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ZEBRAFISH AS AN INNOVATIVE MODEL TO SCREEN THE BEHAVIOURAL EFFECTS OF NOVEL DRUGS

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Indice

Abstract

Zebrafish (*Danio rerio*) is an emerging animal model alternative to rodents for studying human diseases. Its typical shoaling behaviour (tight aggregation of individuals) consisting of forming a tight group in which fish swim together, may represent an excellent model to study social behaviour. Zebrafish appear to be a good model to study learning and memory, too.

The neuropeptides oxytocin (OT) and arginine vasopressin (AVP) are two of the most-studied brain signaling molecules encoding information relevant to social behaviour. Isotocin (ISO) and vasotocin (AVT) are the equivalent neurohypophiseal hormones in fish, regulating reproductive and social behaviour.

On this basis, we studied the effect of both OT and AVP in comparison with ISO and AVT, on shoaling, fear response to predator and learning and memory. Social behaviour was studied using mutant zebrafish *Nacre*. Since these peptides are known to affect anxiety in humans and rodents, the same compounds were also tested on fear response to predator, using *Astronotus Ocellatus* as stimulus fish. OT (2-40 ng/kg), ISO (0.1-10 ng/kg), AVP (0.5-40 ng/kg) and AVT (0.001-20 ng/kg) were given i.m. 10 min before each test. AVT/AVP were more potent to elicit anxiolytic than social effect while ISO and OT were equally potent. To investigate the mechanism of action, different antagonists were given 10 min before each peptide: the OT receptor antagonist Desgly (0.00001-1 ng/kg), the V1a receptor subtype AVP antagonist SR 49059 (0.00001-20 ng/kg) and the V1b receptor subtype antagonist SSR 149415 (0.00001-1 ng/kg). In both tests, treatment with all the peptides increased social preference and decreased fear response in a dose-dependent manner interpolated by symmetrical parabolas. Pre-treatment with SR 49059, SSR 149415 and Desgly dose-dependently blocked the pro-social and anxiolytic effect induced by each peptide. The less selective antagonist appeared to be SSR 149415. All the neuropeptides did not induce any change in swimming activity.

Neuronal nicotinic acetylcholine receptors (nAChRs) play a modulatory role in cognition and zebrafish provide a preclinical model to study these cognitive processes. On the other hand, nicotinic receptor has been characterized in this teleost fish.

Abstract

Using a T-maze task, we investigated the effect of cholinergic drugs on spatial memory in zebrafish. Nicotine (0.0002-0.2 mg/kg), given i.p. 20 min before the test, improved the mean running time difference, showing an inverted U dose-response function. Selective and non selective nAChR antagonists, injected i.p. 10 min before nicotine, were used to study the receptor subunits, involved in spatial memory. Nicotine-induced cognitive enhancement was reduced by the selective nAChR subtype antagonists, MLA (0.01 mg/kg) for α7 subunit, MII (0.1 mg/kg) for α6ß2 subunit, DhßE (0.01 mg/kg) for the α4ß2 subunit, the non selective antagonist mecamylamine (0.1 mg/kg) and the muscarinic antagonist scopolamine (0.025 mg/kg), with DhßE being more active than MLA or MII. No change in swimming activity was observed for all the nicotinic drugs.

Another important cognitive process is the selective attention. It can be assessed in rodents with the novel object recognition (NOR) test. In the standard version of this test, the selection of objects to be used is critical. To overcome the limitation of NOR, we created a modified version of NOR, the virtual object recognition test (VORT) in mice where 3D objects were replaced with stationary geometrical 2D shapes and presented on two Ipods 3.5-inch widescreen displays.

A comparable discrimination index as NOR was shown in VORT. 2D shapes that could be highly discriminated and some which could not, were identified. Mice were able to distinguish among different movements (horizontal, vertical or oblique). In fact, the shapes previously found not distinguishable when stationary were better discriminated when moving. Secondly, we focused our attention on zebrafish, which have a good capability to learn and a better visual acuity. Based on this abilities, we investigated in VORT if zebrafish, like mice, were able to discriminate different geometrical 2D shapes (circle, square or triangle), when presented on Ipod-screens, placed at the sides of a water tank. To evaluate the possibility that moving 2D shapes increased the attention of zebrafish, specific movements were applied to the same geometrical shapes. We found that zebrafish, like mice, were able to discriminate different geometrical 2D shapes both stationary and with different movements. In particular, the discrimination index of shapes, previously not discriminate, increased when they were moving. Finally, we investigated if

memory performance could be improved by treatment with nicotine both in mice (0.1 mg/kg) and in zebrafish (0.02 mg/kg) or worsened by scopolamine (0.25 mg/kg for mice and 0.025 mg/kg for zebrafish) or by mecamylamine (1 mg/kg). Nicotine improved discrimination index for stationary shapes previously not discriminated while anticholinergic drugs impaired episodic memory in both species.

Taken together, these findings showed the pro-social and anxiolytic properties of OT/AVP system mediated by different receptors and confirmed the important role of cholinergic system in the processes of acquisition and memory consolidation in zebrafish similar to mammals. Moreover, we showed, for the first time, both mice and zebrafish could discriminate not only geometrical shapes but also different movements in VORT, allowing a direct comparison between animal model and human to study attention. Zebrafish opens a new avenue of research to rapidly screen new compounds for the treatment of abnormal social behaviours (including autism or schizophrenia) and neurodegenerative diseases.

Introduction

2. *INTRODUCTION*

2.1. *ZEBRAFISH: a new alternative model*

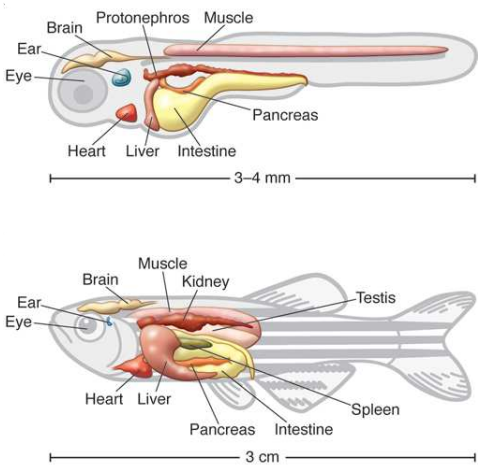
The zebrafish, Danio Rerio, is a tropical freshwater fish belonging to *Cyprinidae* family. The zebrafish is native to the streams of the south-eastern Himalayan region and it is found in parts of India, Pakistan, Bangladesh, Nepal, and Burma. This specie arose in the Ganges region in eastern India and commonly inhabits streams, canals, ditches, ponds, and slow-moving or stagnant water bodies, including rice fields. The zebrafish is named for the five uniform, pigmented, horizontal, blue stripes on the side of the body, which are reminiscent of a zebra's stripes and which extend to the end of the caudal fin. Its shape is fusiform and laterally compressed, with its mouth directed upwards. The male is torpedo-shaped, with gold stripes between the blue stripes; the female has a larger, whitish belly and silver stripes instead of gold. The zebrafish can grow to 2.5 in length, although it seldom grows larger than 4 cm in captivity. Its lifespan in captivity is around two to three years, although in ideal conditions, this may be extended to five years (Spence et al., 2008).

The approximate generation time for zebrafish is three to four months. A male must be present for ovulation and spawning to occur. Females are able to spawn at intervals of two to three days, laying hundreds of eggs in each clutch. Upon release, embryonic development begins; growth stops after the first few cell divisions. Fertilized eggs almost immediately become transparent, a characteristic that makes Danio rerio a convenient research model species in genetics. Development progresses rapidly and precursors to all major organs appear within 36 hours of fertilization. Hatching takes place 12 - 36 hours later, depending on the embryo's internal conditions and the external temperature, which is ideally 28.5 °C. Swimming and feeding behavior begin about 36 hours later. The sex of juveniles cannot be distinguished, except by dissection and sex determinants are not clearly understood. Zebrafish are omnivorous, primarily eating zooplankton, insects, insect larvae, and phytoplankton or worms and small crustaceans.

The small body size, large brood size, external development and optical transparency of zebrafish allows to overcome the limitations of mammalian models and it provides a powerful tool, which includes large-scale genome mutagenesis and gene mapping, transgenesis, protein overexpression or knockdown, cell transplantation and chimeric embryo analysis and chemical screens (Veldman & Lin, 2008), making this organism particularly well-suitable for the molecular genetic analysis of vertebrate neurodevelopmental mechanisms relevant to neuropsychiatric disorders.

It is possible to visualize complete neurotransmitter systems in the whole zebrafish brain at 5 days post fertilization when the fish already displays a sophisticated repertoire of behaviours (Panula et al., 2006). Magnetic Resonance imaging (MRI) on living zebrafish embryos for molecular screening or molecular tracking over time (Canaple et al., 2008) and Magnetic Resonance Histology (MRH) of the zebrafish brain have also been carried out (Ullmann et al., 2010). Therefore, the relative simplicity of the nervous system in fish larvae facilitate circuit analysis (Burgess & Granato, 2007).

Larval zebrafish have emerged as a popular model for a number of brain pathologies. Larvae display learning, slep, drug addiction and other quantifiable neurobehavioral phenotypes (Best & Alderton, 2008). Another advantage of using zebrafish larva is the ability to study multiple animlals simultaneously within a high-throughput battery. However, such models have some limitations, since they do not exhibit the rich behaviour of adult animals. Also, larval models have somewhat limited developmental applications, for example, lacking fully established mediatory and endocrine system (Mahler et al., 2010), as well as some neural circuits and projections.



Likewise, behavioural endpoints observed in larval animals may not be fully translated to adult subject'behaviour. Thus, larval research is unable to fully replace the adult zebrafish studies.

Although simplified compared to human behaviors, adult zebrafish can exhibit higher behaviors and integrated neural functions including social behaviors, anxiety, memory, conditioned responses and addiction (Lieschke & Currie, 2007).

2.1.1. *Modelling human disease in zebrafish*

Research with zebrafish has yielded advances in the fields of developmental biology, oncology, toxicology, reproductive studies, teratology, genetics, neurobiology, environmental sciences, stem cell and regenerative medicine and evolutionary theory.

As a model *genetic system*, the zebrafish possesses numerous advantages. Its genome has been fully sequenced and it has well-understood, easily observable and testable developmental behaviors. Its embryonic development is very rapid, and its embryos are relatively large, robust, and transparent, and it is able to develop outside their mother. Furthermore, well-characterized mutant strains are readily available. Moreover, other advantages include the species' nearly constant size during early development, which facilitates simple staining techniques and the fact that its two-celled embryo can be fused into a single cell to create a homozygous embryo. The zebrafish is also demonstrably similar to mammalian models and humans in toxicity testing and it exhibits a diurnal sleep cycle with similarities to mammalian sleep behavior. Due to their short lifecycles and relatively large sizes, zebrafish are a useful model for genetic studies. A common reverse genetics technique is to use antisense oligonucleotides that inhibit translation or affect splicing: Morpholino antisense technology (Lan et al., 2011). Morpholino oligonucleotides (MO) are stable synthetic macromolecules that contain the same bases as DNA or RNA; by binding to complementary RNA sequences, they reduce the expression of specific genes. MO can be injected into one cell of an embryo after the 32-cell stage, reducing gene expression in cells descended from that cell. However, cells in the early embryo (less than 32 cells) are interpermeable to large molecules, allowing diffusion between cells. A known problem with gene knockdowns is that, because the genome underwent a duplication after the divergence of ray-finned fishes and lobe-finned fishes, it is not always easy to silence

the activity one of the two gene paralogs reliably, due to complementation by the other paralog. However this technology permits a quick and easy probing of specific gene function in vivo; in contrast, this technology cannot be used to study gene function in mice because antisense oligonucleotides are rapidly diluted during mouse development. The Wellcome Trust Sanger Institute started the zebrafish genome sequencing project in 2001 and the full genome sequence of the Tuebingen reference strain is publicly available at the National Center for Biotechnology Information (NCBI)'s Zebrafish Genome Page (Clark MS, 2003). In October 2001, researchers from the University of Oklahoma published Danio rerio's completed mitochondrial DNA sequence (Broughton et al., 2001). Its length is 16,596 base pairs. This is within 100 base pairs of other related species of fish and it is notably only 18 pairs longer than the goldfish (Carassius auratus) and 21 longer than the carp (Cyprinus carpio). Its gene order and content are identical to the common vertebrate form of mitochondrial DNA. It contains 13 protein-coding genes and a noncoding control region containing the origin of replication for the heavy strand. Transgenesis is a popular approach to study the function of genes in zebrafish. Construction of transgenic zebrafish permits to better understand the genes involved in the etio-patology of unknown genetic diseases. In 2009, researchers at the Institute of Genomics and Integrative Biology in Delhi announced the sequencing of the genome of a wild zebrafish strain, containing 1.7 billion genetic letters (Petzold et al., 2009).

Zebrafish have been used to make several transgenic models of *cancer*, including melanoma, acute lymphoblastic leukemia (ALL), pancreatic cancer and hepatocellular carcinoma. 50%–60% of melanoma tumor samples carry the activating mutation BRAFV600E, which results in sustained activation of the BRAF/MEK1/2/ERK1/2 MAP kinase pathway (Davies et al., 2002). In addition to BRAF, another member of the MAP kinase pathway, the RAS oncogene (rat sarcoma viral oncogene homolog), is found mutated in approximately 20% of metastatic melanoma. In 2005, Patton et al. generated the first zebrafish melanoma model, in which human BRAFV600E was expressed in melanocytes using the promoter of microphthalmia-associated transcription factor a (mitfa) (Patton et al., 2005). Fish injected with this mutant form of BRAF developed moles similar to humans, whereas

injection of BRAF into p53M214K mutant fish led to the formation of melanoma starting at four months of age. Although p53 mutations are rare in human melanoma tumors, the gene is frequently functionally inactivated by loss of CDKN2A or other tumor suppressor pathways (Avery-Kiejda et al., 2011). More recently, a model of melanoma formation independent of p53 activity was developed using a UAS\GAL4 system. A cross between a driver line expressing the transactivator GAL4 driven by the c-kit (kit-a in fish) promoter and a responder line expressing the human activated form of H-RASG12V led to the generation of fish that display a hyperpigmented phenotype and the formation of tumors beginning at four weeks after fertilization (Santoriello et al., 2010).

Ceol et al. used the BRAFV600E/p53M214K fish model to develop a method for testing the role of genes in a recurrently amplified region of human chromosome 1 in melanoma. They found that SETDB1, a histone methyltransferase that is amplified in as many as 30% of human melanomas, accelerated tumor formation (Ceol et al., 2011). This model recapitulate the pathology of the human melanoma in order to be utilized as a cost-effective system for screening of anticancer compounds. Zebrafish, expressing mutated forms of either the BRAF or NRAS oncogenes, develops melanoma when placed into a p53 deficient background. Histologically, these tumors strongly resemble the human disease, are fully transplantable and they exhibit large-scale genomic alterations. The BRAF melanoma model was utilized as a platform for two screens. In one study, (Ceol et al., 2011) this model was used as a tool to understand the functional importance of genes known to be amplified and overexpressed in human melanoma. One gene, SETDB1, markedly accelerated tumor formation in the zebrafish system, demonstrating its importance as a new melanoma oncogene. This was particularly significant because SETDB1 is known to be involved in the epigenetic regulation that is increasingly appreciated to be central to tumor cell biology. In another study (White et al., 2011), an effort was made to therapeutically target the genetic program present in the tumor's origin neural crest cell, using a chemical screening approach. This revealed that an inhibition of the DHODH protein (by a small molecule called leflunomide) prevented development of the neural crest stem cells which ultimately give rise to melanoma via interference with the process

of transcriptional elongation. Because this approach would aim to target the "identity" of the melanoma cell rather than a single genetic mutation, leflunomide may have utility in treating human melanoma. In 2012, a group of researchers (Hoage et al., 2012) developed a new strain of zebrafish, named “Casper”, whose adult bodies had transparent skin. This allows for detailed visualization of cellular activity, circulation, metastasis and many other phenomena. Because many gene functions are shared between fish and humans, the Casper strain is expected to yield insights into human diseases such as leukemia and other cancers.

A common type of childhood leukemia is the *acute lymphoblastic leukemia* (ALL) (Linet et al., 1999). The first transgenic cancer model established in zebrafish was a T cell leukemia model generated using a chimeric transgene encoding the mouse c-Myc gene fused to GFP driven by a rag2 promoter (Langenau et al., 2003). Within two months, injected zebrafish developed tumors in the thymus that spread to the gills, eye, abdominal organs, and muscle. Later, a double-transgenic line was developed carrying the lox-dsRED2-lox EGFP:mMyc and Cre driven by a heat shock promoter. After induction, these fish developed tumors with 81% penetrance (Feng et al., 2007). Another T-ALL leukemia model was established using an activated form of NOTCH1, which is mutated in over 50% of all human T-ALL cases. Zebrafish, carrying the mutated NOTCH1, developped tumors with late onset, but when crossed to a line overexpressing bcl2, a more rapid tumor onset was observed, suggesting

cooperation between the Notch pathway and bcl2-mediated apoptosis (Chen et al., 2007). bcl2 overexpression in both Notch- and Myc-induced T-ALL led to more aggressive tumors that were resistant to radiation (Feng et al., 2010). Their studies demonstrate the utility of modifier screens in the identification of genetic interactions. These zebrafish T-ALL models are sensitive to the same chemotherapeutic drugs used currently in patients (Mizgirev & Revskoy, 2010) and thus may be used in drug screens to identify novel therapeutics. In addition, a comparative study on copy number aberrations (CNAs) in zebrafish and humans revealed an overlap between T-ALL CNA genes across species,

supporting zebrafish as a relevant model for studying human leukemias (Rudner et al., 2011).

