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Zebrafish embryos and larvae as a complementary model for behavioural research

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Chapter 8

Summary and Discussion

The zebrafish (*Danio rerio*) is a small teleost which has emerged as a prominent model in various fields of research. Its rapid development, high fecundity, external fertilization, transparent body at early stages, low maintenance cost and short life cycle are some of the key advantages making it a successful model [43]. The favourable cost-benefit ratio of zebrafish embryos or larvae makes it possible to screen many compounds in a high-throughput context [46,87]. The larval zebrafish is also able to bridge the gap between whole organism phenotypic assays and *in vitro* high-throughput screening techniques because it helps in data collection with minimal resources [465]. Thus, if properly assessed, we can benefit from it in the field of drug discovery and it may also help in quick assessment of drugs effects.

One of the main areas of interest lies in the central nervous system of zebrafish. The behavioural assays have been developed into screening platforms for the assessment of locomotor activity, impaired visual and auditory functions, cognitive impairment and drug dependence potential [55,161,260]. The use of these behavioural assays holds great promise for high-throughput screening of thousands of small molecules as new drug candidates. A wealth of information generated from larval zebrafish can be used to evaluate the safety liabilities of compounds making it a useful tool for advancing drug discovery and saving time and money [70,89,254,255,286]. The use of inexpensive, automated video-tracking equipment has made the screening assays more efficient in terms of time and cost [466]. In this thesis, we used automated video-tracking to analyse the behavioural repertoire of zebrafish larvae, and the effect of compounds, changes in lighting, light conditions during rearing, and novel fields, including fields of varying colour or light intensity, on locomotor activity.

In **chapter 2** we reviewed the literature relating to the use of zebrafish embryos and early larvae as animal models in biomedical research. The functional domains of drug targets, and other functionally important proteins, are often highly conserved between the zebrafish and mammals. Furthermore, the zebrafish embryo or larva shows a complex behavioural repertoire only a few days after fertilization. We also showed how behavioural studies in mammals are being translated to the zebrafish embryo/larva model. We gave emphasis to behavioural studies that may be relevant to drug screening or safety toxicology. The use of video tracking hardware and software in these studies provided an automated, high data-content readout for zebrafish behavioural responses. Published behavioural

assays using zebrafish developmental stages include visual motor response, acoustic stimuli, effect of novel environments and various pharmacological compounds.

In **chapter 3**, we screened pesticides, insecticides and metals for their effects on zebrafish development and locomotion using visual motor response test. The LC_{50} of these compounds in zebrafish was also calculated and compared with publish data on rodent LD_{50} . The results showed that different compounds have selective effects on hatching rate and development. Some compounds were teratogenic, producing malformations. On the basis of effects on locomotor activity, compounds caused four different types of responses. (i) monotonic suppression, whereby the locomotor activity of the zebrafish larvae was suppressed in a dose-dependent manner; (ii) monotonic stimulation, whereby the locomotor activity increased at higher doses; (iii) biphasic (suppression and stimulation), whereby the locomotor activity increased at lower doses but was suppressed at higher doses (iv) neutral response, where the locomotor activity was neither increased nor decreased.

This type of assay might be useful not only for screening of compounds but also for the discovery of new drugs. It also helps to monitor developmental malformations and functional impairment by using simple visual motor response test. It could well be scalable to high-throughput, saving time and money in drug discovery and the evaluation of drug safety. The LC_{50} values obtained from the assay did not correlate well with the published rodent LD_{50} data. Some compounds were more toxic in zebrafish than in rodents or vice versa. However, other studies did find a correlation between zebrafish LC_{50} values and rodent LD_{50} values, depending on the class of compounds [98,467].

Previous work has shown that adult zebrafish prefer dark, while zebrafish larvae prefer the light compartment in an open field test [80]. Zebrafish larvae also show a startle response and greatly increased locomotion at the sudden onset of darkness [49,122]. However, less is known about whether this response is intrinsic or learned. To distinguish between these alternatives, we raised zebrafish larvae under different lighting conditions from the onset of fertilization until the day of testing when effects on hatching, mortality and locomotor activity were recorded. (i) The zebrafish embryos were provided with 14:10 h light/dark (ii) the embryos were raised in constant darkness (iii) the embryos were raised in constant light.

