has expressed doubts about the adaptogen concept [140].

The Challenges in the Research of Herbal Medicine

Plant-based anxiolytics have for many years been assayed on rodent-based behavioral models (reviewed by Sarris *et al.*[26]), Moreover, many preclinical[141-147] and clinical studies[148-153] have identified the anxiolytic activity of herbal medicines. Nevertheless, there are several challenges hampering the progress of herbal psychopharmacology (summarized in **Table 2**).

Table 2. Current challenges present in research involving herbal psychopharmacology. Adapted from information in Sarris *et al.* (published in *Eur. Neuropsychopharmacol*. with permission of the publisher, Elseiver©, Amsterdam, Netherlands).

Challenges	Explanations
Predictability of model	• Evidence from *in vitro* model cannot be extrapolated to human clinical applications [26] • *In vivo*, herbal constituents undergo biotransformation [26]
Experimental design	• Difficult to standardize experimental design [26] • Improper standardization causes poor translation between different studies [26]
Incomplete studies	• Therapeutic effects of main active ingredients do not guarantee same effect for crude extracts [154] • Traditional medicinal systems assume synergy to be the main reason for therapeutic effects [137]
Low bioequivalence	• Different commercial herbal preparations have different bioequivalence that needs to be evaluated [26] • Difficult to assess safety and efficacy due to low bioequivalence [26]
Poor replication	• Therapeutic effect is not reproducible in different laboratories [26] • For example, *Piper methysticum* in Europe yielded positive results, however, similar results are not replicable in the United States.
Practical flaws	• Herbal extracts have flaws that hamper translation into therapeutic application [26], some examples are: ▪ Inability to cross the blood-brain barrier [155] ▪ Poor aqueous solubility [155] ▪ Propensity to degrade easily [155]
Safety and efficacy	• These are highly dependent on the chemical composition of the extract, which is influenced by numerous factors [26] including: ▪ Phytochemical variability ▪ Environmental conditions (temperature, rainfall and etc.) ▪ Exposure to pests and microbial infections ▪ Parts of the plant used for extraction ▪ Preparation method (harvest time, storage, and extraction) ▪ Quality of soil

Despite all these bottlenecks, scientists assume that the integration of omics-technology (systems biology) into phytomedicine will advance this field since this will pave the way to explore different areas of this field. This approach often includes

various biochemical inter-disciplines, such as pharmacogenomics, proteomics, and metabolomics [26]. An example of an application of omics-technology is studying the epigenetic effects of herbal extracts using proteomic analysis [26]. *Hypericum perforatum* was used in two epigenetic studies [156, 157], which revealed the regulation of different genes and proteins involved in synaptic and energy metabolism function. Therefore, systems biology may provide answers to many questions in phytomedicine, such as clinical efficacy, pharmacodynamics, synergy effects, and toxicity.

The zebrafish in Neurobehavioral Research

The zebrafish (*Danio rerio*) is now an important model organism in neuropharmacology. Both adults and larvae are extensively studied to increase our understanding of the brain function, dysfunction and their genetic and pharmacological modulation [158]. Neurobehavioral tests to assess anxiety in zebrafish are adopted from rodent models [159]. Such tests include open field tank, light-dark tank, and novel tank diving test [158].

Zebrafish has high genetic and physiological homology to humans [158]. Other features of zebrafish are a central nervous system (CNS) similar to that of mammals including mouse and humans[160-162], high fecundity (a single female can produce up to 300 eggs at a time[163]), rapid embryonic development (major organs form within 1 day post fertilization (dpf)[158], easy maintenance at high densities in the laboratory [164], sexual maturation within four months [164], and external development of optically transparent early embryos [164]. This latter feature facilitates the direct observation of tissue and organs development, as well as the *in vivo* injection of drugs or genetic constructs [158]. External development of a transparent embryo is not a feature of development found in mice (*Mus musculus*) and rats (*Rattus rattus*) [164].

The close similarity between mammalian and zebrafish behavioral paradigms can be exploited to study anxiety, fear, post-traumatic disorders, and other stress-related human psychiatric conditions. Previous findings suggest that behavioral studies using zebrafish as the model organism can span multiple behavioral domains including anxiety[165-170], depression[171, 172], neurodegeneration[162], serotonin

syndrome[173], and sleep disorders [174-177]. In fact, former studies have already shown that both larval and adult zebrafish are sensitive to all major classes of neurotropic drugs, including antipsychotics[178, 179], anxiolytics[168, 170], and antidepressants [180, 181].