Zebrafish are well suitable for studying *hematological disorders*. The first zebrafish model of a human disease derived from positional cloning was estabilished in 1998 (Brownlie et al., 1998). Isolated from a large forward genetic screen, the zebrafish *mutant sauternes* (sau) has a defect in hemoglobin production. The mutated gene encodes erythroid synthase δ-aminolevulinate synthase (ALAS-2), which regulates the first step in heme biosynthesis; inactivation of this gene leads to congenital sideroblastic anemia in zebrafish and humans. Since then, several other mutants have been isolated from genetic screens that resemble human hematological diseases, including anemia (Taylor et al., 2011), polycythemia (van Rooijen et al., 2009), and porphyria (Dooley et al., 2008). The positional cloning of the hypochromic anemia mutant weissherbst (weh) identified ferroportin 1 as a novel iron transporter. The human ortholog was subsequently found mutated in patients affected by hemochromatosis, a disorder characterized by iron absorption defects (Montosi et al., 2001). Other anemias have been phenocopied using morpholino-mediated knockdown (Danilova et al., 2008), including diamond blackfan anemia (DBA), which is modeled by knockdown of ribosomal protein RSP19 (Tamary et al., 2007). Characterization of the RSP19 morphants and other ribosomal mutants revealed an activation of the p53 pathway, raising the possibility that a p53 family member could be targeted for DBA treatment (Taylor et al., 2012).

Another focus of zebrafish research is to investigate about heart failure, congenital and *cardiovascular diseases*. When human heart are subjected to physiological and pathological stress, individual ventricular cardiomyocytes undergo hypertrophy to increase in size, resulting in a larger heart with a thickened left ventricular wall (Alcalai et al., 2008). Although cardiomyocytes regeneration occur at some low rate in human hearts, this regenerative ability is unfortunately inefficient and neither adequately repairs damaged cardiac tissue nor responds to demands of overload stress with an efficiently compensated heart (Perrino et al., 2006). In contrast, adult zebrafish efficiently and completely regenerate working heart tissue via hyperplasia proliferation of existing cardiac cells. Jean

and collaborators (2012) used a novel approach that was an excessive intermittent forced swimming exercise to study molecular and cellular pathways, involved in the proliferative stress response of zebrafish hearts. They discovered that the exposure of zebrafish to long-term excessive cardiac overload stress, through intermittent forced swimming exercise, elicited a proliferative response similar to the regeneration process seen with cardiac injury models. According to them, this exercise regimen results in overall heart enlargement and this one can be explained by either collective increase in size of single cardiac cells (hypertrophy) or increase in the cardiac cell number (hyperplasia). So, this model can be used to investigate, in zebrafish hearts, proliferation initiation factors and the molecular targets for specifically treating human pathological cardiac overload stress conditions such as diabetes and hypertension.

Zebrafish have been used to model *kidney disease* (Swanhart et al., 2011). Zebrafish have become a popular model for studying renal diseases thanks to the anatomical simplicity of their kidneys. Polycystic kidney disease (PKD), nephronophthisis, acute kidney injury (AKI) and a range of ciliopathies have been modeled in zebrafish. Several studies suggest that cilia (microtubule-based hair like organelles) play a central role in the etiology of PKD. The proper function of cilia prevents cystic formation and this hypothesis has been largely supported by the characterization of several zebrafish mutants, carrying mutations in cilia proteins, such as intraflagellar transport proteins and LRRC50 (van Rooijen et al., 2008; Cao et al., 2010). Several ciliopathies including Bardet-Biedl syndrome (BBS), nephronophthisis (NPHP), Jeune, Joubert, oro-facial-digital (OFD1), and Meckel (MKS) syndromes have been modeled in zebrafish using morpholinos for ciliopathy candidate genes. The use of drugs such as rapamycin and roscovitine ameliorate the renal phenotype observed in these morphant embryos, suggesting that zebrafish can be used to identify potential therapeutic agents for renal cystitis (Tobin & Beales, 2008). The zebrafish kidney is also a valuable system for studying acute kidney injury because, as opposed to mammals, fish can generate new nephrons throughout their life and regenerate new nephrons after injury (Diep et al., 2011). Diep et al., in a series of transplantation experiments, were able to identify LIM homeobox 1a–positive (lhx1a-positive) cells as adult

self-renewing nephron stem/progenitor cells. These findings pave the way for isolating similar cells in mammals with the aim of developing novel renal regenerative therapies.

A model of *muscle disorders* has been responded for zebrafish. Duchenne muscular dystrophy (DMD) is a lethal genetic disorder characterized by wasting of muscle tissue, caused by mutations in the dystrophin gene. Zebrafish strains, with mutations in the dystrophin gene (called dmd or sapje), were identified from a large forward genetic screen (Bassett et al., 2003) and they have a phenotype similar to the human disease, displaying progressive degeneration of skeletal muscle. Currently, there is no cure for DMD and the treatments, employed so far, are aimed at controlling symptoms to maximize quality of life. Zebrafish dmd mutants have been used in a chemical suppressor screen to identify potential compounds that can correct the disease pathology, as detected by alterations in birefringence, which measures muscle integrity (Kawahara et al., 2011). This screen revealed a number of compounds that appear to effectively reduce dystrophic symptoms in zebrafish. In particular, PDE5 inhibitors appear to be useful and they have also been shown to be effective in the mdx mouse model of muscular dystrophy (Asai et al., 2007).

Another notable characteristic of the zebrafish is that it possesses four types of *cone cell*, with ultraviolet supplementing the red, green and blue cone cell subtypes found in humans. Zebrafish can thus recognize a wide spectrum of colours. The zebrafish eye is similar in morphology, physiology, gene expression and function to the human eye. Several zebrafish mutants displaying eye defects and visual impairment have been identified, revealing that signaling pathways including sonic hedgehog (Shh), nodal, and retinoic acid are involved in eye development and disease (Bibliowicz et al., 2011). Pharmacological intervention in these mutants has shed light on the mechanisms of these diseases. For example, the *blowout mutant*, which harbors a mutation in patched1 (a negative regulator of Shh) revealed that pharmacologic inhibition of the Hedgehog pathway rescues the coloboma phenotype characterized by open choroid fissure (Lee et al., 2008). Rather surprisingly, *lamb* (laminin beta 1) and *pax2 mutants* displaying coloboma are rescued upon treatment with gentamicin and paromycin, two aminoglycoside drugs that most likely allow translational read-through of nonsense mutations (Moosajee et al., 2008).

Neuhauss et al. used for the first time optokinetic and optomotor behavioral assays to analyze more than 400 mutants previously identified, based on defects in organ formation, tissue patterning, and pigment formation. This study uncovered mutations that led to lens degeneration, melanin deficiency, lack of ganglion cells, ipsilateral misrouting of axons and optic-nerve disorganization (Neuhauss et al., 1999). Several other ocular diseases, including glaucoma and retinal degeneration have also been modeled in zebrafish (Morris, 2011). Retinal degeneration is observed in several ciliopathies such as Leber's congenital amaurosis (LCA), BBS, Senior-Loken syndrome, Joubert syndrome, and MKS (Lancaster et al., 2009). The phenotype of the zebrafish mutants (*oval*, *elipsa*, *fleer*) closely resembles some of these human ciliopathies, including defects in photoreceptor outer segment formation. Positional cloning of the *oval* locus identified a mutation in the intraflagellar transport protein 88 (IFT88), which is a component of the IFT complex. This complex is involved in the generation and maintenance of ciliated structures (Morris AC, 2011). The further characterization of these mutants should help to clarify the roles of individual IFT particle members in the formation and survival of photoreceptor cilia.

This species is also studied to better understand the development of the *retina*; in particular, how the cone cells of the retina become arranged into the so-called 'cone mosaic'. Zebrafish, in addition to certain other teleost fish, are particularly noted for having extreme precision of cone cell arrangement (Allison et al., 2010). In 2007, researchers (Lawrence et al., 2007) grew a type of zebrafish adult stem cell found in the eyes of fish and mammals that develops into neurons in the retina. These could be injected into the eye to treat diseases that damage retinal neurons or in general different diseases of the eye, including macular degeneration, glaucoma, and diabetes-related blindness. The researchers studied Müller glial cells in the eyes of humans aged from 18 months to 91 years, and they were able to develop them into all types of retinal neurons. The stem cells successfully migrated into diseased rats' retinas and they took on the characteristics of the surrounding neurons. The team is working to develop the same approach in humans.

2.2. *CENTRAL NERVOUS SYSTEM DISORDERS*

Zebrafish show similarity to humans in terms of the structure of the nervous system, which includes a fore-, mid-and hind-brain, including diencephalon, telencephalon (Mueller & Wullimann, 2009), cerebellum and a peripheral nervous system with motor and sensory components, enteric and autonomic nervous systems. However, the telencephalon has only arudimentary cortex. For these reasons, many studies have focused on the use of zebrafish as a model for neurological, neurodegenerative and behavioral diseases. Human neurological diseases, including Parkinson, Huntington, Alzheimer, Schizofrenia and addiction diseases have been successfully modeled in zebrafish and the orthologs of major disease-associated genes have been identified in zebrafish (Xi et al., 2011). Specific region of zebrafish brain are strikingly conserved with the human counterparts, making zebrafish an excellent organism to model human neurodegenerative conditions. Many approaches to generate transgenic zebrafish are currently available, including microinjection or electroporation of plasmid DNA or transgenic cassettes into the cytoplasm of fertilized eggs (I-SceI meganuclease or the Tol2 trasposon) (Kawakami et al., 2000; Thermes et al., 2002). Other techniques include sperm-mediated gene transfer, pseudotyped retroviral infection, retroviral infection of in vitro-cultured sperm and BAC transgenis (Sager et al., 2010). However, a well-estabilished gene knockdown technology in zebrafish uses morpholinos, as already explained before, which are synthetic oligonucleotides of approximately 25 bases that are hybridize specifically to complementary sequences of mRNA to disrupt translation initiation or splicing (Bill et al., 2009).

The majority of Parkinson's disease (PD) cases are forms, which may suggest a role of environmental factors or more complex gene-environment interactions. The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrapyridine (MPTP), known to induce PD in humans, has been successfully used in rodent and primate models of PD. MPTP can interrupt the electron transport chain in mitochondria at complex I, which will eventually result in cell death. In adult zebrafish, MPTP induced a transient decrease in dopamine levels as well as behavioral defects (Anichtchik et al., 2004). Earlier studies in larval zebrafish showed a

significant reduction of dopamine neurons in the ventral diencephalon following MPTP treatment (McKinley et al., 2005). More extensive studies have suggested that different groups of dopaminergic neurons in the ventral diencephalon show different sensitivities to MPTP, with those neurons with ascending projections being more sensitive (Wen et al., 2008; Sallinen et al., 2009). Dopamine levels also declined after MPTP treatment, which is consistent with findings in humans. Thus, similar to mammals, MPTP can induce a significantly functional deficit of dopaminergic neurons in zebrafish. These studies strongly indicate that the dopaminergic neurons in the ventral diencephalon of zebrafish are analogous to those in the mammalian nigrostriatal pathway. Zebrafish orthologs of PD-related genes have highly conserved functions in the development and survival of dopaminergic neurons and in motor behavior. Toxin-induced loss of dopaminergic neurons in zebrafish is also a relevant model of human PD.

A fatal neurodegenerative disorder is the Huntington's disease (HD). HD follows an autosomal-dominant inheritance pattern of a mutant form of the huntingtin gene (*HTT*), coding for an abnormal expansion of a trinucleotide repeat encoding glutamine (CAG) at the amino terminal of the HTT protein. The length of the polyglutamine tract correlates with the penetrance, age of onset and severity of HD. At the moment, the normal function of the *HTT* gene is not well understood. Despite the ubiquitous expression of *HTT*, specific brain areas of HD patients are affected earlier than others. The neuronal atrophy characteristics of HD are seen in regions of the striatum (caudate and putamen) and cortex. Medium spiny neurons of the striatum, which use γ-aminobutyric acid and project to the substantia nigra and globus pallidus, are the most vulnerable to loss (Graveland et al., 1985). What puts these neurons at risk is largely unknown. The zebrafish Htt protein shares about 70% identity with the human HTT ortholog and encodes four glutamines (vs 35 glutamines in the normal human HTT) (Karlovich et al., 1998). Zebrafish could prove to be a useful model, as the early embryonic death of mice with a targeted null mutation in *Htt* provide very limited insight into the role of HTT (Zeitlin et al., 1995). Zebrafish antisense morpholino (MO) knockdown, which provides a deficiency but not a total depletion of the Htt protein, might be a better strategy. Observations made in zebrafish *htt* morphant

implicate wild-type Htt function in cellular iron utilization (Lumsden et al., 2007). During gastrulation, the *htt* mRNA is expressed uniformly throughout the embryo; however, as development progresses, its expression decreases in non-neuronal tissues but remains strong in the head region. High concentration of MOs resulted in a large variety of developmental defects (slight growth delay, brain necrosis, lack of brain ventricle enlargement, and thinned yolk extension) possibly due to the off-target effects of the MOs; at lower doses of MO, a thin yolk extension and blood hypochromia were the most common morphologic defects remaining. The hypochromic blood was associated with deficits in hemoglobin production, caused by altered iron metabolism. Making iron available to the embryo restored hemoglobin production in Htt-deficient embryos. Signs of iron deficiency, including defects in iron homeostasis and energy metabolism, are features of HD pathogenesis (Morrison & Nevin, 1994). Using the same Htt-deficient zebrafish model, Henshall and collaborators (Henshall et al., 2009) focused on CNS defects to explain the specificity of neuropathology in HD brains. Although *htt* is ubiquitously expressed in the zebrafish brain, it seems to have a specific function within the forebrain that enables formation of telencephalic progenitor cells and preplacodal cells. Because the zebrafish telencephalon is believed to house the structures analogous to the human striatum, this study may have found a way of implicating a striatal-specific loss of medium-sized spiny neurons to the expression of Htt protein. Zebrafish deficient in Htt lose placode-derived tissue including olfactory and lateral line sensory neurons and they have a reduction in telencephalic tissue. The finding that sensory neurons are perturbed by the reduction of Htt in the zebrafish model is consistent with the clinical observation that HD patients present with impaired olfactory function (Pirogovsky et al., 2007). Another in vivo study, investigating Htt loss-of-function in zebrafish, observed massive apoptosis of neuronal cells by 24 hpf, which was accompanied by impaired neuronal development, small eyes and heads, as well as an enlargement of brain ventricles (Diekmann et al., 2009). Later in development, these Htt-deficient zebrafish develop lower jaw abnormalities with most branchial arches missing. Most notably, brain-derived neurotrophic factor (BDNF) expression was reduced. BDNF enhances the differentiation of sensory and sympathetic

neurons. The observation that Htt-MO and BDNF-MO produce similar phenotypes suggests that Htt regulates BDNF function. Moreover, treatment of Htt-deficient embryos with exogenous BDNF significantly rescued these defects. Therefore, it may be the loss of normal *htt* function that contributes to the symptoms of HD pathology, and not exclusively the toxic gain-of-function caused by an expansion of the polyglutamine tract; moreover increasing expression of the prosurvival neurotrophin, BDNF, could be a therapeutic approach in the treatment of HD.

The pathologic hallmarks of Alzheimer's disease (AD) are extracellular amyloid-ß (Aß) protein-containing neuritic plaques and intracellular hyperphosphorylated tau-containing neurofibrillary tangles. Early-onset AD is associated with mutations in three genes involved in Aß proteolysis displaying autosomal-dominant inheritance patterns in humans: amyloid-ß precursor protein (*APP*), Presenilin 1 (*PSEN1*), and Presenilin 2 (*PSEN2*). Late-onset AD is linked to a number of genetic risk factors including the apolipoprotein E (ApoE), which is a cholesterol transport protein, and the neuronal sortilin-related receptor (*SORL1*), which acts as a sorting receptor for APP. In humans, APP undergoes post-translational processing. If APP is cleaved by α-secretase, a benign Aß peptide is produced. Alternatively, APP can undergo two sequential cleavages by ß-secretase and γ-secretase to generate the pathogenic Aß peptide pushing it toward the late endosomal pathway. Depending on where the γ-secretase cleaves the Aß C-terminal, either Aß42 or Aß40 will be generated. Aß42 is the longer form of Aß, which is more cytotoxic than the shorter Aß40 peptide. Deposits of Aß outside the neuron are the underlying cause of AD. The hyperphosphorylated tau protein, which forms neurofibrillary tangles inside neurons, is the catalyst for AD disease progression. This leads to the disassembly of microtubules essential for neuronal transport, disrupting neurotransmitter communication between neurons, and ultimately resulting in cell death (Lehmann et al., 2013).

Several of the human genes encoding the enzymes required for the post-translational modifications of APP have been found with a high percent of amino acid similarity in zebrafish. It has two genes similar to human APP: *appa* and *appb (*Musa et al., 2001). During gastrulation, both app genes are expressed in the entire embryo, whereas at 24

hours-post-fertilization, the *appa* and *appb* paralogs are expressed in the telencephalon, the ventral diencephalon, the trigeminal ganglia and the posterior lateral line ganglia. Orthologs of the ß-secretase and γ-secretase complexes are found in zebrafish and are expressed in the CNS (Groth et al., 2002). Despite conservation of the Aß domain and of the secretases between zebrafish and humans, a zebrafish Aß peptide has yet to be found and it is not known if the above post-translational modifications that occur in human APP processing also occur in zebrafish.