The results showed that constant light greatly accelerated hatching, while constant darkness delayed it, compared to control. There was no significant effect on mortality in any lighting regime. However, the larvae raised in constant dark showed a number of

malformations such as yolk sac oedema, pericardial oedema and dispersed pigmentation. The zebrafish embryos raised in normal lighting regime, or constant light, did not show any excess of abnormalities over controls. The visual motor response test at 5 dpf showed that larvae raised in constant dark failed to respond to sudden onset of darkness. The larvae raised in constant light had a high basal activity and hence response to sudden darkness was not very high as compared to zebrafish larvae raised in normal light-dark cycle.

To see whether zebrafish embryos can recover from the effects of abnormal lighting conditions, the visual motor response test was done two hours after taking out zebrafish embryos from abnormal lighting regimes. The results showed that zebrafish embryos raised in constant dark regained normal locomotor activity and responded to sudden onset of darkness as control. This assay confirmed that displaying hyperactivity response to sudden onset of darkness is not influenced by the environmental conditions. However, normal light-dark cycle is essential for normal development (**chapter 4**).

In **chapter 5** we showed that choosing the time of day and developmental stage to run a particular assay is very important. Although, there was no effect of time of day on the locomotor activity of the zebrafish embryos, there was a significant difference in the activity between all age groups tested. Compared with 6 dpf larvae, the 4 dpf larvae were less active and 5 dpf larvae were hyperactive. The zebrafish larvae display non-associative learning. This was demonstrated by presenting the zebrafish larvae with alternating light and dark stimuli with different intervals for three hours. The 10 min interval produced no habituation in 4 dpf larvae. However, although 5 and 6 dpf larvae did not show a decrease in activity to sudden darkness, they showed decreased activity within the 10 min dark phase.

The alternating light and dark with 5 min interval showed decline in the activity after 5 cycles and did not decrease for rest of the test period in case of 4 and 5 dpf while 6 dpf larvae did not show habituation. The zebrafish larvae of all age groups tested showed a rapid decline in activity when presented with 1 min alternating light and dark phases for three hours. at. However, to distinguish between rapid habituation and fatigue, a sound stimulus was presented after habituation to light/dark challenge. A spike in activity confirmed that zebrafish larvae are able to habituate to a certain stimulus with short interstimulus interval. In principle, this type of assay could be used to identify drugs which play a role in learning and memory and might also be used as screen for effects of compounds on cognition.

In **chapter 6**, we used the exploratory behaviour of zebrafish larvae to examine their colour preference. The zebrafish larvae showed a preference for orange and green, but displayed avoidance behaviour towards yellow, blue, red and black. This shows that zebrafish larvae are able to discriminate between different colours, and that a complex environment triggers the expression of anxiety-like behaviours. In this way, we developed and adapted a traditional rodent behavioural assay that serves as gold standard in preclinical drug screening. Our assay also provides a version of the same test that affords the possibility to investigate the impact of environmental stress on larval zebrafish behaviour. This assay could contribute in the future to advance our knowledge of molecular mechanisms underlying behavioural stress, anxiety states, and learning and memory. It therefore has the potential to be relevant to drug discovery.

In **chapter 7**, we further tested different shades of greyscale filters for the preference and avoidance patterns of zebrafish larvae. The results showed that zebrafish larvae avoided the dark and preferred to remain in the white or less dark areas. We further investigated the role of abnormal lighting conditions on preference and avoidance patterns. The zebrafish larvae raised in constant dark until the day of testing moved more in the black, yellow and blue zones while zebrafish larvae raised in constant light avoided these colour zones. However, preference for orange and aversion to red was unchanged. For pharmacological manipulations, the anxiolytic diazepam was used to ascertain the behaviour observed in the colour-enriched field was an anxiety-related phenomenon. The colour preferences and aversions, as well as the freezing behaviour displayed by zebrafish larvae in a complex environment, was abolished after exposure to diazepam. This study shows that anxiety may be an important determinant of the colour preferences, and exploratory behaviour, of zebrafish larvae. It also shows that these preferences can be modified by lighting conditions during rearing.

In summary, we have shown that zebrafish larvae are able to display behavioural repertoire which can be used to study toxicology, pharmacology, adaptation patterns, stress, anxiety, memory and learning. However, the mouse model continues to be the dominant model in most of the research fields. For example, examining drug effect on metabolism would be more reliable in mammals than on zebrafish because the sequence homology conservation is lower in case of zebrafish and human as compared to homology between rodents and humans [468]. However, research on mammalian species is expensive with lower throughput. Therefore, screening large number of compounds on mammals would cost more money, time and compound quantity. So it will be worth to screen compounds in zebrafish before further testing which will be cost and time efficient with minute quantity

of drugs needed. In this way, zebrafish model can serve as a complementary model in various fields.