Last but not least, another major advantage of zebrafish is that they are cost-effective model. They are good candidates for high throughput screening (HTS)[178, 182] and the costs of *in vivo* screening of one drug in the zebrafish are approximately US$300, which is 500 times cheaper than similar rat assays [183].

Zebrafish Larvae in Neurobehavioral Research for Anxiety

Here the use of zebrafish larvae in anxiety research is discussed. There are several behavioral phenotypes that can be considered as an anxiety-like behavioral domain in zebrafish. Kalueff *et al.* made an extensive catalog of zebrafish behavioral phenotypes for multiple behavioral domains, including for anxiety [184]. Although this catalog is primarily based on adult zebrafish, it can be a good reference to study similar behavior in larval zebrafish. Relevant behavioral phenotypes include: (i) alarm reaction, (ii) burst swimming, (iii) corkscrew swimming, (iv) erratic movement, (v) escape behavior, (vi) freezing, (vi) hyperactivity burst, (vii) immobility, (viii), meander, (ix) photokinesis, (x) startle response, and (xi) thigmotaxis.

Often there is an overlap between zebrafish anxiety-like behavior and fear-related behavior [166, 168, 169]. The fear response is cue-oriented, due to a direct reaction to a currently present stimulus [166, 169, 185-187]. By contrast, the anxiety response is more diffuse since it is produced by potential (but not present) aversive stimuli. Currently, there is no clear distinction in larval zebrafish between these two behavioral phenotypes; however, some phenotypes (e.g. the alarm reaction) are more relevant for assessing fear; others (e.g. withdrawal) more closely represent anxiety-like behavior [184].

Most experimental models of anxiety in zebrafish larvae are based on (i) the visual motor response (VMR) test, (ii) thigmotaxis (inner/outer zone preference), and (iii) scototaxis (light/dark zone preference). The VMR test is an example of a response to startle stimuli and is often measured as the distance that larvae swim following stimuli. Zebrafish larvae can be startled by different stimuli such as acoustic, tactile and visual stimuli [188-190]. All these stimuli create different

responses in the larvae; for example, a sudden transition to darkness is characterized by large-angle (‘O-bend’ shaped) turns [189].

The theory behind scototaxis test is similar to the rodent experimental model; however, zebrafish larvae showed a natural preference for a bright environment instead of for a dark environment [183]. The authors justified this observation based on the fact that zebrafish are diurnal and rodents are nocturnal. However, there is a problem in their justification since adult zebrafish, though also being a diurnal displayed preference to the dark compartment in LDPT [115, 165, 191, 192]. Contradicting to these findings, some authors have reported adult zebrafish showing a preference for the light environment [159, 193]. Reasons for these discrepancies seen in adult zebrafish are not clear but are likely due to different experimental designs used by these different laboratories [194]. Miklósi and Andrew suggested that maturation of melanophores in zebrafish could be the reason for the age-related switch in the preference for light/ dark[195], but this claim needs further clarifications. In essence, how the age-related switch changes the preference of light/ dark in zebrafish is still not thoroughly studied.

Stephenson and colleagues have shown that zebrafish preference for light/ dark is dependent on ambient light levels and olfactory stimulation [196]. Results from this study could provide a potential explanation for the contradicting observations in adult zebrafish. According to the authors, at lower light intensity levels, zebrafish devoid of food odor preferred lighter environment than darker and this preference reversed with increasing light intensity. These highly interesting observations suggest a trade-off between food foraging and the risk of predation. Moreover, this study is a good example showing approach-avoidance motivational conflict suggested by Maximino and colleagues [168]. Scototaxis behavior in zebrafish cannot be explained solely based on avoidance of the white compartment alone or approach to the black compartment alone.

Results from behavioral studies using cavefish have suggested that thigmotaxis is linked to exploration or predator avoidance (approach-avoidance motivational conflict as seen in scototaxis) [197]. Schnörr *et al*. published interesting results for thigmotaxis analysis using zebrafish larvae [198, 199] They found that zebrafish larvae (as young as 5 dpf) express anxiety by showing thigmotactic behavior. In that study, anxiolytics (diazepam) attenuated the wall-hugging (thigmotactic) behavior,

while anxiogenic drugs (e.g. caffeine) enhanced that behavior.