Loss of zebrafish Appa and Appb function by MO knockdown resulted in reduced body length and defective convergent-extension movements during gastrulation (Joshi et al., 2009). Interestingly, these defects are rescued by wild-type human APP mRNA, but not by the Swedish mutant APP, known to cause familial AD. Both zebrafish *psen1* and *psen2* are expressed ubiquitously during embryogenesis. However, *psen2* is more restricted to the CNS, eye and spinal cord at 1 day-post-fertilization (Groth et al., 2002). Another candidate gene that requires more in-depth characterization is the ApoE ε4 susceptibility gene, whose function in zebrafish is not well understood. ApoE is expressed in the zebrafish eyes, and some cells of the mesencephalic, telencephalic and rhombencephalic brain areas, suggesting that it may play a significant function in the CNS (Babin et al., 1997).

Similar to early neurodegenerative diseases, Schizophrenia is a devastating disorder caused by both genetic and environmental factors that disrupt brain development and function. It is distinguished as a neurodevelopmental disorder in part due to early cognitive impairments, behavioural dysfunction in childhood and adolescence, and abnormalities in CNS development with no neurodegenerative component (Lewis & Levitt; 2002). Neuropsychiatric disorders, including schizophrenia, are associated with disturbances in pre-pulse inhibition (PPI), which affects the individual' s ability to filter out external information from the environment (Braff et al., 2001). Larval zebrafish have been found to exhibit PPI which modulate the acoustic startle response in a manner similar to mammalian PPI. Larval PPI is disrupted by dopamine agonists and this disruption can be corrected by antipsychotic drugs similar to the mammalian situation. A zebrafish screen has isolated the *Ophelia* mutant, which has reduced PPI (Burgess & Granato, 2007) and further high-

throughput zebrafish screens could help understand the genetic basis for defects in PPI observed in schizophrenia. A study employing both *in-vitro* and *in-vivo* studies in zebrafish found that antipsychotic drugs produce alterations in acetylcholinesterase activity and this could reveal molecular mechanisms related to cholinergic signaling in schizophrenia (Seibt et al., 2009). A well-documented schizophrenia-susceptibility gene, which has recently been characterized in zebrafish cranial neural crest (CNC), is d*isrupted in schizophrenia 1* (*disc1*). Disc1 is a potent regulator of *sox10* which is an oligodendrocyte related schizophrenia susceptibility gene. Understanding the basic functions of Disc1 in transcriptional regulation, cell migration and cell differentiation in the zebrafish neural crest might give insight into the cellular processes that, when disrupted, predispose individuals to mental illness (Drerup et al., 2009). Other recent findings in the zebrafish embryo reveal neurodevelopmental connections that may exist between key candidate schizophrenia susceptibility genes like DISC1 (Drerup et al., 2009), NudE-Like (NDEL1/NUDEL) (Drerup et al., 2007), NRG1, OLIG2 and ERBB4 and they suggest that disc1 and nrg1 function in common or related pathways controlling development of oligodendrocytes and neurons from olig2-expressing precursor cells (Wood et al., 2009).

The zebrafish model has been widely used to better understand the complex processes involved in the development of Addiction. Numerous studies, in recent years, have focused upon modeling alcohol addiction in zebrafish and the findings have striking parallels with the effect of ethanol in mammals. Acute alcohol exposure has been shown to result in significant changes in dopamine, serotonin and related metabolites in adult zebrafish brain (Chatterjee & Gerlai, 2009). Different strains of zebrafish have been found to show differences in response to a zebrafish shoal and predator while undergoing alcohol withdrawal and this is correlated with differences in neurochemical (including dopamine) responses. Withdrawal after chronic exposure to ethanol in adult, zebrafish has been profiled in terms of freezing bouts and erratic movements in the novel tank diving test and correlated with changes in whole body cortisol (Cachat et al., 2009). In addition to alcohol, the effects of drugs of abuse including cocaine, amphetamine, and morphine have also been studied in zebrafish. Withdrawal symptoms following euphoria are part of the

cycle of drug abuse for drugs like cocaine. In zebrafish, cocaine withdrawal produces an anxiety-like state which develops earlier in the female but is more robust and persistent in males and is accompanied by a decrease in dopamine transporter and an increase in dopamine levels (Lopez Patino et al., 2008). Thus the zebrafish provides new opportunities to study genetic and endocrine gender-related factors involved in cocaine withdrawal symptoms. Studies in zebrafish have also shown that exposure to drugs such as morphine, during development, changes the expression level and localization of nociception receptors, indicating that nociception (NOP) plays a role in development of dependence/addiction to drugs (Macho Sanchez-Simon & Rodriguez, 2009). Additionally, the effects of a potent psychoactive drug Salvinorin-A have been documented in zebrafish. Salvinorin-A produces rewarding effects independently of its effects on zebrafish locomotor activity and these effects are mediated by kappa-opioid and cannabinoid receptors (Braida et al., 2007), confirming similarities between zebrafish and mammals in terms of their behavioral responses to alcohol and drugs of abuse and possibly also the underlying neural substrates. Larval zebrafish have been used to explain how genetic variation accounts for differential predisposition to nicotine dependence (Petzold et al., 2009). Four-day old larval zebrafish show increased locomotor activity in response to a water stimulus and pretreatment with nicotine (2.5–50 μM) results in significantly greater locomotor activation. Zebrafish display sensitization due to prior nicotine exposure and the nicotine response is blocked by nicotinic receptor antagonists. This nicotine behavioral assay has been coupled with zebrafish forward genetic screens, using gene-breaking transposon mutagenesis to identify mutants with defects in Chaperonin Containing Protein 8 (CCT8) and GABA-B receptor Subunit 1. According to them, these genes provide candidates for human association studies about predisposition to nicotine addiction.

Two different teams of researches (Cadet, 2009; Webb et al., 2009).tried to combine a zebrafish forward genetic screen with CPP and micro-array analysis led to the discovery of the “no-addiction” (*nad*) mutant which does not exhibit preference for the compartment paired with amphetamine. They discovered that many of the genes involved in reward pathways, in the brain development and associated with neurogenic zones of the adult

brain responded inappropriately to amphetamine in the *nad* mutant, indicating that drugs of abuse can change course of the brain natural reward system. In addition, the transcription factors identified in their study can be used to study the link between neurogenesis and addiction (Kily et al. 2008; Cadet, 2009; Webb et al., 2009) coupled microarray analysis with the Conditioned Place Preference (CPP) assay in adult zebrafish to study whether prolonged nicotine/ethanol exposure in zebrafish results in changes in behavior as well as changes in gene expression at the level of the brain. Using zebrafish as a model system, candidate molecules and pathways that underlie neuro-adaptation to both ethanol and nicotine were identified like glutamate receptors, benzodiazepine receptors and molecules associated with synaptic plasticity (Kily et al., 2008).

2.2.1. *Autism Spectrum Disorder* (ASD)

A non neurodegenerative CNS disorder is Autism Spectrum Disorder (ASD). Autism spectrum disorder encompasses a range of psychiatric diseases including autism (the severest form of ASD), Rett's syndrome and Asperger's syndrome and Pervasive Developmental Disorder not otherwise specified (PDD-NOS or atypical autism). The symptoms of ASD are highly variable between patients, making it difficult uncover the genetic and neurobiological changes that underlie these disorders. The ASDs are complex, multisystem disorders that are defined by unifying core abnormalities in the development of language, social and repetitive behaviours. Hundreds of single-gene causes and chromosomal copy-number variations (CNVs) are known to confer risk, but in aggregate account for less than 20% of children with ASD (Kou et al., 2012).

However, the number of people thought to suffer from ASD is increasing over time which might be due to a shift in the diagnostic criteria used (Lord, 2010). High-functioning individuals may communicate with moderate-to-high language skills, although difficulties in social skills may result in communication deficits. Low-functioning individuals may have severe deficiencies in language, resulting in poor communication between the individual and others. Behavioural intervention programs have been developed for ASD and are

frequently adjusted to accommodate specific individual needs. Many of these programs are school-based and aim to support the child in the development of their skills, for use outside the classroom with family and friends. Strides are being made in understanding the factors contributing to the development of ASD, particularly the genetic contributions that may underlie these disorders. More than 80% of children with ASD do not have a monogenic or copy-number variations cause. The majority of children with ASD develops disease as the result of interactions between large sets of genes and environmental factors. Common comorbidities in non-single-gene forms of ASD provide important clues to shared mechanisms of disease. Comorbidities include epilepsy and sleep disturbances (Kohane et al., 2012), gastrointestinal abnormalities (Buie et al., 2010), abnormalities in tryptophan metabolism and platelet hyperserotonemia (Mulder et al., 2004), altered intracellular calcium and mitochondrial dynamics (Palmieri et al., 2010), hypoimmunoglobulinemia, hyperuricosuria (Page & Coleman, 2000), methylation disturbances (James et al., 2008), disturbances in sulfur and glutathione metabolism (James et al., 2008), neuroinflammation (Vargas et al., 2005), cerebellar vermis hypoplasia (Courchesne et al., 1994) and Purkinje cell loss (Bailey et al., 2008).

Analysis of synaptic plasticity together with behavioural and molecular studies have become a popular approach to model autism spectrum disorders in order to gain insight into the pathophysiological mechanisms and to find therapeutic targets. Abnormalities of specific types of synaptic plasticity have been revealed in numerous genetically modified mice that have molecular construct validity to human autism spectrum disorders.

Mutant mouse models provide powerful research tools to investigate the genetic factors associated with ASD and its co-morbid disorders. Transgenic mice are developed to test hypotheses about causes of disease and potential treatments. These models with mutations in candidate genes, that have been identified in people diagnosed with ASD, provide useful tools to address different aspects of the genetic factors which may contribute to expression of ASD in both the human and potentially animal model. Briefly, human candidate mutations of the GABA receptor ß 3 subunit, as well as SHANK3, TSC1, NEUROLIGIN3 and NEUROLIGIN4, PTEN and CNTNAP2, the serotonin transporter short

polymorphism, and tyrosine kinase, which is found in the lymphatic system are introduced into the mouse genome (Yoo et al., 2009).

In the area of social abnormalities, there are ways to measure social approach and reciprocal social interactions in juvenile and adult mice. Impairments in communication may be investigated in mice by tests analyzing responses to social cues or ultrasonic vocalizations, made by mice in the presence of socially-relevant stimuli. Using this model, communication skills of mice with genetic or behavioural phenotypes similar to ASD can be studied to better understand how changes in genes may mediate change in humans behaviours.

Several different mouse strains which express ASD-like behaviours have been used to identify factors that may be involved in ASD. Neuroligin 4 *(nlgn4)* mutations have been detected in some autistic individuals. Nlgn4 is a synaptic cell adhesion protein (Buxbaum, 2009). Synapses are essential to all brain activities, such as perception, behaviour, memory, and thinking. Proper function of the brain's neuronal networks depends on a delicate balance between excitatory and inhibitory electrophysiological signaling among neurons. *Nlgn4 knockout* mice exhibit deficits in social interactions and communication similar to social and communication difficulties observed in humans diagnosed with ASD, such as decreased sociability and decreased communication with others.

Another mouse model that may be relevant for studying the neurobiological aspects of ASD is the phosphatase and tensin homolog (*Pten)* mouse. Five mutations in *Pten gene* sequencing, a lipid phosphatase disrupted in some cancers, have been implicated in patients diagnosed with ASD with a prevalence of 8.3% (Varga et al., 2009) and it is important for mediation of cell size and number, although the link to ASD is currently unclear. *Pten knockout mice* display abnormal social interactions and enhanced responses to sensory stimuli, analogous to those observed in humans with ASD (Kwon et al., 2006). Another mouse model, heterozygotes for the tuberous sclerosis gene *Tsc2*, display deficits in learning and memory (Ehninger et al., 2008). Thus disruption in genes, such as *Pten,* tuberous sclerosis complex *(Tsc1* and *Tsc2)* may be related to ASD, as observed in humans and animal models of ASD. Another proposed model of ASD is the *reelin haploinufficient*

(+/-) reeler mouse (HR), which may have neural deficits similar to those observed in ASD. It has been demonstrated that HR mice have reduced expression of oxytocin receptors, particularly in the cortex and part of the hippocampus (Liu et al., 2005). Disruptions in oxytocin or its receptor activation early in development could alter gene expression or genetic variations. *Reelin* plays a role in the development of the oxytocin system. This model may help to elucidate the role of hormonal factors, such as oxytocin, in the development and/or expression of ASD.

Crawley and collaborators (Crawley et al., 2010) used two lines of transgenic knockout (KO) mice lacking the oxytocin receptor (OTR) or the vasopressin receptor V1a (V1AR), respectively, to investigate the role of these receptors in ASD. In a series of behavioral and pharmacological experiments, baseline nociceptive sensitivity and oxytocin (OT)-induced analgesia were compared between KO and wildtype (WT) littermates. The finding that OT-induced analgesia is normal in OTR KO mice but impaired in V1AR KO mice suggested a V1AR-mediated effect of OT, and this was confirmed by experiments using OTR- and V1AR-selective antagonists. In order to determine the putative site of action of OT, they mapped the autoradiographic distribution of OTRs and V1ARs in mouse spinal cord and determined gene expression levels in dorsal root ganglia (DRGs) using real-time quantitative RT-PCR as well as a single-cell based RT-PCR approach. Naviaux (2013), for example, hypothesizes that all of these clinical comorbidities can result from a single, evolutionarily conserved, metabolic state associated with a cellular danger response (CDR). Since mitochondria are located at the hub of the wheel of metabolism and play a central role in non-infectious cellular stress (Jaeschke et al., 2012), innate immunity, inflammasome activation (Zhou et al., 2011) and the stereotyped antiviral response (Tal & Iwasaki, 2011), the scientists search for a signaling system that is both traceable to mitochondria and critical for innate immunity. Purinergic signaling via extracellular nucleotides like ATP and ADP satisfied these requirements. They test the role of purinergic signaling in the maternal immune activation mouse model of ASD and they show that antipurinergic therapy reverses the abnormalities found in this model.

Easily replicable assays have been developed to quantify social behaviours relevant to ASD in mouse models. One such task is an automated three-chambered apparatus to measure social approach (Nadler et al, 2004; Crawley et al., 2007). In this task, following a habituation period, the mouse is allowed to freely explore the entire apparatus. One compartment contains a novel object and the other compartment contains a novel mouse that the subject has never been in physical or olfactory contact with before. The novel mouse is enclosed in an inverted wire cup that allows visual, olfactory, auditory and some tactile contact with a novel mouse, but ensures that all social approach is initiated only by the subject mouse. Typically, the wild-type mouse start in the middle and quickly approach the novel mouse. There are many visits to each compartment during which, beam-breaks, number of visits to each compartment and time spent in each compartment are recorded. Most normal, inbred strains of mice that have been investigated will spend more time with the novel mouse, than with the novel object (Moy et al., 2004; Nadler et al., 2004). Time spent sniffing the novel mouse is also measured. Strong correlations are observed between time spent in the chamber and time spent sniffing the conspecific mouse. In all probability, this single automated parameter is sufficient to capture true social behaviors.

2.2.2. *Use of zebrafish to study ASD*

In recent years, zebrafish are a useful tool to study the *in vivo* function of genes implicated in autism, since the larvae develop externally and rapidly, large number of larvae can be generated and used for molecular and genetic screens and the larvae are transparent enabling observation of brain development in living embryos at single cell resolution. Zebrafish behavioural assay could be used to study social behaviours affected in autism include so-called “measures of personality” including: social distance, activity in open field under social isolation and distance from predator (Dadda et al., 2010), novelty induced responses, inhibitory avoidance (Blank et al., 2009), social interaction (Delaney et al., 2002), courtship behaviour (Colman et al., 2009), dominant-subordinate relationships (Larson et al., 2006) but also repetitive behavioural patterns with no obvious function and

comorbid conditions in autism including aggression and seizure disorders (Colman et al., 2009). Although it is not possible to study complex behaviours such as language development in fish, a few recent studies have provided information about the function of ASD-linked genes during neural development.

Approximately, one percent of cases of autism are associated with deletions within a single region of chromosome 16, which contains nearly 30 genes (Blaker-Lee et al., 2012; Golzio et al., 2012). Of these 30 genes, at least 25 have clear homologs in zebrafish and using morpholino antisense oligonucleotides, which inhibit maturation of the mRNA corresponding to each gene, it may be possible to identify genes required for the formation of normal brain structure or neurones and asses interaction between genes. An example can be a zebrafish morpholino knockdown experiments, which provided the first insight into the important physiological roles of Sushi domain-containing protein 4 (SUSD4), which is deleted in patiens with autism. SUSD4 is highly expressed in the CNS in different species including human, mouse and zebrafish and it plays an essential role in zebrafish development (Tu et al., 2010).

Golzio and collaborators (2012) analyzed 24 of the genes contained within this CNV and identified one, *KCDT13*, as most causative for the disease. Over- expression of *kcdt13* (by mRNA injection) led to microcephaly, whereas gene-specific morpholino knock-down caused an increase in head size, likely due to alteration of the cell cycle in both cases. This study provides a demonstration of the use of zebrafish to identify disease-causing variants within large genomic regions.

Gauthier et al. (2010) have used morpholinos to knock- down *SHANK3*, a gene linked to schizophrenia and ASD in human patients. S*hank3* morphant zebrafish show decreased swimming after being touched, a phenotype which can be rescued with a wild-type (but not a mutant) version of the corresponding rat gene. Therefore, this study validated alterations to *shank3* as being important in the control of behavior, with potential implications for ASD.

