Zebrafish embryos and larvae as a complementary model for behavioural research
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Chapter 1

General Introduction
Introduction

Animal models in biological research

In the early 20th century, scientists often used simple organisms such as bacteria and bacteriophages to explain how cells work at the molecular level. With the rise of genetics, researchers focused on more complex systems by using higher organisms such as the fruit fly *Drosophila melanogaster* which has a short generation time (7-9 days from egg to adult; [1]). Furthermore, it has four pairs of large chromosomes which facilitate the study of eukaryotic genetics [2]. Use of the nematode *Caenorhabditis elegans*, yeast, mice, rats and the chicken embryo as animal models increased in various fields of science [3,4]. These model organisms, especially mice, remained the focal point for fundamental biological and clinical research and were mainstays of biomedical research [5].

*In vitro* assays also helped in various disciplines of science. However, due to their lack of a complex physiological environment, cell-culture studies sometimes correlate poorly with results in the whole animal [6]. A good example is provided by behaviour, a phenomenon which cannot easily be studied with the help of *in vitro* assays. The study of behaviour dates back to the time of Aristotle, when he had many interesting observations concerning animal behaviour [7]. In the modern era, Charles Darwin (1809-82) wrote a whole chapter ‘instincts’ in his book *Origin of Species* [8]. In the twentieth century, animal behaviour was studied in the context of learning by Ivan Pavlov and Edward Thorndike. Later it became an independent scientific discipline ‘ethology’ due to the efforts of Konard Lorenz and the Dutchman Niko Tinbergen [9].

The process of developing an animal model

The first step in developing an animal model is to define the purposes of the model. In the next step (validation), the model is developed and tested to see how well it serves its purpose. The validation of animal models depends on two criteria: i) scientific; ii) ethical [10,11]. With the help of a multidisciplinary approach, scientists set the scientific criteria on the basis of reliability, reproducibility and relevance of the model. Unfortunately, the
ethical criteria is not easily quantified. If animal welfare is compromised, or pre-set scientific criteria are not met, the animal model is discontinued [10].

A key objective in developing an animal for studying behaviour is to understand genetic factors, environmental factors and the underlying mechanisms responsible for abnormal behaviour. This is done by studying dissociations between processes in the animals with natural deficits; or by inducing them pharmacologically [12]. The next step is to translate insights from the preclinical study to the clinical and vice versa [13]. This step is achieved by studying the effects of compounds involved in cognition and neuroprotection, and also by assessing the effects of experimental manipulations or drug treatments [14-16]. Risk assessment related to these treatment is also carried out by studying toxicology [17].

**The value of rodent models in research**

Rodent models have excelled in modelling human diseases due to the striking homology between mammalian genomes [18] as well as anatomy, cell biology and physiology. Genetically modified mice has proven effective in number of cases in biomedical research resulting in development of new treatments of various diseases [19]. This has made the mouse the most widely used model of human disease due to availability of gene knockouts and knockins [20].

**The need for alternative models**

Among all animal models, rodents (rats and mice) have been the most used for studying behaviour [21-24]. However, use of animals in research also raised ethical concerns especially in the field of toxicology, biomedical science and also in behavioural research despite less invasive studies [25]. Russell and Burch's 3Rs (Replacement, Reduction and Refinement) provided a means to improve animal welfare [26]. So efforts were made to replace the animals models specially mammals [27]. One such model is the zebrafish, which is much cheaper to work with than rodents and which is subject to less stringent legal restrictions under EU legislation.
The zebrafish as an alternative animal model

The zebrafish (Danio rerio) is a freshwater teleost fish, native to Bangladesh, India, Nepal and Sri Lanka [28,29]. It has many attributes which make it an good model in various fields of research. The zebrafish model has some advantages over rodent models. It is very easy to keep large numbers in a small, space owing to its small size and it is a more cost-efficient model than rodents [30]. External fertilisation and development of the eggs outside of the mother (which reduces maternal behavioural influences) and the transparency of the zebrafish embryo (which allows live cell imaging) are other advantages when studying multiple developmental processes [31]. Females lay eggs every 2-3 days and each clutch may contain hundreds of eggs. Zebrafish embryos kept at 28.5°C hatch between 48-72 hours post fertilization (hpf), when they become free-swimming larvae with a complex behavioural repertoire [32].

The early life stages of zebrafish embryo are optically transparent can be monitored easily with the help of a dissecting microscope or with confocal microscopy [33]. The development of zebrafish embryos is rapid and organogenesis is completed within 3 days post fertilization (dpf) [33,34]. The body plan of zebrafish embryos is homologous with that of other vertebrates and includes, for example, a liver for metabolic activation [35,36], a thyroid gland [37,38] and a blood-brain barrier [39,40]. Researchers in the basic medical sciences have taken advantage of the high degree of genomic similarity between zebrafish and mammals [41], the small size of zebrafish, and the fact that their phenotype can be so rapidly assessed in high-throughput [42] to use the species in many research contexts [43,44].