The HPA axis, which governs stress responses, is also conserved in teleost fish such as the zebrafish (where it is referred to as the hypothalamic-pituitary interrenal (HPI) axis). The homology between the HPA and HPI axes in terms of anatomy and molecular constituents could result in similar functional organization and physiology of the stress response [200]. Due to these remarkable qualities, a range of complementary assays can be developed to assess the correlation between behavioral and endocrine systems in relation to stress response in larval zebrafish [200]. For instance, it is possible to translate chronic mild stress (CMS) paradigm in zebrafish larvae to evaluate their behavioral profile.

There are a few important factors that need to be addressed when screening for potential anxiolytics using zebrafish larvae (explained in **Table 3**). Relevant example studies for each of these factors included. These factors can have a huge influence on the outcome of research and warrant proper attention. Anxiolytic drugs have been reported to have side effects, such as impairment of visual sensitivity [201, 202]. Benzodiazepines have been previously shown to affect visual sensitivity at high concentrations [201]. Since the light/dark preference test relies on an intact vision, larval zebrafish could struggle to distinguish white and dark zones when exposed to such drugs.

Airhart *et al.*[203] studied the effect of fluoxetine (an SSRI) on larval zebrafish locomotion and found that larvae exposed to fluoxetine on 4 or 5 dpf showed reduced spontaneous swimming activity (SSA) at 6 dpf with no recovery until 14 dpf. Fluoxetine increases postsynaptic concentrations of serotonin, due to the inhibitory effect on serotonin transporter protein (SERT) [204]. This study also showed a significant reduction of SERT and 5-HT_{1A}-receptor transcripts in the spinal cord but not in the brain after fluoxetine treatment. The authors also suggested that fluoxetine neurotoxicity on intraspinal ventromedial neurons could have caused cessations of movement.

Table 3. Factors that may influence the outcome of larval zebrafish behavioral assays.

1. Age • Behavioral assays are performed at 5–7 dpf as most organs are already fully developed. • Same compounds may yield different results if bioassays are performed using larvae of different ages. Example study (I) Ali *et al*.: LC_{50} values of 60 water-soluble compounds declined as the embryo developed.
2. Individual variation • Coping style is the individual difference in response to stress exposure. Example study (I) Tudorache *et al.*: Individual zebrafish larvae can be classified into early and late emerges.
3. Route of delivery • Lipophilic compounds are difficult to dissolve in water (immersion exposure) and affects bioavailability and uptake mechanisms. • Immersion exposure technique of hydrophilic compounds can cause unwanted side effects. Example study (I) Bailey *et al.*; Nilsson & Fange; Stray-Pederson; Finney *et al*.: Dissolving compounds in water may affect oxygen exchange in the gills and swim bladder of aquatic organism. (II) Ordas *et al*.: Rifampin and moxifloxacin adhere to the skin of larvae.
4. Strain difference • Strain type influences general locomotor activity and thigmotactic behavior. • Different strains respond differently to anxiolytic compounds. Example study (I) Egan *et al*.: Adult leopard strain has a higher baseline anxiety level and can be useful in screening anxiolytic compounds. (II) Norton: Analyzed behavior of different wild type strains [AB, Casper, Tubiengen (TU), and Wild Indian Karyotype (WIK)]. TU strain spent lesser time in the outer zone than others.
5. Solvent • Solvents that used to dissolve the lipophilic compounds can alter locomotor activity at a very lower concentration. Example study (I) Hallare *et al*.: Sub-toxic levels of DMSO increased hsp70 levels in zebrafish embryo and larvae.
6. Temporal factor • Time frame of a day highly influences behavioral profile of zebrafish larvae Example study (I) Burgess & Granto; MacPhail *et al*.: Zebrafish larvae are hyperactive in the beginning of a day and have stable baseline activity in the afternoon.

The study by Airhart *et al.* shows significant side effects of fluoxetine that can be mistaken for an anxiolytic effect. Moreover, clinical studies have shown that SSRIs exposure at therapeutic levels during the third trimester of pregnancy causes children

to have lower APGAR (appearance, pulse, grimace, activity, and respiration[205, 206]) scores at birth compared to control group without exposure. The APGAR score provides a quick summary of the health of a newborn baby [206, 207]. Therefore, it is essential to know if the effects of any anxiolytic compounds represent a therapeutic effect or a toxic effect.

Zebrafish Larvae in Natural Product Research

Unlike the rodent counterpart, zebrafish larvae have not been used extensively for plant-based anxiolytic activity research. Nevertheless, some researchers have exploited zebrafish larvae for other bioassays using plant extracts or even plant-based pure compounds. One of the earliest reports of a plant-based product bioassay using zebrafish as the animal model was on characterizing pro-angiogenic properties of *Angelica sinensis* using transgenic lines of zebrafish [208]. In another study, anti-angiogenic properties of East African medicinal plants were investigated using zebrafish bioassay-guided fractionation. Crawford *et al.* used thin-layer chromatographic (TLC) to fractionate and isolate bioactive compounds responsible for the anti-angiogenic effect [209].