Mutations in *Nlgn3* and *Nlgn4* genes in humans have also been linked to mental retardation and autism and *Nlgn* along with neurexin are involved in synaptic function and

maturation (Boucard et al., 2005). Studies in zebrafish have revealed that seven genes in the zebrafish neuroligin family are very similar to their human homologs, suggesting that these genes are subjected to a very strong evolutionary pressure to preserve their function. Similarly to mouse, *Nlgn* are expressed throughout the nervous system of the zebrafish and also this model system provides an excellent opportunity to further explore the role of neurexins and neuroligins in the development of ASD. However, changes in cerebellar structure and disrupted cerebellar gene expression has also been associated with ASD (Fatemi et al., 2008). The role of the Met (proto-oncogene associated with cellular metastatic cancer) / HGF (hepatocyte growth factor) signalling pathway in cerebellar development has been studied in zebrafish to understand the developmental basis of autism (Elsen et al., 2009). In this study, the authors revealed that in zebrafish Met signalling is critical for cerebellar morphogenesis, including normal growth and cell type specification, and it plays an important role in hindbrain cell migration. For example, altered connections between the frontal lobe (the orbitofrontal cortex) and temporal lobe (including the superior temporal gyrus and temporal polar cortex) may mediate some symptoms of the disease (Geschwind, 2011). Other important brain areas include the cerebellum, brainstem, and limbic areas including the hippocampus, amygdala, septal nuclei, and the anterior cingulate cortex (Lord et al., 2000, Norton, 2013). Finally, autism has also been related to megalencephaly, an overall increase in brain size in some patients.

2.2.3. ***Shoaling and social behaviour in zebrafish***

The characterization of social interactions in both humans and animals is a key approach for studying social behavior. However, the biological mechanisms underlying social behavior in vertebrates are complex and remain poorly understood. Deficits in social interactions represent a common endophenotype of various neurobehavioural disorders, including schizophrenia and autism (Saverino & Gerlai, 2008; Veness et al., 2012). Due to their fully characterized genome, robust behavioral responses and high-throughput nature,

zebrafish have emerged as a complementary model in biomedical research. While primates and rodents have traditionally been utilized to study the genetic and neural underpinning of social interactions (Ribeiro Do Couto et al., 2009), zebrafish can also be used for examining both normal and aberrant social behavior (Miller & Gerlai, 2007). Shoaling, an important evolutionarily conserved behavior, has long been identified in zebrafish representing the interaction of a number of animals moving together in coordinated movements (Buske & Gerlai, 2011). In zebrafish, shoaling is an innate behaviour maintained at a relatively stable and high level throughout the lifespan but it is also modulated by social learning (Engeszer et al., 2004). Shoaling is thought to provide the individual fish with multiple benefits, including access to mates, efficient foraging and defense against predators. It is known that zebrafish are not indifferent to the fish around them. They prefer individuals that exhibit characteristics similar to their own; they show preference towards their own conspecifics and this feature is not unique to zebrafish. Saverino and Gerlai (2008) showed that zebrafish prefer individuals (computer animated zebrafish images) that exhibit characteristics similar to their own and they show preference towards their own conspecifics. The preference manifests as mixing with the stimulus fish and swimming closer to them than to heterospecific fish. According to them, shoaling allows for earlier predator detection through increased vigilance, facilitates coordinated anti-predator behaviour and divides the attention of the predator. It is also known that greater similarity among group members reduces predation by minimizing phenotypic oddity. Thus it is possible that the preference for shoaling with its own conspecifics allows zebrafish to minimize predation. Alternatively, or in addition, forming a shoal with conspecifics can increase reproductive success and may also facilitate foraging. Irrespective of the evolutionary reasons, zebrafish appear to shoal best with its own fellow. The reasons why zebrafish like shoaling with conspecifics than with other fish are for some specific features zebrafish may ignore and some to which they may be sensitive. In a previous study, stripe orientation was found to have an effect on preference in zebrafish (Turnell et al., 2003) but in an another study (Saverino & Gerlai, 2008), the stripe orientation (the presence or absence of stripes) did not seem to matter to zebrafish

test. They spent the same amount of time near the altered and the unaltered images. On the other hand, color has been found to be an important factor affecting choice behavior in fish. In fact, red altered zebrafish image were avoided while yellow colored images elicited a strong preference from zebrafish. The explanation of this choice is probably that zebrafish possess yellow xantophores (Parichy, 2007). Similarly to other fish species, zebrafish are capable of rapid color change, and for example, they show their most vivid coloration during courtship and spawning; conversely, fish that are in fear inducing situations or those that are not healthy show pale coloration, so they are avoided. Another important features that affect the shoaling behaviour is the body shape. While the compressed ("fat" looking) image did not elicit any differential response from zebrafish as compared to the unaltered image, the stretched image ("longer and narrower" fish) induced a robust avoidance reaction. This not preference is probably because in this second case, zebrafish identifies a predatory species (*Xenentodon cancila* or *Nandus nandus)* that has an elongated body shape similar to our stretched zebrafish image.

Extensively used in zebrafish research, shoaling assays have been used to study ontogenesis, effects of environmental stressors (Brierley & Cox, 2010), behavioral organization (Krause et al., 2000), genetic factors (Wright & Krause, 2006) and pharmacological modulation (Buske & Gerlai, 2011).

Conventional shoaling tests have long relied upon manual analysis of easily quantifiable endpoints collected from photographs or video-captured static images. Simple paradigms have included measuring the preference of a single zebrafish placed in a central compartment of a test tank flanked by two adjacent compartments which contain a shoal of conspecifics or are empty (Wright & Krause, 2006). In these conditions, for the first time, it has been measured shoaling develops and if changes with age, in zebrafish appears (Buske & Gerlai, 2011). Newly hatched zebrafish disperse while adult zebrafish have been documented to exhibit robust shoaling, a strong preference for staying close to conspecifics. A previous study investigating the effects of kin exposure on preference for conspecifics in zebrafish revealed an imprinting-like effect of olfactory cues at an early age of the fish (6 days post fertilization-dpf) and suggested that some preference for

conspecifics already exists at this stage of development (Gerlach et al., 2007). In another study (Engeszer et al., 2007), preference for conspecifics was also demonstrated to be based solely upon visual cues (as mentioned before in the study of Saverino and Gerlai) which found measurable preference for conspecifics at post flexion stage (about 12 dpf) of zebrafish. Social behavior (preference for particular conspecific color variant) was also shown to be influenced by early exposure to the given color variant. So, Buske and Gerlai describe, for the first time, that the maturation of social behavior changes in shoaling, in developing zebrafish. Shoal cohesion significantly increases in zebrafish from the first few days of free swimming stage to adulthood.

More recent attempts to assess the internal dynamics of association among fish in free-swimming shoals have focused predominantly on measuring inter- and intra-fish distances over the course of a series of video frames (Pham et al., 2012). While computationally based programs have been developed to quantify several parameters of group cohesion in zebrafish, locations of the animals must still be coded manually (Miller & Gerlai, 2007). Thus, the previous applications of video-tracking technology in shoaling tests have focused on increasing the efficiency and speed of manual coding, and not full-scale automation. In particular, no methodology was available that enables an automatic identification of freely swimming fish location within a shoal from a video source. Pham and colleagues applied the Social Interaction Module of EthoVision XT8.5 software (Noldus Information Technology, Wageningen, Netherlands) for detecting multiple unmarked animals in a social context, able to assess zebrafish shoaling behavior by simultaneously tracking all fish and recording dynamic changes in social behavior between the subjects. Overall, this approach offers a novel, high-throughput method of measuring zebrafish shoaling with its temporal dynamics on par with traditional manual analyses, as well as the ability to track alterations in shoaling behavior evoked by various experimental manipulations.

Similarly, Saverino and Gerlai (2008) in their study suggested that the precision with which the speed of movement, the shape, size, color and pattern of the images one may present to zebrafish will not only allow a systematic and detailed analysis of how zebrafish responds to certain cues in the context of social or other (predatory) encounters, but is

also a prerequisite for high throughput behavioral screening. So, computer aided quantification of behavior, for example measuring the location and swim path characteristics of zebrafish using video-tracking will allow automated quantification of behavioral responses. Given that the experimenter is not required to control the image presentations or to be present throughout the experimental session to quantify behavior; one can run multiple equipments at a time and thus decrease the amount of time required to test a set number of zebrafish.

Recently, Green et al., (2012) have shown a comparison between manual results and softwer generated data. The ability of zebrafish to swim in groups presents some limitations to computer-based shoaling analysis, mainly due to overlapping of objects, if tested in groups of 6, 7, or 8 zebrafish. For example, as fish travel behind each other, the computer can lose track of subjects if too many are present, ultimately resulting in higher percentage of subject loss. However, in their study, this percentage was rather low (<2% subject loss), confirming the optimal detection settings used here. Groups of 5 fish and under proved to be easier to track in SIM softwer (showing no difference in inter-fish distance between groups of 3, 4, 5, 6, 7 and 8 fish) support the notion that smaller groups display similar shoaling tendencies to the larger (8 fish) shoals. Comparing the manual results with the software-generated data, there is high correlation among all groups, especially the 4-fish group, indicating that automated recordings of zebrafish group behavior can be as effective as manual recording, and that 4-fish shoals can be optimal for such studies. Overall, also this recent study presents a novel protocol for analyzing zebrafish social behavior – a paradigm previously limited to time-intensive manual analyses. This method described demonstrates the ability of video-tracking technology to assess zebrafish shoals of various sizes, as well as bi-directional modulation of shoaling behavior by various treatments.

It is also essential to emphasize that the zebrafish has become a widely utilized model organism in pharmacological and toxicological research, particularly due to evidence that they may share with humans and other mammals some key receptors targeted by drugs of abuse (for example acetylcholine nicotinic and dopaminergic receptors (Bencan & Levin,

2008). It is thus feasible to suggest that the behavioral effects and mechanisms of action of drugs such as nicotine and alcohol (ethyl alcohol or ethanol) can be usefully studied using zebrafish. A number of such studies have found that alcohol exposure affects zebrafish locomotion, aggression and stress (Gerlai et al., 2006), startle responses and responses to a predator (Gerlai et al., 2008), all in a dose-dependent manner. Nicotine has also been shown to reduce stress in zebrafish and improve learning at low doses (Levin et al., 2007).

Shoaling behavior is affected by both environmental and internal conditions (such as the presence of predators or the level of hunger). Studies have demonstrated disruption of shoaling by, for example, LSD (Grossman et al., 2010), MK-801 (NMDA receptor antagonist) and SKF38393 (a dopamine receptor agonist) (Maaswinkel et al., 2013). On the contrary, buspirone (a 5-HT receptor antagonist) has been found to increase social preference (Barba-Escobedo & Gould, 2012). For example, several studies of the effects of alcohol on zebrafish shoaling, each using a different measure of social preference (but, interestingly, identical concentrations of alcohol), have concluded that alcohol exposure either reduces shoaling with increasing concentration (Gerlai et al., 2008), has an inverted-U-shaped dose-response effect (decreasing and then increasing shoaling), or has no effect at all on shoaling tendency (Echevarria et al., 2011).

Miller et al. (2013) suggested, with a simple set of defined behavioral measures to characterize zebrafish shoaling, how this shoal behaviour is affected by exposure to various concentrations of nicotine or ethyl-alcohol. Groups of 8 zebrafish each were exposed to either alcohol or nicotine and were subsequently allowed to freely swim in a large circular tank. From the trajectories of the fish, they demonstrated that both low and high concentrations of alcohol and nicotine affected shoaling behaviour, showing anxiolytic effect and reducing motor speed. Intermediate doses produced behaviour similar to control group.

2.2.4. *Anxiety and fear response to stress in zebrafish*

Anxiety disorders and phobias represent a large unmet medical need in the human population (Garner et al., 2009). The response to danger can vary, describing the multiple states: panicked, nervous, anxious, worried, and frightened are different examples. The concept of predatory imminence describes the continuum of responses to danger, ranging from sensing a potential threat, such as when an animal is in an area where there was previous encounter with a predator, to sighting a predator and to imminent attack. In humans, fMRI (Functional Magnetic Resonance Imaging) analysis indicates that the ventral prefrontal cortex and other forebrain regions are activated by distal threats, whereas proximal threats activate midbrain regions such as the periaqueductal gray (Mobbs et al., 2007). An intriguing question is how the brain computes the threat level and thereby activates the appropriate regions to control fear responses. The fact that the response changes when a hiding place is available indicates that sensory information is integrated into the computation.

Another variable that can alter fear responses is the amount of anxiety. Although the terms fear and anxiety are sometimes used interchangeably, there are clear distinctions. Fear is the response to immediate danger and it is transitory. It appears quickly when specific cues are sensed and dissipates when the danger has passed. Anxiety, in contrast, is interpreted as a more sustained state, caused by the anticipation of danger (Davis et al., 1994). Ethologically, this may correspond to the situation when an animal is in an area where a predator had been previously encountered. An anxious animal is likely to freeze to a cue that is innately recognized as dangerous, while a non-anxious animal will flee (Mongeau et al., 2003). The effect of anxiety can also be seen in rodents by examining acoustic startle under different illumination conditions: when transferred from dark to light environment (which increases anxiety), there is an increase in the startle amplitude (Walker & Davis, 1997).

Fear and anxiety utilize different neural substrates. The difference is reflected by the selective effects of drugs on one state but not the other (Blanchard et al., 1993).

Disruption of emotion in humans causes mental disorders, with fear disorders being related with phobias while de-regulated anxiety is linked to post-traumatic stress disorder (PTSD), generalized anxiety disorder (GAD), and also with depression. It is thus important to understand how anxiety is generated and perhaps more importantly, how it is normally reduced. While a person may be aware of feeling fearful or anxious, much of the mental processes generating these emotions lie outside the realm of consciousness. The neural circuits involved are highly conserved in evolution and so fear and anxiety in humans can be investigated by studying the corresponding emotion in animals. From studies, which have mostly been conducted in mammals, it has emerged, as emphasized by LeDoux (2000), that the brain regions involved are not separate from those involved in cognition, as suggested by the limbic theory of emotions. Rather, there is a complex interplay and the term "limbic system," usually taken to refer to as a specialized set of primitive structures responsible solely for emotion, does not reflect the architectural reality of the brain. One of the earliest brain regions identified as being critical for fear in mammals is the amygdala, which consists of several nuclei. Lesions that target the central amydala cause a reduction or loss of behavior associated with fear. Electrical recordings indicate that the central amygdala is activated during fear conditioning. Information enters the amygdala via the lateral nucleus (Clugnet & LeDoux, 1990). When stimulation of pyramidal neurons in the lateral amygdala of rats is paired with a neutral stimulus such as a tone, the animal develops a fear response to the auditory cue, confirming the critical role of this region (Johansen et al., 2010). The central amygdala controls various fear responses, including autonomic and behavioral changes, via its projection to multiple sites. These include the periaquaductal gray, which regulates freezing, the lateral hypothalamus, which mediates autonomic changes such as arterial pressure and the locus coeruleus, which releases norepinephrine and thereby increases the level of arousal. The bed nucleus of the stria terminalis (BNST) is another structure that receives input from the central amygdala. It appears to be unnecessary for phasic fear responses (Hitchcock & Davis, 1991), but is required for anxiety (Davis et al., 2010). This is suggested, among others, by the finding that local infusion of the AMPA antagonist NBQX into the central amygdala fails to block

light enhanced startle; infusion into the BNST, in contrast, blocks this startle (Walker & Davis, 1997). A critical mediator of anxiety in rodents is the corticotropin releasing factor (CRF). CRF antagonists block many effects of stress (Dunn & Berridge, 1990), while infusion of CRF can magnify the amplitude of acoustic startle (Liang et al., 1992) and also trigger anxiety-associated behavior in the elevated plus maze (Sahuque et al., 2006). CRF appears to act via CRF1 receptors in the BNST, being released by fibers from the lateral nucleus of the central amygdala. Another region where CRF acts is the dorsal raphe nucleus in the midbrain. When exposed to a strong, uncontrollable stressor, high concentrations of CRF activate the receptor CRF2, which then sensitize the raphe nucleus such that subsequent aversive stimuli cause the release of high amounts of serotonin; low concentrations, which activate CRF1, do not have this consequence. Uncontrollable stressors thus have a distinct effect; they cause a phenomenon known as "learned helplessness" (Vollmayr & Gass, 2013) or "behavioral depression" (Weiss et al., 1994). This behavior is characterized by poor avoidance and escape; in rodents it also causes ulcers, reduced aggression and exaggerated fear. Although often considered a model for depression, it can be prevented by anxiolytics, consistent with the overlap between the two conditions and a role for anxiety in its genesis.

In contrast to the large work that has been done in mammalian systems, little it is known on fear and anxiety in the zebrafish. Considerable interest has been generated by the possibility of identifying new drugs by screening compound libraries using zebrafish (Kokel & Peterson, 2008; Guo, 2009). Drugs can be delivered to fish simply by addition to the water they swim in, and commonly used anxiolytics such as nicotine, buspirone and diazepam are effective on zebrafish (Bencan et al., 2009), suggesting that the molecular basis of anxiety may be evolutionarily conserved between fish and mammals. Another factor usually cited in support of using zebrafish is the fact that it is a genetic system (Gerlai, 2010). Hence, screens of ENU-mutagenized animals can be carried out to identify genes that predispose an individual to excessive fear responses. Large-scale behavioral screens have been done with mice, where they have led to the discovery of a few mutants affecting fear and anxiety (Cook et al., 2007). A major advantage of the fish system is the

transparency of juvenile stages. With appropriate techniques (Grewe & Helmchen, 2009), such as high speed two photon calcium imaging, it will be possible to map cellular networks involved in fear and anxiety at a far higher resolution than is possible with mammals, especially if the studies are done in intact larvae/juveniles. Additionally, specific subsets of neurons can be manipulated, for example by expression of the light chain of tetanus toxin (TeTXlc), (Asakawa et al., 2008) and the use of GAL4 drivers (Scheer & Campos-Ortega, 1999), with the transparency enabling precise identification of neurons expressing the transgene. On the other hand, several behaviours of zebrafish can be used to investigate fear and anxiety. An example of an immediate threat is the strike of a predator. Larval zebrafish are able to sense the movement of water generated by the predator via the lateral line organ, and respond with an escape movement that is characterized by a "C" bend (McHenry et al., 2009). A fear response can also be reliably elicited by the alarm substance "Schreckstoff" (Jesuthasan & Mathuru, 2008). Schreckstoff is released from the skin of injured conspecifics and it is detected by the olfactory system. Schreckstoff triggers a rapid increase in swimming behavior. Intriguingly, higher concentrations of the substance cause a stronger reaction (Speedie & Gerlai, 2008).

