

# Using Zebrafish for Screening and Development of New Nicotinic and Dopaminergic Drugs

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Zebrafish are a new vertebrate animal model with advantages for screening and development of therapeutic agents. Many biochemical pathways present in humans are conserved in zebrafish. Zebrafish embryos develop rapidly (first somite at about 10 hours of development compared to 9-10 days in the rat) outside of the mother with the potential for generating hundreds of embryos from a single mating. Zebrafish can be grown in plates for several days to allow easy observation and use in High-Throughput Screening (HTS). The early embryos are also transparent, providing the ability to observe specific cells or brain regions during development without the need to kill the animals. Embryogenesis is complete by 72 hours post fertilization (hpf) and most organs are fully developed at 96 hpf [1]. A large number of mutants are available and much of the genome has been sequenced (<http://www.sanger.ac.uk>) which allows the genetic advantages of mice, but with a short generation time (2-3 months). Zebrafish neural development occurs in a well characterized pattern and well-defined molecular markers (antibodies, DNA probes) are available to help identify specific cells and brain regions. Transgenic zebrafish have been developed which express GFP in specific cells [2] and allow for the activity of specific neurons to be monitored *in vivo* [3]. The small size of zebrafish makes them ideal for use in 96 or 384 well plates. The cost to maintain zebrafish is a fraction of that for mice since they don't require feeding until 6-7 days post fertilization.

The use of zebrafish allows one to combine the ability to perform behavioral assays with HTS [4]. Many behavioral assays developed in other animals, and which are used to assay drugs targeted to several neurological diseases, are available in zebrafish [5]. These include Prepulse Inhibition (PPI), startle response, locomotor and learning assays. These can be performed on a higher scale and at much lower cost than in other vertebrates. The data collection and analysis from many of these tests can be automated [4]. Indeed, zebrafish can be used at several points in the drug discovery process. Zebrafish are ideal for testing drug toxicity on a large scale, thus saving much time, money and effort to further develop a compound with toxicity in vertebrates. In many cases, compounds with known toxicity in humans have a similar response in zebrafish [1]. Zebrafish have also been used in target confirmation after a lead compound has been identified. *In vivo* Structure Activity Relationship (SAR) studies in zebrafish have been performed [6]. Using *in vivo* screens during early stages of drug discovery could produce great savings in time, money and efficiency. Zebrafish have been used already in a large number of small molecule screens in both wild-type and mutant fish [1]. Zebrafish have also been used for targeted screens as well.

Methodologies are being developed for rapid behavior-based identification of neuroactive small molecules in zebrafish [7]. Thus it may be useful to screen and develop drugs to treat illnesses related to cholinergic and dopaminergic systems in zebrafish. Neuronal Nicotinic Acetylcholine Receptors (nAChRs) are involved in several CNS disorders such as addiction, Alzheimer's, epilepsy, and schizophrenia period [8].

nAChRs are pentameric structures [9,10] which function as ligand-gated ion channels permeable to Na<sup>+</sup>, K<sup>+</sup> and Ca<sup>2+</sup> and are composed of multiple  $\alpha$  and  $\beta$  subunits. There are nine neuronal nAChR  $\alpha$  subunit

genes ( $\alpha 2$ - $\alpha 10$ ) and three nAChR  $\beta$  subunit genes ( $\beta 2$ - $\beta 4$ ) and distinct subtypes are formed from combinations of  $\alpha$  and  $\beta$  subunits. nAChR subtypes are designated as homo- or hetero-pentameric, depending on the subunit composition. Heteromeric receptors contain both  $\alpha$  and  $\beta$  subunits with the  $\alpha 4\beta 2$  receptor being the most prominent heteromer in the brain. Homomeric receptors are comprised of only  $\alpha$  subunits with the  $\alpha 7$  nAChR being the primary homomer. Each neuronal nAChR subtype also has a distinct pharmacology with both the  $\alpha$  and  $\beta$  subunits determining the sensitivity to agonists and antagonists [11,12]. Individual subtypes also have distinct ion channel properties such as conductance, rate of desensitization and channel-open time [13]. Neuronal nAChRs are often located presynaptically in the CNS and modulate release of important neurotransmitters such as norepinephrine, serotonin, Gamma Aminobutyric Acid (GABA), glutamate, and dopamine [14].

Cholinergic cells are distributed in zebrafish in a pattern similar to that seen in other vertebrate animals, present in sites including autonomic ganglia, the telencephalon, cranial motor nuclei, spinal cord, olfactory bulb, retina, tegmentum, and cerebellum [15]. We have cloned nine zebrafish neuronal nAChR cDNAs ( $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 6$ ,  $\alpha 7$ ,  $\alpha 8$ ,  $\beta 2$ ,  $\beta 3$ ,  $\beta 4$ ). The structure of each nAChR gene has been determined by comparison of the cDNAs to the genome sequence. The  $\alpha 2$ - $\alpha 6$  and  $\beta 2$ - $\beta 4$  genes each have six exons as in other vertebrates while the  $\alpha 7$  gene has ten as in other species. The expression patterns of the  $\alpha 2$ ,  $\alpha 4$ ,  $\alpha 6$ ,  $\alpha 7$ ,  $\beta 3$  genes have been determined [16,17]. Many of these are expressed in regions analogous to structures in rats and mice. For example, the  $\alpha 6$  nAChR subunit gene is expressed in zebrafish dopaminergic neurons as in mice. Zebrafish neuronal nAChRs subunits have been expressed in oocytes and the pharmacology of several combinations including  $\alpha 3\beta 4$ ,  $\alpha 4\beta 2$  and  $\alpha 7$  determined [18]. For the most part, our work has shown that the pharmacology of cholinergic compounds in zebrafish is similar to that in other vertebrates and can be used to interpret behavioral assays used in HTS. Zebrafish are amenable to high throughput screening approaches to test compounds for effects on learning, memory, and anxiety. Zebrafish have been used to examine the anxiolytic effects of nicotine [19], spatial discrimination learning [20] and delayed spatial alternation [21].

Dopamine signaling is involved in addiction, schizophrenia and Parkinson's disease. Zebrafish orthologs of all four human dopamine receptors and associated enzymes have been identified [22]. Zebrafish have a well characterized set of catecholaminergic neurons, but the

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homolog to the human mesencephalic dopamine neurons, has not been identified, although a possible region has been put forward [23]. Zebrafish have been used to screen for potential neuroprotective compounds in a zebrafish model of Parkinson's [24]. There is great potential to develop zebrafish disease models and behavioral assays which can be used in HTS for drugs which affect dopaminergic pharmacology.

The advantages of zebrafish for pharmaceutical screening [25] can be exploited to complement existing cell culture and mouse studies to test and develop new therapeutic compounds. Development and testing of compounds targeted to cholinergic and dopaminergic systems can take advantage of the use of zebrafish for HTS and development of disease models.

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