Earlier, zebrafish larvae were used in behavioral assays to identify herbal medicines with antiepileptic (anticonvulsant) properties. These studies involved extracts of *Valeriana officinalis*[210], *Solanum torvum*[211], and *Salvia miltiorrhiza* [212]. Although these three studies focus on antiepileptic activity, they can yield useful information for anxiety research. For example, in those studies, they used pentylenetetrazole (PTZ) to induce epilepsy-like seizures in zebrafish larvae. This compound is known to inhibit $GABA_A$ receptors [213, 214]. Studies with *in vitro* assays revealed that crude herbal valerian extracts and their active constituents such as valerenic acid[215], alkaloids, and lignans, could interact with $GABA_A$[216], glutamates[217], adenosine[218], and serotonin receptors [34]. These receptors are important in neurochemical modulation of anxiety. This study shows that zebrafish larvae can, in principle, be effectively used for screening plant extracts for anxiolytic effects.

Torres-Hernandez and colleagues[210] also used PTZ as an agent to stimulate epileptic seizures in zebrafish larvae. Swimming speed in light and dark conditions, together with light-dependent zone preference, were used as measures of behavioral

activity. The study revealed that crude extract at all concentration tested alleviated PTZ-induced epilepsy. Moreover, the authors reported that valproic acid (VPA: a synthetic analog of valeric acid, which is naturally found in the valerian plant) also reversed the effects of PTZ. However, there are many concerns on the observations from this paper. The main lacking information is the phytochemical profile of the plant extract. Therefore, it is not practical to compare the behavioral changes induced by a crude extract with a pure compound known to be present in the plant (VPA).

Another surprising outcome of the previously mentioned study is that the crude valerian extract alone did not produce any toxic effects after 24 hours of exposure at concentrations 7 mg/ml. This concentration is higher compared to the concentrations used in an unpublished pilot study done at Leiden University (Plant Sciences and Natural Product Laboratory). This study revealed that dried methanol extracts of valerian root were extremely toxic to the larvae (exposure at 4 dpf) even at a very low dosage (~62.5 µg/ml; Muniandy and colleagues, unpublished data). Moreover, according to the authors, the pure compound valerenic acid showed extreme toxicity in their bioassays even at low concentrations. This prompts us to wonder whether valerenic acid was really extracted completely, or even present in the plant samples used in this study. The method of extraction could be the reason for the toxicity differences in both studies.

Torres-Hernandez and colleagues used 48 well plates in the zone preference analysis. This choice of well plate has potential issues. Previously, thigmotaxis behavior in wild-type zebrafish larvae was published using 24 well plates [198, 199]. According to these studies, the width of the inner and outer zone of an arena should be at least equivalent to, or higher than, the length of the larva (c. 4mm at 5 dpf). Based on this criterion, 48 well plate arenas are too small for thigmotaxis analysis. Furthermore, the larvae in that study were acclimatized in darkness for 27 minutes prior to the onset of alternating light and dark conditions. Starting with an aversive condition before recording the behavioral pattern is not optimal as it can influence the basal behavioral pattern and may induce freezing in animals with a high anxiety level [109].

Other studies have examined the anticonvulsant activity of the plants *Solanum torvum*[211] and *Salvia miltiorrhiza*[212] in zebrafish larvae using a new strategy that combines high-performance liquid chromatography (HPLC) microfractionation with

at-line anticonvulsant bioassay. Challal *et al.* reported that both *S. torvum* and its isolated active constituents (triterpene glycosides) showed anticonvulsant activity in PTZ-induced activity. Unlike the valerian study, here the phytochemical profiles of different extracts were reported. A methanol extract of *S. torvum* was chosen for further fractionation since it reduced PTZ-induced activity. Furthermore, the larvae were chronically exposed (18 hours) to crude methanol extract or to isolated compounds before subsequent treatment with PTZ and behavioral analysis. Unfortunately, the study did not include a behavioral profile for the larvae treated with plant extract alone. This information would have been useful because, for example, it is possible that the plant extract alone might cause some physiological sensation that reduces the motor response; this, in turn, could be misinterpreted as an anticonvulsant activity.