At the behavioral level, fear reactions such as jumping or erratic movements but also increased shoal cohesion with conspecific can be elicited in adult zebrafish by exposing them to alarm substance extracted from the skin of zebrafish or also to synthetic substances such as Hypoxanthine 3-N Oxide (Parra et al., 2009) or to a natural predator or other fish species upon their first exposure to these fish (Bass & Gerlai, 2008). The zebrafish alarm response provides the opportunity to analyze fear at multiple levels from genes to circuits (Jesuthasan & Mathuru, 2008). The combination between this assay with a genetic screen could allow the discovery of mutations leading to enhanced or reduced fear and it could also be used to screen chemicals which alter the fear response. Zebrafish have been observed to exhibit anti-predatory responses including erratic movements, freezing, increase of time on the bottom, leaping, a robust decrease of activity and jumping when exposed to animated images or live predators (for example Indian leaf fish or Astronotus Ocellatus fish) on the basis of visual cues alone and these responses can be quantified by

automated methods (Gerlai, 2010). Both direct and visual contact with predators has been shown to increase whole body cortisol in zebrafish (Barcellos et al., 2013). Cortisol levels, which also increase following exposure to a stressor, can be measured relatively easily with an ELISA-based test for human cortisol (Egan et al., 2009). As in mammals, stress can modify a fear response and increase anxiety in zebrafish (Champagne et al., 2010). Zebrafish have a single glucocorticoid and mineralocorticoid receptor which have been cloned and sequenced and the zebrafish corticoid signaling pathway which is involved in stress is similar to that of mammals (Denver, 2009).

Previously, animated image of a sympatric predator of zebrafish was shown to induce fear responses. Gerlai and Luca (2012) expanded on this gained knowledge and they investigated whether other moving images may induce more robust fear responses. The images investigated included the original sympatric predator, the Indian leaf fish, another sympatric predator, the needle fish, a bird silhouette moved on the side or above the tank, an expanding dot mimicking rapid approach of an object shown on the side and from above the tank, as well as non-fear inducing images including a single and a group of zebrafish. Their results indicated that although the sympatric predators do induce some fear responses, the other images, particularly the expanding dot but also the bird silhouette shown from above are more effective. The results also revealed a stimulus dependent motor pattern response repertoire of zebrafish demonstrating that perhaps univariate quantification methods might not be appropriate for uncovering the complexity of fear or anxiety related phenotypical changes in this species.

Another index of anxiety in zebrafish is the preference for dark over light environments or scototaxis (Maximino et al., 2010). When anxious, fish display a preference for dark surroundings. When forced into light surroundings, these fish will freeze. When given a choice between light and dark compartments, adult zebrafish show a significant preference for the dark and avoidance of the light compartment. (Serra et al., 1999; Maximino et al., 2010). This response is only seen if the tanks are open at the top and only the walls and floor are light/dark. Zebrafish, which show higher avoidance of the light compartment, display the characteristic freezing response if confined to the light side

and this response may be a reliable behavioral measure of anxiety (Blaser et al., 2010). Moreover, Blaser and Peñalosa's (2011) purpose was to determine the effects of acute ethanol exposure on behavior in the light/dark task. In their test, subjects exhibited phototaxis (preference for light) when illumination was manipulated, but scototaxis (preference for dark) when wall and substrate color were manipulated. There was a clear interaction between locomotor activity and color preference, with animals preferentially freezing in darker locations. Secondly, zebrafish were exposed to ethanol (0.25%, 0.5%, or 1.0%) or water for 30 minutes, and then placed in a black/white preference tank containing either ethanol (same doses) or water for a 30-minute test. Ethanol exposure increased locomotor activity and reduced freezing. Additionally, there was a significant interaction between ethanol treatment and locomotor activity on side preference. Low doses of ethanol increased white avoidance in normally swimming fish, while high doses did not. On the other hand, another stimulus that causes anxiety in zebrafish is novelty and this is the basis of the novel tank assay; anxiety is indicated by the presence of the fish at the bottom of the tank (Sackerman et al., 2010). When exposed to a new tank, zebrafish dive to the bottom and do not initially explore the environment. In the wild, this response could help zebrafish escape from predators. The novel tank diving response (Bencan & Levin, 2008) has been developed to characterize the fear/anxiety response in zebrafish (Bencan et al., 2009; Bergner, 2009). Fish spending more time at the bottom of the tank and freezing or swimming slowly or exhibiting increased erratic movements are considered to be fearful or anxious. Pre-treatment with anxiolytic substances like nicotine decreases bottom-dwelling (Levin et al., 2007). Acute exposure of adult zebrafish to substances like ethanol, nicotine, fluoxetine or diazepam results in anxiolytic effects in this assay (Egan et al., 2009), while exposure to alarm substance or caffeine had anxiogenic effects. Chronic ethanol and fluoxetine treatment improve habituation to novelty in zebrafish while anxiogenic agents like pentylenetetrazole and alarm pheromone attenuate habituation (Wong et al., 2010). Thus, zebrafish novel tank diving response can serve as an inexpensive, high-throughput model for screening anxiolytic drugs.

A major goal in using the zebrafish should be to deepen our understanding of how the brain generates and also terminates emotion. This understanding can occur at several levels. At the circuit level is the identification of all cells involved (neural and glia, from the sensory system to the motor output neurons and stress circuitry), as well as characterization of how information is transmitted and stored in the network. Investigations at the molecular level can address the question of how responses can be modified by experience and disease to become maladaptive. Clearly, one step in using the zebrafish is to identify the brain circuits involved, analogous to what has been done for decades in mammals. Homology provides a fast track but can be difficult to utilize given the differences in the anatomy of fish and mammalian brains. A major difference is the eversion of the fish brain during development, in contrast to the evagination of tetrapod brains (Wullimann & Mueller, 2004; Yamamoto et al., 2007). The result is that homologous structures occupy different areas. The notion of homology itself requires some consideration. Because vertebrate brains that exist now are the result of diversification from a common ancestor over millennia, there are many differences between species. A structure can be said to be homologous if there is shared development, as reflected in the pattern of transcription factor expression and if there is similar connectivity and function. Based on these criteria, the teleost amygdala is thought to reside in the medial region of the dorsal pallium (Dm) (Wullimann & Rink, 2002; Northcutt, 2006). Tracing studies indicate that this region receives indirect input from multiple different sensory systems, while efferent neurons project to the hypothalamus and other regions (Northcutt, 2006). Lesioning the Dm in goldfish disrupts acquisition and retention (Portavella et al., 2004) of avoidance but does not affect spatial learning, consistent with this being the amygdala equivalent. The bed-nucleus of the stria terminalis has not been explicitly defined in the zebrafish. It may be a derivative of the eminentia thalamus (Wullimann & Mueller, 2004) and it would be expected to express Dlx-2, Nkx2.1 and Tbr1 (Puelles et al., 2000).

In contrast to the paucity of knowledge on telencephalic structures, there is substantial information on the brain stem structures involved in the response to danger. These include the locus coeruleus (Ma, 1994), raphe nuclei (McLean & Fetcho, 2004;

Lillesaar et al., 2009), and the periaquaductal gray (Agetsuma et al., 2010). The structures responsible for CRF and cortisol increase, the hypothalamic-pituitary-interrenal (HPI) axis (homologous to the mammalian HP-adrenal (HPA) axis) are also well defined (Flik et al., 2006; Alderman & Bernier, 2007). As satisfying as it is to be able to pin down some structures on the basis of homology, thereby confirming the evolutionary conservation of fear circuits, a more important achievement would be to use the zebrafish to discover new facets about the mechanisms of fear and anxiety. Direct imaging of neural activity using calcium indicators, at single cell resolution, across the whole brain, would be one important step. It would be useful not only to probe depolarization but also hyperpolarization, which could be done using fluorescent probes for chloride (Markova et al., 2008), in a fish that is presented with a cue signifying threat. Two zebrafish studies have provided a fresh look at the role of the habenula in fear. The habenula is a part of the epithalamus that regulates a number of brain stem nuclei, including raphe nuclei and the ventral tegmentum (Bianco & Wilson, 2009). It is well conserved in evolution. In mammals, it consists of two major subdivisions, the medial and lateral habenula. These areas receive input primarily from the posterior septum and entopeduncular nucleus respectively; the medial habenula projects to the interpeduncular nucleus (IPN), while the

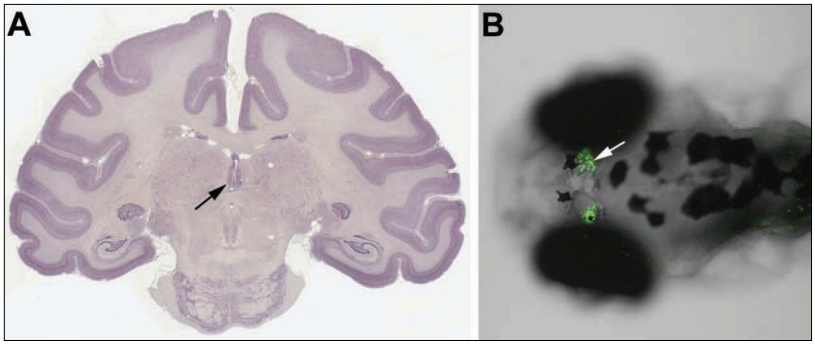


Figure A & B: Comparison of the location of the habenula in a mammalian, an adult *Macaca mulatta*, (A) and *zebrafish* (B) brain.

lateral habenula projects to raphe nuclei and the rostromedial tegmental nucleus, which regulates the dopaminergic system (Jhou et al., 2009). This pattern of connectivity is also seen in zebrafish, although the lateral habenula occupies a ventral location in adults (Amo

et al., 2010). Hence, it has been termed the ventral habenula; the dorsal habenula of the zebrafish is homologous to the mammalian medial habenula.

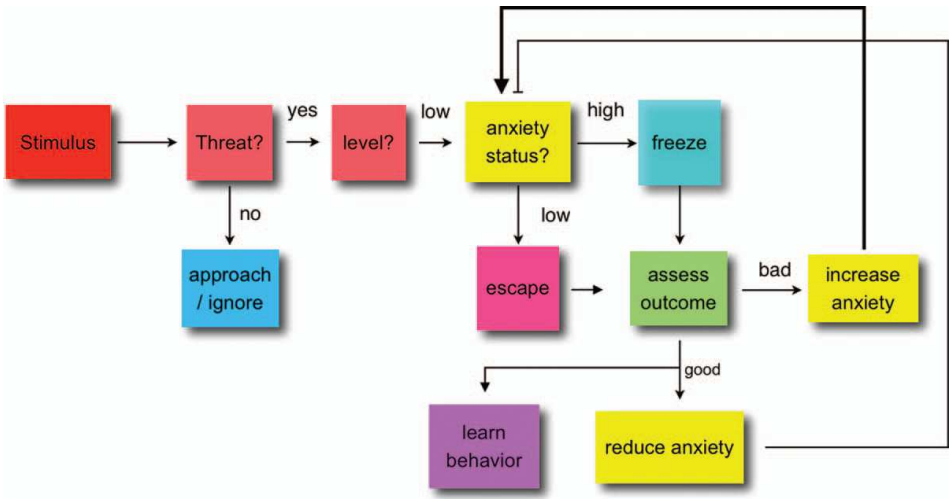
There has been a surge of interest in the lateral habenula recently, a result of work in primates showing that this is activated when an animal receives a punishment or when a reward is withheld (Matsumoto & Hikosaka, 2007, 2009).

In other words, negative reward causes firing of EN (or globus pallidus in primates), which would then activate the lateral habenula (Hong & Hikosaka, 2008). This causes an inhibition of dopaminergic neurons, which can provide a "teaching signal". The function of the medial habenula is more obscure, primarily because of its relatively small size and very deep location in mammals. It has been implicated in many processes, from nociception to addiction and anxiety but how it does so is unclear (Klemm, 2004). In human fMRI studies, the medial habenula would normally not be resolvable. In zebrafish, because of the different mode of brain development, the medial habenula is large and easily accessible. In larval stages, especially, the medial habenula is close to the epithelium and not yet encased by the skull.

Disruption of habenula afferents from septal neurons, by photobleaching of membrane-targeted KillerRed (Lee et al., 2010), or expression of TeTXlc in the dorsal (homologous to medial) habenula (Agetsuma et al., 2010) was found to have a striking effect on fear responses in zebrafish. Instead of swimming to avoid or escape from an aversive cue (shock), both adult and juvenile fish froze, indicative of increased fear. A key observation is that the freezing response only occurred after several conditioning trials, where light was paired with shock. Even if they managed to avoid the shock in early trials, they still developed the tendency to freeze; this is in contrast to control animals, which continue to display avoidance once they have successfully done so. The response of fish with disrupted habenulae is seen only in normal fish that cannot escape a shock (Lee et al., 2010). Hence, in the absence of a functional medial habenula, fish behave as though they cannot register that they have successfully avoided shock. Their behavior is reminiscent of the behavior of helpless dogs in the classic experiments of Seligman and colleagues (1967). The concept of *control* over a stressor has been at the heart of some interpretations of learned

helplessness/behavioral depression. Animals that receive a strong stressor but they are able to terminate it by pressing a lever, do not display helpless behavior. Animals that have received the same amount of the stressor, but they are unable to terminate it, display helpless behavior. Lack of control thus correlates with subsequent helplessness, which is characterized by passive acceptance of a painful shock. The sensitized release of serotonin, mediated by CRF, appears to be a critical component of the circuit that registers lack of control (Amat et al., 1998; Hammack et al., 2002).

Amat and coworkers proposed that there is a circuit that performs the opposite function, which is to compute control over a stressor and mediate the appropriate effects (Amat et al., 2005). This was based on their finding that lesions to the ventral medial pre-frontal cortex (mPFCv) of rats cause helpless behavior even when the animal has control over the outcome. They suggest that the mPFCv detects contingency between a behavior and the outcome. If the outcome is favorable, raphe nuclei are inhibited; in the absence of this regulation, a favorable outcome can never be detected. Zebrafish with the habenula lesions, described above, also behave as though they cannot detect a favorable outcome. It is thus possible that the pathway involving the posterior septum-medial habenula-IPN is a part of the network that detects control over a stressor. Given the connections from the IPN to the raphe nuclei, one target of this pathway may be modulation of serotonin release. This function of the habenula is likely to be evolutionarily conserved. Despite the contradictory results from lesioning the rodent habenula, the cleanest study provides evidence that lesioning the medial, but not the lateral habenula, causes deficits in avoidance. The findings from zebrafish, where lesions can be precisely targeted and visualized, combined with rodent work; this suggest the existence of a feedback system that functions to



modulate fear responses. The major proposal is that the brain contains a circuit for reducing anxiety, which is triggered the ability to control a stressor. This is, effectively, an off-switch for anxiety. The notion that it operates via the medial habenula is consistent with the finding that the anxiolytic nicotine has the largest number of receptors in the medial habenula (Marks et al., 1998).

2.3 *LEARNING AND MEMORY*

Learning involves a change in behavior in response to environmental stimuli, and it depends critically on plasticity within the nervous system. Learning-related changes include modulation of synaptic and non-synaptic (intrinsic) ion channels and receptors, dendritic branching, spine density, and plasticity through genetic and epigenetic mechanisms (Bosch & Hayashi, 2012; Mayford et al., 2012; Zovkic et al., 2013). Much research has focused on understanding exactly how the brain changes as a function of experience. Such experience-dependent plasticity involves both structural and functional alterations that contribute to adaptive behaviors, such as learning and memory, as well as maladaptive behaviors, including anxiety disorders, phobias, and post-traumatic stress disorder. On the other hand, memory is the process by which experience-based information is encoded, stored and finally retrieved. The duration that a memory is stored, (the time between memory acquisition and retrieval), is used to differentiate between short-term and long-term memories (Atkinson & Shiffrin, 1971; Baddeley & Warrington, 1973; Milner, 1972). Short-term memory has a limited capacity (amount of information that can be stored and retrieved) and has a short-term temporal decay, usually defined between several seconds and one minute (Eysenck, 1988). Often, short-term memory is confused with working memory, although the two concepts are generally believed to be distinct. Working memory is a term that was used by Miller (1973) to refer to memory while it is being used to plan and carry out behavior. Specifically, working memory is considered a cognitive process that includes attention, short-term memory and information processing (Baddeley, 1992; Becker & Morris, 1999). Information stored in short-term

memory can be transferred into long-term memory by a process called consolidation (Dudai, 2004; Wang & Morris, 2010). In contrast to short-term memory, long-term memory can store unlimited amounts of information for potentially unlimited duration.