Conclusions

- The larval zebrafish is a useful model in toxicology and drug discovery. However, its predictivity (power to predict toxic effects in rodents or humans) is restricted by compound class (**chapter 3**).
- Light-dark cycle plays an important role in the normal development of the zebrafish embryo, and abnormal lighting regimes during rearing can result in different kinds of malformations (**chapter 4**).
- The hyperactivity displayed by zebrafish larvae following the onset of sudden darkness is an intrinsic characteristic because zebrafish larvae raised in constant dark still displayed hyperactivity after sudden dark challenge (**chapter 4**).
- The basal locomotor activity of 4 dpf zebrafish larvae is significantly lower than either 5 or 6 dpf larvae. There was no effect of time of day on basal locomotor activity (**chapter 5**).
- Zebrafish larvae displayed habituation to a repeated stimulus of sudden dark with a short interstimulus interval of 1 min; longer interstimulus intervals (5 min, 10 min) resulted in little or no habituation. The hyperactivity was always higher resulting from light-to-dark transition than with that of dark-to-light transition (**chapter 5**).
- The 4 dpf zebrafish larvae did not show habituation with longer ISI intervals whereas 5 and 6 dpf zebrafish larvae adapted to the darkness after brief period of hyperactivity. However, the activity in the dark remained always higher than activity in the light period (**chapter 5**).
- Zebrafish larvae are able to discriminate colours, and they show a preference for orange and green, but aversion towards red, yellow, blue and black. The larvae also show freezing behaviour in the complex environment (**chapter 6**).
- Zebrafish larvae (4-6 dpf) prefer the light and avoid dark regardless of the degree of darkness. The colour preference and avoidance displayed by zebrafish larvae was abolished after exposure to diazepam (**chapter 6**).
- Zebrafish larvae raised in an abnormal lighting regime showed changes in some aspects of their colour preference, although orange and red remained as preferred and avoided colours, respectively (**chapter 7**).
- The zebrafish larva is a useful complementary animal model in behavioural research amenable to high-throughput screening of compounds and drug discovery (**general conclusion**).

Future Perspectives

The studies in this thesis demonstrate that zebrafish can be used as a complimentary model to rodents in toxicological screening, drug discovery and behaviour research. However, there is a need to fully characterize and understand the internal and external factors which can influence the locomotor activity or conclusions drawn from it. Previous studies have already shown that experimental conditions such as temperature, diameter of microtiter plates, presence of malformed larvae and raising larvae in abnormal lighting conditions can affect locomotor activity of the zebrafish larvae. Thus, there is a need to look for the factors which can affect the behaviour of zebrafish larvae. The physiological changes taking place due to these factors (pharmacodynamics), and molecular mechanisms underlying them, also need to be investigated.

With the increasing ethical concerns about using mammals as research models, and the increasing legislation that restricts such research, there is likely to be a growth in demand for alternative models such as the zebrafish larva. However, the locomotor assays used to assess effect of different compounds on development, raise the question of bioavailability [469]. The most convenient and straight-forward method used is to dissolve the test compound in the embryo buffer. In this case, one is unsure of the dose of chemical reached inside the embryo and the time at which it enters the body of zebrafish embryo. In fact, the absorption, distribution, metabolism and excretion (ADME, pharmacokinetics) of compounds by zebrafish early life stages is very little known. To complicate things further, the zebrafish embryo has a chorion during first 2-3 dpf which is 1.5-3.5 μm thick and composed of three acellular layers [274,470]. Mechanical or chemical dechoriation may be carried out but it can affect behaviour displayed by the larval zebrafish [134,471]. Thus, there is a need to work on the quantification of bioavailability of compounds inside the zebrafish embryo to compare the effects on zebrafish and other animal models. Indeed, the lack of information on ADME is one reason why the pharmaceutical industry has been slow to take up the zebrafish model.

The study involving colour preference can be used to work on the learning and memory capabilities of zebrafish larvae. These assays will also be useful in screening drugs for effects on anxiety. The freezing behaviour and colour preference displayed by zebrafish larvae should also be validated with drugs which can either increase or decrease learning or memory. In general, the studies in the present thesis will help us understand the behavioural repertoire of zebrafish, impact of light-dark cycle and could be used in screening of compounds and drug discovery.