The study using *S. miltiorrhiza* reported similar anticonvulsant activity in both crude extract and with purified active constituents (tanshione II and militrone). Unlike the previous study with *S. torvum*, the authors chose an acute exposure (1 h) regime since chronic exposure (3 hours) caused bradycardia, loss of posture, and delayed touch responses to the larvae beyond maximum tolerated concentrations. The toxicity differences at different time exposure in these two studies might be explained in terms of the chemical structure of the purified compounds. Another feature of the study is that the larvae were pre-incubated in 1% DMSO before subsequent exposure to either PTZ or the plant extract. At this concentration, DMSO was not toxic to the larvae; however, care must be taken in interpreting behavioral data when DMSO used as a solvent. This is because DMSO is shown to increase heat shock protein 70 (hsp70, a marker for stress response[219]) levels even at low concentrations in larval zebrafish [220].

Though some studies have shown that zebrafish larvae can be used to explore the therapeutic potentials of plant extracts, this promising animal model needs much more evaluation and optimization. Indeed, plant-based extracts and purified compounds have been reported to be toxic to fish (ichthyotoxic). For example, ichthyotoxicity has been reported for flavonoids[221, 222] and saponins [223-225]. Furthermore, it has been reported that flavonoids exhibit developmental toxicity in developing zebrafish embryos [226]. Saponins are considered to be extremely toxic for poikilothermic (cold-blooded) animals even though they have low oral toxicity for mammals [227,

228] This phenomenon may be attributed to the poor absorption from the gut of mammals [227].

The main reason why plant extracts could be toxic to aquatic organisms, including the zebrafish, is that they disrupt the balance of water chemistry. As plant compounds decompose in the water, dissolved oxygen in the water may be depleted and the fish can become stressed. When under stress, fish exhaust the energy reserves devoted to maintaining the immune system [229]. This may eventually lead to the death of the fish. Another potential reason for plant toxicity towards fish is provided by the example of saponins. Saponins have been shown to damage the gills of fish and are traditionally used as fish toxins [223]. For example, saponins of *Camellia sinensis* (tea) seed cake resulted in the death of tilapia within 5-6 hours of exposure in the water [230]. Rio *et al.* found that saponin toxicity to mummichog fish (*Fundulus heteroclitus*) increased in the water than when injected intraperitoneally[231], which implies that the saponins were actively absorbed by gill membranes. Moreover, saponins induced toxic effects in different fish species (rainbow trout and Chinook salmon) through damages to the intestinal mucosa [232]. A very recent study done in the Philippines showed that water extracts from *Ocimum sanctum* L. (holy basil) and *Tamarindus indica* L. (Tamarind) leaves were highly embryotoxic and teratogenic for zebrafish embryo [233].

Conclusion

Plant-based therapy for anxiety and other neurological disorders have existed for a long time. However, as with any therapeutic drugs, there are also some issues that need to be addressed when herbal medicines are considered as a means of treatment. The most important issues are efficacy and safety. The high-throughput nature of zebrafish assays can be exploited to investigate herbal medicines. Although zebrafish larvae cannot completely replace the rodent model, they can be a good complement [159].

Bioactivity-guided fractionation is an essential technique to isolate and identify both active and toxic compound in a natural product. Yet, analyzing natural products such as plant extracts is a challenging task since they are made up of a complex matrix with several closely related compounds [234]. Classical bioactivity guided

fractionation is time-consuming[235], labor-intensive[235], and requires multiple chromatographic procedures and large quantities of plant material [236]. On the other hand, zebrafish larvae based screening paradigms only need lower amounts of material at microgram scale [237]. Challal *et al.* have established a new method by combining zebrafish behavioral assay with microfractionation technique to identify bioactive compound from traditional herbal medicine [238].

Therefore, microfractionation technique hyphenated with zebrafish larvae bioassay could be useful in search of new anxiolytic compounds from natural product. However, the hyphenation of these two techniques needs to be further validated in different behavioral paradigms. Moreover, the zebrafish bioassay-guided fractionation technique requires further optimization as well to account for the complexity of herbal extract. Finally, it should be noted that there is evidence that plant extracts can be toxic to fish, even when their toxicity in mammals is low. In a nutshell, plant product screening using zebrafish-based bioassays is still at its infancy stage. However, the vastly growing different -omics technologies (metabolomics, genomics, proteomics etc.) should be hyphenated with different zebrafish-based *in vivo* assays (behavioral, physiological, and toxicology) in order to improve herbal drug discovery efforts. Furthermore, this approach could be an efficient way of identifying novel bioactive molecules in traditionally used herbal medicines.

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