Memories are classified into various subtypes also depending on the type of information stored and cognitive processes underlying its encoding. Cognitive psychologists identify two major classes of long-term memory: declarative (or explicit) and non-declarative (or implicit) memory (Anderson, 1968). Non-declarative memories, as indicated by the name, are unconsciously recalled without awareness of the experiences that have formed them. Non-declarative memories are, for instance, procedural memories required for the execution of integrated procedures involved in both cognitive and motor skills, for example for typing on the computer keyboard or riding a bicycle. In contrast, declarative memory is consciously activated and includes the knowledge or recollection of facts and the meaning of these facts. Declarative memory is further subdivided into semantic and episodic memories (Tulving, 2004). Semantic memories store general factual knowledge independent of personal experience or recollection thereof, (for example knowing that "Romeo and Juliet" is a tragedy written by William Shakespeare). In contrast, episodic memories are recollections of personal experiences, (for example remembering when you went to the theater to see a performance of "Romeo and Juliet"). The repeated encoding of self-experienced episodic memory has been postulated to lead to experience- and context-independent semantic memory (Buzsáki & Moser, 2013). All these forms of memories have been defined based primarily on studies in human patients with brain lesion. These studies are relevant not only for the definition of the different types of memories but also for identifying the neuroanatomical regions involved. These definitions of different types of memory apply to non-human animals and specifically to rats and mice, the most popular animal models used in cognitive neuroscience. Whereas rats and mice are accepted as possessing non-declarative memories, whether these species process and store complex information similar to human semantic and declarative memory is still under debate. In particular, episodic memory is considered to be an exclusively human cognitive function because its definition has been linked to autonoetic consciousness

(Tulving, 2004), a feature that can be demonstrated only in humans (Griffiths et al., 1999). For non-human animals, the definition of episodic-like memory has been proposed. The criterion for episodic-like memory is that a behavioral response should be based on "what" occurred "where" and "when" during a past experience (Clayton & Dickinson, 1998). Although classical paradigms to measure spatial memory cannot be strictly interpreted in terms of episodic-like memory, human and rodent spatial memory are generally accepted to show several similarities to semantic and episodic memory (Buzsáki & Moser, 2013). The major brain structure involved in the encoding, storage and retrieval of declarative memory is the entorhinal cortex–hippocampal system (Hasselmo, 2012). Furthermore, declarative memories are constantly consolidated in the neocortex (Wang & Morris, 2010) and whether the hippocampus is involved in the retrieval of long-term memories that have previously been consolidated in the neocortex remains unclear (Manns et al., 2003). Studies in rats and mice have shown that memory consolidation involves a relatively simple cascade of molecular and cellular events that alter synaptic efficacy and the interaction between the hippocampus and cerebral cortex (Dudai, 2004). Human and animal studies have demonstrated that the same anatomical structures underlying declarative memory are also involved in spatial memory (Stella et al., 2012). Numerous lines of evidence in humans and rodents indicate that non-declarative procedural memory is mediated by the neostriatum and cerebellum (Nagao & Kitazawa, 2008) and, in the case of non-declarative emotional memory, by the amygdala (Adolphs et al., 2005). Thus, declarative and non-declarative memories seem to be processed by the same brain regions in humans, rats and mice.

Other cases of patients with medial temporal lobe damage had intact working memory functions (Cave & Squire, 1992) suggest that brain structures in the medial temporal lobes, including the hippocampus, are involved in long-term memory, whereas working memory is independent of these structures. Functional neuroimaging studies in humans indicate that the prefrontal cortex plays a major role in working memory function (Smith & Jonides, 1998). Recently, the perirhinal cortex and hippocampus have been proposed to be involved in some forms of human working memory related to visual perception (Graham et al.,

2010). Whereas maintenance of visual information occurs in the inferior temporal cortex, the retrieval of goal-directed associative memory depends on top-down signals from the anterior prefrontal cortex (Ranganath et al., 2004). Moreover, spatial working memory tasks activate both the parietal cortex and the prefrontal cortex in humans (Cohen et al., 1997). Thus, working memory has been postulated to involve the interaction between many networks throughout the brain, among which the prefrontal cortex seems to be important for processing information (Gazzaley et al., 2005). Studies in rats indicate that, similarly to humans, working memory and executive functions are controlled by the prefrontal cortex (Kesner & Churchwell, 2011).

2.3.1. *Spatial memory and learning in zebrafish*

Even relatively simple, instances of learning in vertebrates can involve complex interactions of hundreds of molecules, each with distinct spatial and temporal kinetics, as well as neural circuits containing hundreds to thousands of neurons, and thousands to tens of thousands of synapses, which must first be identified and then monitored over time. A proven strategy for reducing this daunting complexity to a manageable level has been to study forms of learning and memory that involve restricted neural circuits. One vertebrate that possesses a nervous system that may be better suited to reductionist analyses of behavior, however, is the zebrafish.

Zebrafish display a rich repertoire of behaviors, including associative learning (Sison & Gerlai, 2010; Valente et al., 2012), social learning (Zala & Määttänen, 2013), and shoaling, the type of group behavior (Engeszer et al., 2007). Zebrafish offer important advantages for relating the molecular events to behavioral response and the the molecular basis of neurobiology: ready accessibility to genetic analysis makes the zebrafish an excellent model system for studies of the molecular bases of neurodevelopment. On the one hand, it is a vertebrate with a sophisticated brain whose basic layout (Tropepe & Silve, 2003) and neurochemical properties (Chatterjee & Gerlai, 2009) are similar to those of higher order vertebrates including mammals.

Standardized validated tests of learning, memory and reinforcement are essential for determining the neural bases for cognitive function. These tests have been developed for mouse, rat and primate species used in traditional experimental models. These tests have also been enormously valuable as tools for determining the neural bases of cognitive function, the sources of cognitive dysfunction and novel treatments for cognitive improvement. Learning in zebrafish is beginning to be studied.

Different laboratory have developed methods to characterize discrimination learning. Williams et al.(2002) have reported that zebrafish would learn to swim to one side of the tank in response to a tapping on that side, which signaled the delivery of food. Generally, all of the fish learned this response within three weeks of training. They tested fish in groups of 5–6. Recognition learning in fish involves primarily the extension to new stimuli of control over the releasing of pre-organized innate responses. Hall and Suboski (1995) showed in zebrafish that visual or olfactory stimuli can elicit escape responses in a learning paradigm.

Recent studies with zebrafish suggested that similarly to another cyprinid, the gold fish (Salas et al., 1996), zebrafish too are capable of performing well in spatial tasks and that their learning performance is dependent upon the NMDA-receptor similarly to what has been found with mammals (Sison & Gerlai, 2011). These zebrafish studies showed that the subjects could locate a reward and show a preference for a particular location in their environment which previously contained the rewarding stimulus. Similar results are often regarded as sufficient evidence for spatial learning ability in the mammalian literature and indeed this is how the gold fish and zebrafish results have been interpreted too. However, the possibility exists that a subject that shows good spatial learning performance identifies the location of the reward not by being able to develop a dynamic spatial map of the external environment but by associating the reinforcer's location with a single cue. In this latter case, performance in the spatial learning task would appear excellent (the subject finds the location well) but this performance would not reflect true relational (spatial) learning but rather simple significant cue associative learning.

Zebrafish are able to associate the sight of conspecifics (reward) with a visual cue. (Sison & Gerlai, 2011) These zebrafish responded to the cue alone during a probe trial by staying in close proximity of the cue. This result confirms that zebrafish have the ability to perform well in associative learning tasks.

It is known (Phillips & LeDoux, 1994) that without the hippocampus, rodents are unable to learn two things at a time and thus can only remember the salient experimentally provided cue but not the spatial information associated with the reinforcer. Fish do not possess a hippocampus, at least not one that has the typical mammalian tri-synaptic circuit, but they do posses a region, the lateral pallium, believed to be the evolutionary precursor of the mammalian hippocampus (Friedrich et al., 2010). It is thought that an intact tri-synaptic circuit is crucial for the hippocampus to perform its function in mammals. Based upon finding no classical hippocampal circuitry in the fish thus it may argue that these simple vertebrates should not be able to learn two pieces of information associated with the reinforcer but these results clearly suggest otherwise. However, conditioning procedures using positive outcomes are also effective in modifying zebrafish behavior. Williams et al., (2002) used a simple spatial alternation paradigm in which fish were fed on alternating sides of a divided fish tank. Ahmad & Richardson (2013) obtained reliable evidence that larval and adult zebrafish could learn which one of a pair of colors (for example blue and orange, blue and green, blue and yellow, yellow and orange) and which one of a pair of line orientations (horizontal and vertical) was paired with food.

Another interesting study belongs to Colwill et al., (2005). In this study they gave a demonstration of visual discrimination learning based on food reward reported. In these experiments, zebrafish were rewarded with brine shrimp for entering a compartment in which the correct stimulus was illuminated on the back wall, but given no food for entering an adjacent compartment, where the incorrect stimulus was displayed on the back wall. They report three experiments demonstrating acquisition of a visual discrimination using a food reward in adult zebrafish. All three experiments used a T-maze in which the arms and goal boxes could be fitted with different colored or patterned

sleeves. Subjects were given a food reward for selecting the correct color (Experiments 1 and 2) or pattern (Experiment 3).

Firstly, they confirmed the findings of other studies that have demonstrated acquisition of a color discrimination, reversal of a color discrimination and acquisition of a line orientation discrimination. Secondly, they extended reports of long-term retention of spatial alternation behavior and active avoidance learning to color discrimination learning. Third, the findings were consistent with other work showing that the suspension of food rewards was an effective extinction procedure. These studies provided clear evidence that zebrafish display rapid and reliable conditioning.

While it might seem peculiar to consider or argue that zebrafish are an excellent model for toxicological studies, there is an increasing number of observations that support this. From the neurological standpoint, zebrafish have been a very popular vertebrate for studying the development of the nervous system. Zebrafish are being developed as sensitive models for studying the neurobehavioral impact of neurotoxicants, as well as pharmacological agents. In the process of developing drugs, chemists synthesize thousands of compounds, hundreds of which pass on to high throughput screening assays. However, the behavioral screening of viable compounds represents a bottleneck in the drug discovery process. A small inexpensive vertebrate model for the rapid assessment of cognitive function could be an asset for the process of drug development. Zebrafish can play a useful role for this commitment.

Considerable effort is being exerted in drug development to produce novel nicotinic agonists, which could effectively treat cognitive impairments of Alzheimer's disease, attention deficit hyperactive disorder, and schizophrenia (Levin & Rezvani, 2002).

Nicotine has a wide variety of pharmacological and toxicological effects, and its reinforcing effects underlie tobacco use addiction (Benowitz, 1996). Nicotine and other nicotinic agonists also have been shown to have potentially beneficial effects, such as cognitive enhancement (Levin & Rezvani, 2000), and they may provide a new avenue of treatment for cognitive disorders such as Alzheimer's disease. Numerous studies in humans have demonstrated profound impairing effects of the muscarinic antagonist scopolamine on

learning, memory and attention in humans (Gilles & Luthringer, 2007). Although the cognitive impairing effects of mecamylamine (a nonselective and noncompetitive nAChR antagonist) is less clear in humans (Voss et al., 2010), blockade of nAChRs has repeatedly been shown to worsen performance in a variety of rodent-based assays of cognitive function (Roegge & Levin, 2006). Moreover, nicotine and selective nicotinic agonists can improve information processing, attention and memory in humans as well as in a variety of animal tests of cognitive performance.

Both nicotinic α7 and α4ß2 nicotinic receptors are important for nicotinic involvement in cognitive function (Bancroft & Levin, 2000). The neuromolecular mechanisms for these behavioral effects of nicotine are still unknown. There are limitations to the use of the rat model to determine the neuromolecular mechanisms of drug actions in behaving animals. Zebrafish offer important advantages for relating the molecular events to behavioral response.

An association between nicotine exposure and improved cognitive function has been reported in many studies. Rodent animal models have demonstrated promnesic effects of nicotine following acute exposure, manifest as improvements in memory and selective attention (Swan & Lessov-Schlaggar, 2007). In humans, enhanced cognitive function following nicotine exposure has been reported in case–control studies with patients suffering from schizophrenia (Jubelt et al., 2008). This has led to the consideration of nicotine or other nAChR agonists as therapeutics for the treatment of patients with schizophrenia (Smith et al., 2007). Diminished cognitive function has been observed in tobacco users experiencing tobacco withdrawal followed by an improvement in cognitive function with tobacco re-administration (Bell et al., 1999). The real or perceived effect of nicotine exposure on cognitive function may be an important factor influencing relapse to tobacco use. Literature suggests that nicotine may have positive effects in several motor and cognitive performance domains (Heishman et al., 2010). However, the relationship between tobacco use and cognitive function remains controversial and poorly understood.

Zebrafish are trainable animals (Williams et al., 2002), enabling assays to be developed to measure memory and learning (Levin & Chen, 2004). Acute nicotine exposure has been

observed to improve discrimination and memory function in zebrafish following a 20- to 40-min latency but not immediately following exposure (Eddins et al., 2009). The observed nicotine-induced cognitive enhancement was reduced by mecamylamine administration immediately prior to the learning assays but not when co-administered with nicotine 40 min prior to the testing. This observation led to the hypothesis that nicotine improves cognitive function through nAChR receptor desensitization and resensitization with heighted response to native acetylcholine (Levin et al., 2006). The increased cognitive function was also correlated with increased concentrations of dihydroxyphenylacetic acid (DOPAC). This dopamine (DA) metabolite, created during DA synaptic reuptake, acts as a DA level surrogate and suggests that potentiated release of DA following nicotine exposure is linked to cognitive improvements (Eddins et al., 2009). The cognitive stimulation of nicotine and its mitigation by pre-administration of mecamylamine was also observed in a study of memory using a place preference assay (de Castro et al., 2009). Many similar studies have also been carried out using mammalian models. The α7 nAChR activation has been linked with improved cognitive function in several species, including rodents (Rushforth et al., 2010) and primates, and it is under evaluation as a potential therapeutic focus for Alzheimer's disease (Bitner et al., 2010). Establishment of conserved cognitive effects of nicotine in zebrafish would facilitate the use of this model to complement studies in mammalian systems, providing a rapid and inexpensive method for advancing understanding in this field.

Nicotine acts on nAChRs, which are pentameric, ligand-gated ion channels existing in three functional states: resting, active, and desensitized (Monod, Wyman, & Changeux, 1965). Human neural nAChR receptors consist of alpha and beta subunits encoded by 11 genes (α2–α7, α9, α10, and ß2–ß4) (Jensen et al., 1990). Heteromeric receptors consist of three alpha subunits and two beta subunits with the most common heteromeric nAChR containing three α4 subunits and two ß2 subunits (α4ß2) (Gotti et al., 2009). Physiological responses to nicotine result from receptor activation and receptor desensitization have been shown to stimulate the release of many brain neurotransmitters (McGehee & Role, 1995).

Literature supports the use of the zebrafish model for exploring nicotine effects on the central nervous system and nAChRs. Transcript expression of α2, α7, and ß3 subunits has been observed in early zebrafish embryos between 2 and 8 hours post-fertilization (hpf) by reverse transcription–polymerase chain reaction (Zirger et al., 2003). The α4 and α6 receptor subunits were also characterized in embryonic zebrafish revealing a dynamic pattern of expression across neural regions in fish 3–96 hpf (Ackerman et al., 2009). Expression of the nAChR ß2 subunit was identified in central nervous system elements of embryonic zebrafish via immunohistochemistry using an anti-ß2 nAChR polyclonal antibody (Welsh et al., 2009). These findings suggest that nAChR's in early zebrafish development have common positional and temporal expression patterns between zebrafish and humans (conservation).

Evidence of nAChR conservation in the zebrafish has also been indirectly demonstrated through known nAChR antagonist studies to determine the reversibility of nicotine effects (Bencan & Levin, 2008; Eddins et al., 2009). Mecamylamine (a nonspecific nAChR receptor antagonist) modulates α4ß2, α3ß4, α3ß2, and α7 receptors (Papke et al., 2001) and it has been observed to reverse a broad range of nicotine-mediated effects in zebrafish, including locomotive sensitization, improved learning, and anxiety reduction (Eddins et al., 2009). Specific nAChR antagonists, including DHß3, MLA, and conotoxin ImI (α7 receptor antagonist) (McIntosh et al., 1999), have also effectively reversed nicotine-induced changes in zebrafish (Bencan & Levin, 2008).

The identification of sequence similarity (homology) between human and zebrafish nAChR genes (or proteins) provides evidence of evolutionarily conserved receptor function complementing the direct and indirect experimental findings previously discussed with respect to nAChRs (Fitch, 1970). Computational comparisons of the human nAChR gene sequences to the zebrafish genome identified candidate conserved genes (orthologs) for the 11 genes encoding human neural nAChRs.

In spite of nicotine and nicotinic agonists have been found by many studies to effectively improve cognitive function in a number of species including humans, monkeys, rats, mice, and zebrafish, other studies have not found nicotine-induced improvement or

have documented negative actions (Dunnett & Martel, 1990*)*. This is possibly due to the inverted U-shaped dose–effect function seen with many drugs in which there is a tendency to improve cognitive function at lower doses and impair performance at higher doses where less specific actions become expressed. Eddins et al., (2009) showed that nicotine caused significant improvement in memory function as measured by a delayed task of spatial alternation. In zebrafish nicotine had an inverted U-shaped dose–response curve with moderate doses improving cognitive function and higher doses having less of a positive effect. Peak improvement was seen at 100 mg/L of nicotine. This was quite similar to the effects of nicotine and other cognitive-enhancing drugs in rodents, monkeys, and humans (Levin & Rezvani, 2002). They have developed a more efficient, rapid cognitive test of simple spatial discrimination that can determine cognitive performance in a single session of seven consecutive trials. This test has been shown to be sensitive to the cognitive-enhancing effects of nicotine and its reversal with the nicotinic antagonist mecamylamine. The data presented in this study replicated earlier findings that nicotine significantly improves choice accuracy of zebrafish as assessed by a three-chamber task of spatial discrimination (Levin et al., 2006). These data also showed that nicotine exposure increased DOPAC levels in the zebrafish brain and that there was a significant correlation between choice accuracy and DOPAC levels. Additionally, the authors found that the nicotinic antagonist mecamylamine, which has previously been shown to prevent nicotine-induced cognitive enhancements, prevented nicotine-evoked rises in DOPAC levels. This work not only confirmed earlier study with regards to nicotine-induced improvements in spatial recognition in zebrafish, but it demonstrated that nicotine-evoked improvements might be related to the modulation of dopamine release or affects on dopamine metabolism.