Behavioural assays using the zebrafish

The zebrafish is well-suited for behavioural genetic research and has been extensively used in systematic screens for behavioural mutants providing an unbiased method to find the underlying genes [45]. Zebrafish larvae display a wide range of behaviours including the photomotor response [46], touch-induced escape response [47,48], visual motor response [49], optokinetic response [50,51], optomotor response [52,53], and light/dark avoidance response [54]. Many of these behaviours have been developed as assays and used for high-
throughput screening and for the discovery of novel neuroactive compounds [46,55,56]. The studies cited above suggest that the zebrafish embryo model has promise for safety/toxicity screening.

**Achievements of the zebrafish model**

The zebrafish model has been pioneered as a developmental and genetic tool for the study of toxicology [57-59], behaviour [60-63], physiology [64,65], neuropharmacology [54,66,67], organogenesis [68,69] and several disease conditions [30,31,70]. The use of zebrafish in high-throughput drug screening and discovery has propelled zebrafish forward as a valuable organism for preclinical research [71-73]. Traditionally, rodent models have been employed for discovery of novel neuroactive compounds and have been very effective [74-76]. However, their use raises ethical concerns [77,78] and they are less productive than zebrafish models in terms of throughput, efficiency and sample preparation time [79].

Despite the fact that rodents are still the mainstay of research in many fields, the zebrafish animal model is increasing in popularity. The zebrafish is serving as a complementary model to rodents [80]. There are certain assays which cannot be done in mammals. For example, tests can be done to see the effects of drugs on the internal organs in real time; or specific process such as apoptosis can be examined in a live zebrafish embryo or larva [81]. These advantages mean that the zebrafish is a potentially valuable animal model for assays which are difficult to perform in mammals.

In order to develop a complementary model to rodents in behavioural research, the rodent behavioural repertoire should be translated to other species such as zebrafish. For example, Champagne and colleagues [80] have examined the feasibility of translating rodent-based behavioural assays to the zebrafish model. They found that this could be successfully done, and that the pharmacological responses of the zebrafish were similar to those seen in rodents. The most common methodologies used in rodents are the use of light/dark box and open field test [76,82,83]. These simple, painless and unconditioned tests use the spontaneous or natural tendency of rodents to explore novel environments [76,84]. The adult zebrafish has been shown to have such tendencies, and this behaviour has been used and validated for anxiety-like behaviour [80]. The only difference in that
study was that rodents prefer the dark zone [84] while adult zebrafish preferred the light zone [80]. One study reported that zebrafish show preference for a dark zone [85] although this discrepancy might be due to the different experimental settings. The different preferences for light and dark in rodents and zebrafish might be explained by the fact that zebrafish are diurnal animals and rely on a well-illuminated environment for finding food and mates, and avoiding predators [28]. By contrast, rodents are nocturnal animals and are therefore more active in the dark [84,86].

There are also a number of studies on larval zebrafish behaviour, showing that these young developmental stages might be usefully employed in high-throughput screening [42,46,87]. By using zebrafish larvae in behavioural research, relatively small volumes of test compound are needed, and this helps to save costs of compounds that are sometimes scarce or expensive [88]. In view of the role that the zebrafish larva and its behavioural repertoire can play as a complimentary model in many fields such as drug discovery and high-throughput screening, we studied in this thesis larval zebrafish locomotor activity and the impact of environmental factors, such as light, to help understand how the zebrafish larva as an alternative model in behavioural studies and drug screening.

Aims of the thesis

The main focus of the studies presented in this thesis was to translate rodent behavioural assays to larval zebrafish for better time and resource management in biomedical, behavioural and pharmaceutical research. This was achieved by following steps.

- Studying the behavioural repertoire of zebrafish larvae at different developmental stages (Chapter 2).
- Developing a simple assay for medium-throughput screening the effects of compounds on zebrafish development and locomotor activity (Chapter 3).
- Refining our understanding of zebrafish larval locomotor behaviour with respect to environmental conditions such as light (Chapter 4).
- Assessing the potential of zebrafish larvae for non-associative learning using the onset of darkness as a stimulus leading to hyperactivity (Chapter 5).
• Developing, validating and translating rodent behaviour displayed in an open field to larval zebrafish (Chapter 6).
• Ascertaining larval zebrafish colour preference and the effects of abnormal lighting conditions and anxiolytic (diazepam) on preference and avoidance patterns (Chapter 7)