Finally, these data showed that, as seen in mammals, nicotine exposure induced changes in dopamine systems in the zebrafish brain. Most importantly, the data demonstrated that zebrafish and mammalian brains used similar signaling mechanisms to mediate cognitive behavior. Cholinergic systems and nicotinic receptors were important for cognitive processes and have been implicated in diseases associated with cognitive impairment

(Levin & Rezvani, 2002). This highlights the potential of using zebrafish as a new complimentary behavioral model. The rapid single-session assessment of nicotinic drugs with zebrafish could serve as a useful preliminary screening tool for further development of new promnestic therapeutics. The zebrafish model will be of great use in future studies of the behavioral and neurochemical effects of additional nicotinic and dopaminergic and agonist and antagonist treatments.

2.4 *THE NOR TEST AND VISUAL DISCRIMINATION IN RODENTS*

In animals, investigations of memory in tasks based on spontaneous behaviour is advantageous compared to learned tasks due to closer analogy with memory tests in humans. Over time, the relationship between novelty and behavior has received much attention from researchers. Novelty is an alteration from expected likelihood of an event on the basis of both previous information and internal estimates of conditional probabilities. More important than a definition of novelty is to know that animals can be affected by a novel stimulus. The novel stimuli can change animals' behavior, provoke stress responses, elicit approach behavior, and cause an increase in corticosterone plasma levels, which is a major index of stress and suggests that confinement in a novel environment is stressful (Bevins et al., 2002). Behavioral tests that evaluate the ability of recognizing a previously presented stimulus constitute the core of animals' models of human amnesia (Baxter, 2010).

Ennaceur and Delacour (1988) studied for the first time the novel object and novel location recognition tests (NOR) in animal models. They concluded that these tests are simple behavioral assays of memory that rely primarily on a rodent's innate exploratory behavior in the absence of externally applied rules or reinforcement. The NOR task has become a widely used model for the investigation into memory alterations. However, it can be configured to measure working memory, attention, anxiety, and preference for novelty in rodents. Yet, it has also been used to test the effects of various pharmacological treatments and brain damage.

The way how performance of animals is evaluated in the NOR test may also vary. It can be calculated through different indexes, as discrimination index, index of global habituation, or preference index depending on the aim of each study (Ennaceur & Delacour 1988; Gaskin et al., 2010). It is important to note that the object recognition in animals may be measured by the difference in the exploration time of novel and familiar objects. The recognition measure is influenced by the interval between time spent with novel object and time spent with sample object as well as the time allowed for rats to explore the sample in a first trial. Thus, a wider range of variables can be sensitive to brain lesions and pharmacological treatments (Ennaceur & Delacour 1988).

The NOR task is particularly attractive because it requires no external motivation, reward, or punishment but a little training or habituation is required, and it can be completed in a relatively short time (Silvers et al., 2007). When animals are exposed to a familiar and a novel object, they approach frequently and spend more time exploring the novel than the familiar one (Ennaceur, 2010). However, the environment influences the choice of animal as well. The increased preference produced by object-environment pairings reflects a conditioned association between environmental cues and the appetitive effects of receiving access to novel stimuli (Bevins et al., 2002). Like this, it can note that environmental familiarization interferes with novel object interaction. The preference for a novel object means that presentation of the familiar object exists in animals' memory (Ennaceur, 2010). The recognition of novelty requires more cognitive skills from the subject, relative to tasks measuring exploration of novel environments or a single novel object (Silvers et al., 2007). This concept is the basis of the classical NOR test which has been used in the study of memory functions in rodents. Animal paradigms like the NOR that evaluate recognition memory and object recognition memory in particular have become increasingly useful tools for basic and preclinical research as it allows studying the neural basis of memory.

The NOR task is very useful to study short-term memory, intermediate-term memory, and long-term memory, through manipulation of the retention interval that is the amount of time animals must retain memory of the sample objects presented during the

familiarization phase before to the test phase, when one of the familiar objects is replaced by a novel one (Taglialatela et al., 2009). It is commonly accepted that memory of a single episode would be much more vulnerable than that based on the repetition of some conditions, such as responses to a reinforcer or the association of stimulus (Ennaceur & Delacour 1988).

Moreover, results of the NOR paradigm are influenced by both hippocampal and cortical lesions (Clark et al., 2000; Buckmaster et al., 2004). It is widely accepted that in both monkey and rat brain, the perirhinal cortex plays an important role in object recognition memory (Aggleton et al., 2010), the ability to evaluate a previously encountered item as familiar depending on the integrity of the medial temporal lobe (Hammond et al., 2004). This brain structure plays an important role in recognition memory formation, and when some damage exists, the performance in recognition memory tasks is impaired (Albasser et al., 2009). Studies with primates and rodents have shown that for visual object recognition memory, the parahippocampal regions of the temporal lobe (namely the perirhinal, entorhinal, and inferior temporal cortices) are very important (Hammond et al., 2004).

In summary, the hippocampus and the perirhinal cortex play different roles in object recognition memory. While the perirhinal cortex is involved in object recognition once it is necessary to representing basic information about familiarity or novelty of an object, the hippocampus is involved in object memorization by encoding information about the experience of object. The perirhinal cortex codes object recognition decays fast and is not sufficient for maintaining information about object during longer retention intervals, while the hippocampus, by coding object memory, maintains strong novel object preference after long but not short delays (Hammond et al., 2004).

The NOR task evaluates the rodents' ability to recognize a novel object in the environment. Basically, in the NOR task, there are no positive or negative reinforcers, and this methodology assesses the natural preference for novel objects displayed by rodents. The task procedure consists of three phases: habituation, familiarization, and test phase. In the habituation phase, each animal is allowed freely exploring the open-field arena in the absence of objects. The animal is then removed from the arena and placed in its

holding cage. During the familiarization phase, a single animal is placed in the open-field arena containing two identical sample objects (*A*+*A*), for a few minutes. To prevent coercion to explore the objects, rodents are released against the center of the opposite wall with its back to the objects. The experimental context is not drastically different during the familiarization and the test phase. After a retention interval, during the test phase, the animal is returned to the open-field arena with two objects, one is identical to the sample and the other is novel (*A*+*B*). During both the familiarization and the test phase, objects are located in opposite and symmetrical corners of the arena and location of novel versus familiar object is counterbalanced. Normal rats spend more time exploring the novel object during the first few minutes of the test phase, and when this bias is observed, the animal could remember the sample object. However, if animal repeats brief exposures to the sample object over a period of a few days, it can discriminate the sample from a novel object after delays of several weeks (Mumby et al., 2002). The strongest novel object preference scores tend to occur early in the test phase; while the novel object is still relatively novel, since in the course of time, the novel object became familiar (Broadbent et al., 2010). Despite animals spent more time exploring the novel object, the recognition performance varies according to the delay between the familiarization and the test phase, as well as the time of exploration of the sample during the familiarization phase (Ennaceur & Delacour, 1988). This procedure is the basis of the NOR. However, taking into account the objective of each investigation, some modifications can be made to the original method. An example is the study of Hale and Good (2005), where for a half of mutant mice, the sample object was *A* and the novel object was *B*, while for the other half, the sample object was *B* and the novel object *A*. These modifications were made to reduce object and place preference effects. The objects apparently had no natural significance for mice and had never been associated with reinforcement.

A modification in the number of objects presented in the familiarization and the test phase could be also observed. It is noted in Oliveira et al. (2010) where three distinct objects were presented during the familiarization phase. In the test phase, three objects were also

presented, but one of them had a novel spatial location. Also, in Benice and Raber's study (2008), three objects were used. In the test phase, the location of objects did not change, but one of them was replaced by a novel one. In another study of Hale and Good (2005), the number of objects was different. They used four different objects placed in the center of the four squares of the arena. In the familiarization phase, animals contacted with four objects. In the test phase, two objects were placed in the same position, remaining two objects switched positions. The object position alteration occurred in a diagonal plane. During the familiarization, an object was placed in the top left, while in the test phase, it was placed in the lower right, or vice versa.

Piterkin et al., (2008) who evaluated the role of the hippocampus in the modulation of novel object preference made a modification in the test phase. Animals explored sample objects in one context and, after a retention interval, they returned to either the same context or to a different one, where they encountered sample objects paired with novel objects. However, this different context was also familiar. Only local features proximal to the object changed between sample exposure and test, whereas global features of the context did not change.

Objects that have been used in the NOR test vary widely in shapes, sizes, textures, materials, colors, and appearance. From the familiarization to the test phase, object features change when a novel object that is somehow different from the familiar one is presented. For instance, it can be observed in Nanfaro and collaborators's study (2010), where during the familiarization phase, animal contacted with two pink truncated pyramids (familiar object) while in the test phase with a gray opaque candlestick (novel, unfamiliar object) and a pink truncated pyramid. Thus, novel and familiar objects had different colors, shape, and size which allowed recognizing them as novelty. It is also important to know whether object eliciting abnormally high levels of spontaneous investigation does not influence the outcome of experiments. Thus, Gaskin et al. (2010) preselected novel/sample object pairs on the basis that each object in the pairs elicited the same amount of spontaneous investigation. Many objects have been used in this test. For instance, cans, bottles, tins, glasses, pots, pyramids, candlestick, tower, cylinder, box,

Playmobil toys (man, woman, monkey, horse, and cow), Lego toys, coffee mugs, teacups, socks, PVC pipe, a sheet of newspaper wadded into a ball, Styrofoam dome, tennis ball, bath loofah, shuttlecock, pet toys, and glass vase have been used (Nanfaro et al., 2010). The objects can be made of metal, glass, porcelain, glazed ceramic, rubber, durable nontoxic plastic, aluminum, or wood (Broadbent et al., 2010), materials that cannot be easily gnawed by animals and that can be easily cleaned. However, concerning the weight, the object should be heavy enough that animals cannot move it, as well as height enough to unable animals climbing or resting on it during trials and concerning the object height, this was influenced by kind of object and varied between 4.5 and 24 cm (Gaskin et al., 2010). So objects were carefully selected. Heyser et al. (2012) placed an animal in the open-field arena with four objects belonging to four categories that were differentiated by size and shape. Thus, they defined criteria for different categories such as large (>18 cm tall), small (<12 cm tall), smooth (having a regular, cylindrical shape), and complex (having sharp angles, curves, or extending features). Then, the object categories were as follows: small/smooth objects (small bowl); large/smooth objects (soda can); small/complex objects (teacup); and large/complex objects (coffee mug).

An important prerequisite to discriminate different object, visualizing their characteristics, is to have a good visual capability. The rodents, in particular mice, are frequently adopted as the model system for investigating the normal and diseased eye because they are amenable to genetic manipulations that are not yet possible in other mammals. Despite their widespread use in vision research, relatively little quantitative information is available about the optics of the mouse eye. The mouse's eye is characterized by a large spherical crystalline lens that fills most of the ocular volume and an axial length of about 3.3 mm, 8 times smaller than that of the human eye. Consistent with the mouse's nocturnal habit, its eye has a numerical aperture (NA) of about 0.49 when the pupil is fully dilated, about two times greater than that of the human eye (Remtulla & Hallett, 1985; Schmucker & Schaeffel, 2004). In mice, the cones constitute a smaller portion of photoreceptor unlike diurnal mammals. Moreover, the retina in the

mouse haven't the fovea, a small region at the center of the retina with high density of cones but not rods (Carter-Dawson & La Vail, 1979).

The mice then are not usually considered visual animal models, because they are nocturnal and mainly they use other sensorial abilities such as smell, hearing and touch to explore the environment. Although conditions in the laboratory are not identical to those of the natural habitat, it is noticed that there are no differences in visual acuity between the laboratory mouse compared to the wild. For this reason, the mouse can be useful model to study the properties of visual system (Shupe et al., 2006). Some strains such as BALB (albino), suffer from retinal degeneration induced by light, while other strains such as C57BL/6J mice no (Lavail et al., 1987).

The ability of mice to discriminate visual stimuli has been established with specific behavioral tests, primarily conducted by Hall (1969) in which mice were trained to discriminate between black or white card, receiving a reward (correct choice) or a mild shock (wrong choice). Over time, Wimer and Weller (1965), in the wake of previous works, studied the capability of mice to discriminate color cues swimming in a water T-maze. Mice were trained to exit from or the white either the black arm and the correct comin-out from the arms was considereted a good index of learning. Mice, showing retina's degeneration, made bad performance during the test.

More recently, Prusky and collaborators (2000) developed the "visual water box", a test in which by use a trapezoidal pool, connected with two screens located in the bottom of the tank, could quantify the visual ability in rodents. The behavioural test showed that control mice had a visual acuity between 0.5-0.6 c/deg while albino mice had a lower visual acuity (0.17-0.38 c/deg). These data are different if compared to rat (0.92 c/deg), cat (6-7 c/deg) and human (60 c/deg) visual acuity (Prusky et al.,2000).

In recent years, is growing more and more the interest of shapes visualized on touchscreens in the object recognition' test, for the study of visual discrimination ability; this important modification in NOR is to approach animal model to human studies.

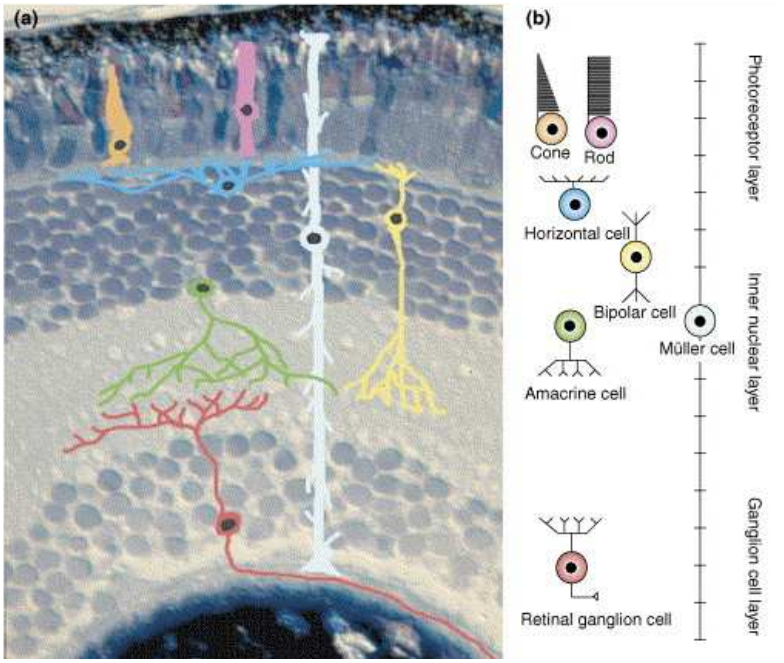
2.4.1. *Zebrafish and vision ability*

As reported before, the zebrafish has rapidly become a favored model vertebrate organism, well suited for studies of developmental processes using large-scale genetic screens. In particular in zebrafish, morphological and behavioral genetic screens have led to the identification of genes important for development of the retinal photoreceptors. This may help clarifying the genetic mechanisms underlying human photoreceptor development and dysfunction in retinal diseases. Genetic screens, using morphological and behavioral criteria, have uncovered numerous mutations, affecting development of the visual system, especially the rod and cone photoreceptors (Neuhauss et al., 1999).

The identification of genes important for rod photoreceptor cell development could increase the knowledge of the mechanisms of rod photoreceptor cell dysfunction and degeneration in human diseases such as retinitis pigmentosa (RP). Retinitis pigmentosa is the most common form of human inherited photoreceptor degeneration and it is an important cause of visual impairment and blindness (Pacione et al., 2003). Patients with RP initially experience night blindness and a loss of peripheral vision in adolescence or early adulthood, due to degeneration of the rod photoreceptors. Degeneration of the cone photoreceptors follows, and patients may become completely blind by 30–60 years of age. Some of the mutations are in genes of obvious importance, such as those involved in the rod phototransduction pathway and in retinoid metabolism. But there are also several genes of unknown function, and genes, such as those that code for pre-mRNA splicing factors, whose role in causing RP is not clear. Furthermore, it is not well understood how mutations in genes expressed solely in rods lead to the death of cones as well. Many of the genes causing RP, perhaps as much as 50% of all cases, remain to be discovered (Rivolta et al., 2002). Because the anatomical organization and development of the retina, including the photoreceptors, are remarkably conserved in virtually all vertebrate species, zebrafish are a good model in which to study photoreceptor development and disease.

Zebrafish retina structure begins as an evagination from the anterior neural keel (a solid rod of cells analogous to the mammalian neural tube) at around 11 hours post-

fertilization (hpf). By 60 hpf, the retina has become fully laminated, with three distinct nuclear layers separated by two plexiform layers. Most retinal cell types can be distinguished by morphological or cytochemical criteria by 72–96 hpf (Schmitt & Dowling, 1999). Zebrafish possess one type of rod photoreceptor and four distinct classes of cones sensitive to UV, blue, red, and green light. As in most teleosts, each cone type is distinguishable by its morphology and spectral sensitivity (Robinson et al., 1993); the short single cones are UV sensitive, the long single cones are sensitive to blue light, and the red and green sensitive cones form a pair in a double-cone structure (with the red cone being the principal or longer member of the pair). Within the photoreceptor cell layer, the rod nuclei are located closer to the inner retina to the cone nuclei, but the rod outer segments project beyond the outer segments of the cones and interdigitate with the retinal pigmented epithelium (RPE). In the photoreceptor cell layer the cones are arranged in a crystalline mosaic pattern. The mosaic consists of alternating rows of double cones and single cones, arranged such that the green cones in a double cone pair always flank an UV cone, and the red cones are always next to the blue cones in the adjacent row. It was formerly thought that the rods were inserted into the cone mosaic with a random distribution, but recent work has shown that rod photoreceptors form a regular mosaic as well, positioning themselves in a square surrounding each UV cone. This mosaic arrangement is apparent by about 10 dpf (Fadool, 2003). Development of photoreceptors, as for the other classes of neurons in the retina, has been shown to be regulated by both intrinsic factors and by environmental cues. One interesting aspect of photoreceptor cell development is that the rods follow a



developmental program that is distinct from the cones. Infact rhodopsin expression precedes cone opsin expression, but in the dorsal and central retina rod differentiation occurs subsequent to the generation of cones (Schmitt et al., 1999).

Zebrafish visual responses can be evaluated behaviorally and measured physiologically by electroretinogram (ERG). The ERG records the summed field potential in the retina in response to light. The physiological and behavioral data suggest that rod photoreceptors do not become functional until almost 2 weeks after the first rods are detectable by immunolabeling and in situ hybridization. It take long for the rods to become functionally mature, whereas cone function is detectable 1–2 days after cones first appear (Morris & Fadool, 2005).

Zebrafish are highly visual animals, and must be able to search for food and avoid predators soon after hatching; so different visual behaviors exist which can be measured in adult zebrafish, for example the optomotor response (OMR). In this test, zebrafish is placed inside a rotating circular chamber containing an alternating pattern of light and dark stripes on the interior. The zebrafish will respond to movement of the stripes swimming to follow moving visual stimuli. Fine resolution of the response can be achieved by altering the intensity or wavelength of illumination, or the velocity of drum rotation. The same test can evaluated the larvae OMR but in this case the movement of the stripes is compared with the larvae eye movements.

One advantage of using the OMR to evaluate visual function is that, by using a computer monitor displaying the moving stripes beneath a rectangular chamber, entire clutches of zebrafish can be tested at the same time. Fish with normal vision will follow the moving stripes and accumulate at one end of the chamber; 90% will respond to the stimulus within 60 s (Morris & Fadool, 2005). Moreover, adult zebrafish can be screened for visual system deficits by measuring the escape response, which is elicited when the fish encounter what they perceive as a threatening object. The fish is placed in a round transparent chamber with a pole in the middle. In response to appearance of a black segment on a rotating drum surrounding the chamber, the fish will hide behind the pole. This assay has been used to test the time course of dark adaptation in adult fish, and to

isolate zebrafish mutants with dominantly inherited retinal degenerations (Li & Dowling, 1997).

Among different visual abilities, the zebrafish can distinguish the colours (Spence & Smith, 2008). It is known that zebrafish have colour vision with peak absorbance in ultraviolet (362 nm), violet (415 nm), blue (480 nm), and yellow (570 nm) which is comparable with that of humans (peak absorbance blue =420 nm, green =536 nm and yellow =564 nm). However, it is not known if the colours that zebrafish can discriminate elicit spontaneous approach or avoidance behaviours.

Avdesh and collaborators (2012) determined the natural colour preference in zebrafish by use gravel and plastic sleeves of four different colours (red, yellow, green, and blue). Zebrafish were tested in two apparatus: the first is the *place preference* where fish, after an habituation, had to make a preference choice between two chambers characterized by two different colours, in six different colours combination. The second is the *T-maze test*, where zebrafish, after an habituation exploring the maze, could decide to enter in one of the coloured short arms, following its personal colour preference. All data were then analysed by a softwer for measuring the reflectance spectrum of different colour. Their results showed that zebrafish had an aversion to the colour blue and the colour red with green were the most preferred; yellow was average tolerated. This information is useful in choosing colours for colour-based learning and memory paradigms. Reds and greens were equally preferred over other colours, and they were good choices for appetitive experiments. Red versus green would be a good choice for discriminative stimuli where there is no natural preference for one over the other. Blue were more aversive than the other colours, and it might be useful for validating experiments involving aversion, anxiety, or fear.

The interest for adult zebrafish and the studies in the visual research are growing more and more, thanks to its excellent visual system with a cone-dominated retina. In the past, measurements of visual function have been performed only in the larval zebrafish. A standardized and reliable method for visual acuity measurements is a prerequisite to analyze genetically modified fish lines and to evaluate the effect of a therapeutic action to

adult zebrafish. Tappeiner and collaborators (2012) developped a new optokinetic reflex (OKR) assessment with a modified and standardized system, indicating the values cycle/degree (c/d), eye movement at varying angular velocities. They determined a mean visual acuity of 0.59 c/d in adult zebrafish and a mean visual acuity of 0.16 c/d in larval zebrafish. The difference in this parameter in mainly explained by the different eye size: due to geometrical reasons a larger eye leads to a larger retinal image with better visual acuity as photoreceptor spacing is comparable between the two development stages. It is clearly known that zebrafish have an optimal visual acuity correlating with a wide eye rotation (Tappeiner et al., 2012) because they are able to discriminate different colours (Avdesh et al., 2012), they can visualize a predator, shunning the danger (Gerlai & Luca, 2012) or they recognize mates for food, coupling or protection (Saverino & Gerlai, 2008). They are able to distinguish from simple cues to different stimuli, sometimes associating them with a reward (Colwill et al., 2005). The development and standardization of new test can extend the studies about this important animal model that is uniquely positioned to bridge the gap between zebrafish and human studies across different research field.

Aim

3. *AIM*

During the last decade, zebrafish, a small teleost fish belonging to *Cyprinidae*, has been used in neurobehavioural toxicology (Bretaud et al., 2004) as a model for human behavioural disorders and drug screening of potential therapeutic application (Levin et al., 2002; Gerlai, 2010). It is a rapidly emerging animal model alternative to classical rodents. Zebrafish is a shoaling fish forming multimember groups and its typical aggregative behaviour represents an excellent model to study social behaviour. There is evidence that oxytocin (OT) and arginine vasopressin (AVP) are two neurohypophyseal hormones involved in the regulation of social behaviour in mammals while isotocin (ISO) and vasotocin (AVT) are the equivalent hormones in fish (Goodson & Bass, 2000). Since no data are available about the effect of these neuropeptides on zebrafish, first aim of this project was to investigate the effect of both OT and AVP on shoaling behaviour in comparison with ISO and AVT. Since these peptides are known to affect anxiety in humans and rodents (Young et al., 2009), the same compounds were also tested on fear response to predator. Then, to investigate the mechanism of action, we established to inject different antagonists before each peptide: the OT receptor antagonist desGly, the most potent selective OT compound reported to be 95 times more potent as an OT than AVP receptor antagonist in the rat (Manning et al., 1995), the V1a receptor subtype AVP antagonist SR 49059 and the V1b receptor subtype antagonist SSR 149415 (Serradeil-Le Gal et al., 2007).

Cognition is highly complex CNS function that includes many components (as memory) that may be compromised by a disease state. New therapies for improving cognitive functions are necessary yet. There is evidence that zebrafish is a good alternative model to study learning and memory (Avdesh et al., 2012). Thus, the second aim of this project was to investigate the effect of nicotine on spatial memory using T-maze task, a simple, rapid and inexpensive assay since nicotinic receptor has been characterized in this teleost fish (Henley et al., 1988). Recently, eight nAChR subunit cDNA (α2, α3, α4, α6, α7, ß2, ß3 and ß4) have been cloned in zebrafish (Zirger et al., 2003; Ackerman et al., 2009) showing an high degree of sequence identity and similarity when compared with rats and human

orthologs (Papke et al., 2012). Thus, in order to better characterize the nAChR subtypes involved in nicotine-induced cognitive facilitation, we decided to use selective (MLA, MII and DHβe) and non selective (scopolamine and mecamylamine) antagonists given alone and in combination with nicotine.

To exclude that treatments with different neurohypophyseal hormones and cholinergic compounds could influence general activity, the swimming behaviour was also tested.

An other important cognitive component of CNS is the selective attention. It can be tested in rodents with NOR test (Pan et al., 2008), but the selection of objects to be used is critical. Thus to overcome this limitation, the third objective of this project was to create a modified version of NOR, called virtual object recognition test (VORT), replacing real objects with 2D white geometrical shapes (circle, square or triangle). VORT was validated firstly on mice, comparing the results with NOR and then on zebrafish. Since no data are available about the object recognition test on zebrafish, both mice and zebrafish were tested in VORT, to identify 2D shapes that could be highly discriminated and which could not. Secondly, to evaluate the possibility that application of motion to 2D shapes could increase attention, specific movements (vertical, horizontal or oblique) were applied to discriminated and not discriminated shapes in mice and zebrafish. To validate VORT, the effect of nicotine and two non-selective nAChR antagonists scopolamine and mecamylamine were also tested.

Materials and Methods

4. MATERIALS AND METHODS

4.1. *Zebrafish model*

Adult AB wild type (WT) zebrafish (*Danio rerio*) (50 % male and 50% female) were purchased from a local pet shop (Aquarium Center, Milan, Italy). Nacre mutant zebrafish were a kind gift of Dr Mione (IFOM-IEO, Milan, Italy). Both strains were kept at approximately 28.5 °C on a 14 h light /10h dark cycle. All fish were raised with their wild-type siblings in visual isolation from other phenotypes. Behavioural testing took place during the light phase between 9.00 and 14.00 hours. Tank water consisted of deionized H_2O and sea salts (0.6g/10l of water; Instant Ocean, Aquarium System, Sarrebourg, France). The home tanks with groups of approximately 30 adult fish were maintained with constant filtration and aeration. Fish were fed daily with brine shrimp and flake fish food (Tropical fish food, Consorzio G5, Italy). All the fish were drug naïve and each fish was used only once. 10 fish per group were used. The experimental protocol was approved by the Italian Governmental Decree No. 28/10. All efforts were made to minimize the number of animals used and their discomfort.

Behavioural assays

Social preference test

Social preference test was carried out according to Engeszer et al. (2007). *Nacre* mutants and WT counterparts were used as stimulus fish shoal. All stimulus shoals comprised two males and two females randomly chosen from each group tank. Shoals of as few as four zebrafish exhibit shoaling behaviours indistinguishable from those of larger groups (Breder, 1946). A glass test tank (122 cm long x 55 cm tall x 32 cm wide) divided into five equal compartments, was used. Outermost compartments, hereafter referred to as stimulus areas, were separated from inner compartments by glass walls, which were sealed with silicon aquarium sealant to isolate water in the stimulus areas from the inner compartment. We further subdivided the inner compartment, marking off three zones of

equal volume comprising a left preference area, a central no-preference area, and a right preference area. The tank was lit by two 250-Whalogen lamps placed above and on either side of the test tank. Light from these lamps then reflected off two sheets of Teflon hung at a 45° angle from the top of the tank. Thin sheets of opaque plastic, as temporary visual barriers to separate the exterior compartments from the interior compartment, were used. The water level in the tank was kept at 25 cm depth. Opaque barriers were placed in the central compartment, to visually isolated the subject fish from the stimuli areas, one containing the stimulus (*nacre*) shoal, the other the WT shoal. Initially, the subject fish was placed in the central compartment. The fish were allowed 5 min to acclimate to the test tank and then the opaque barriers were removed. Fish were allowed up to 15 min to recognize both stimuli. We started recording time spent in association with each stimulus after the first 5 minutes- period in which the subject recognized both stimuli. If the subject did not recognize both stimuli in 15 minutes, the zebrafish was excluded. When the fish subject swam parallel to one of the shoal members, it was considered to recognize the stimulus shoal (Engeszer et al., 2004). Shoaling preference was quantified by recording the total time spent by the WT test fish in proximity of each stimulus shoal.

Fear response to predator test

Fear response to predator test was conducted according to Bass and Gerlai (2008), introducing slight modifications. The tank used for this test is the same of the shoaling preference test. The predator used is a couple of *Astronotus ocellatus fish*, located in the left compartment while the other compartment was empty. This predator stimulus was chosen for its innate aggressiveness (Gonçalves-de-Freitas & Mariguela, 2006) and for its predatory behaviour toward zebrafish, previously studied in our laboratory. Initially, fish subject was placed in the central compartment to visually isolated the subject fish from the stimuli areas. The fish were allowed 10 minutes to acclimate to the test tank and then the opaque barriers were removed. During 10 minutes of the test, fear response to predator were quantified by recording the total time spent by WT zebrafish in proximity of

predator or to empty compartment. At the same time, the zebrafish behaviour was observed, mainly recording four activity: *thrashing* (swimming parallel to the stimulus), *freezing* (fish immobility), *erratic movement* (rapid movement) and *jumping*. As control, a group of ten WT zebrafish was observed without any stimulus, so the tank was completely empty.

T maze test

A transparent Plexiglas T-maze (filled with tank water at a level of 10 cm) was used according to Yu et al. (2006) with slight modifications. The apparatus included a starting zone (30 cm x 10 cm) separated from the rest of the maze by a transparent removable door. Behind the partition, there was a long (50 cm x 10 cm) arm and two short (20 cm x 10 cm) arms, which led to the removable deep water chambers (30 cm x 30 cm). One of two chambers, used as reservoir, contained artificial grass, shells, stones and coloured marbles that offered a favourable habitat for the fish. To prevent viewing of the two chambers, two removable opaque partitions (4.5 cm x 30 cm) were put, in a staggered way, at the beginning of each short arm. To minimize procedural novelty stress, the fish first underwent 2 habituation trials of 1 h every day for three days, which also served to reduce handling stress. During these trials, the fish (in groups of 16) were allowed to freely explore the entire maze. To minimize acute social isolation stress, zebrafish groups were only gradually reduced in size during the experiment according to Sison and Gerlai (2010) starting with 16 fish per group on day 1 to 8 fish per group on day 2, 4 fish per group on day 3, and individual fish testing starting from day 4. Each subject received two training trials of exposure in the T-maze, at an interval of 24 hours. During each trial each fish was placed in the start box for 5 minutes with its door closed.

Then, the start box door was raised and then lowered after the fish had exited. Ten minutes were allowed to reach the reservoir or the other chamber. The location of the reservoir was chosen randomly in each trial. The running time taken to reach the reservoir and stay for at least 20 seconds was recorded by an

experimenter blind of pharmacological treatments. After 20 seconds, the fish returned to their home tank. The fish were then given a second session 24 hours later. Finally, it was calculated the difference between the running time taken to reach the reservoir in the first trial and after 24 hours, (indicated as Δ).

Virtual object recognition test (VORT)

A transparent Plexiglas tank (filled with tank water at a level of 10 cm) was used in VORT. The apparatus was characterized by a single environment (71.5 cm x 31 cm x 11.5 cm), divided by external markings to delimits a left and a right preference areas. Opaque barriers were placed in the central compartment, to visually isolate the subject fish from the stimuli areas, exposing two identical white geometrical shapes on a black background were shown on two 3.5-inch widescreen displays (iPod screens). The shapes moved in equal distances horizontally, vertically or diagonally (distance 320 px) at a constant speed of 120 px/s. The videos were created in Adobe Flash, with a frame size of 320 px ×480 px, frame rate of 30 fps and encoded with the apple native H.264 video codec. The stimuli were looped on a 3rd generation iPod Touch through iTunes for the duration of the experiment (320 px horizontal axis and 480 px vertical axis). The luminosity of the screens was constant across the two screens and testing sessions. The fish were first habituated to the test apparatus in the central closer no-preference area, for 5 minutes. Then, they were subjected to a familiarization trial (T_1-visualing two identical shapes) and finally, after a delay, to a novel shape recognition trial (T_2-visualing familiar shapes vs novel shape). Both T_1 and T_2 consisted of a 10 minutes session. An experimenter blind to the treatment group manually recorded the exploration times to the shapes in each fish. Shape recognition was scored when each zebrafish was in proximity of the iPod screens. Animals were used more than once but specific shapes were never repeated across the experiments. For static cue shapes were simple geometrical shapes (square, triangle, circle, cross, etc.) with equal surface areas (2.5 cm^2). A complete list is given in figure C. For dynamic cue, the same above shapes could move in 8 directions separated by 45°/90°.

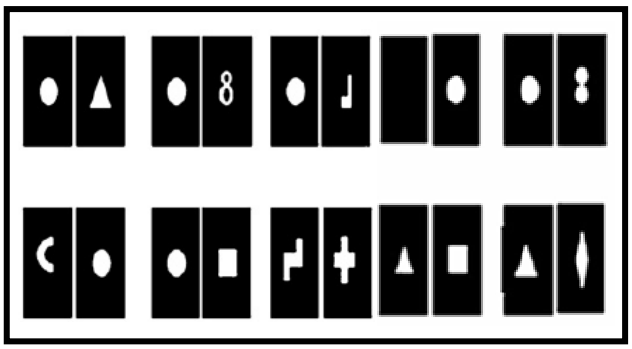


Figure C: Examples of geometric shapes used for the experiment.

Swimming activity test

After treatment, each zebrafish was placed in a transparent observation chamber (20 cm long × 10 cm wide × 15 cm tall) containing home tank water filled at a level of 12 cm. The floor of the chamber was divided into ten equal-sized 2 × 10 cm rectangles. Swimming activity was monitored by counting the number of crossed lines in a 30 seconds observation period every 5 minutes, for a total of six observation bins over 30 minutes (Swain et al., 2004).

Drug and treatment

Body weight was measured as previously described (Braida et al., 2007). Fish were removed from their tank using a net and placed in a container, containing tank water, positioned on a digital balance. Zebrafish weight was determined as the weight of the container plus the fish minus the weight of the container before the fish was added. The mean of three measurement was recorded. Fish were injected intramuscularly (i.m.) in the caudal musculature in the social preference, fear response to predator and swimming activity test. For T-maze, visual object recognition and swimming activity test, zebrafish were injected intraperitoneally (i.p.). Each volume, depending on the fish's weight (2 µl/g), was given using a Hamilton syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland). For i.p. injection,

fish were anaesthetized with ice as previously described (Kinkel et al., 2010) and placed in a supine position. The injection was made in the abdominal cavity. No more than the tip of the needle was inserted into the abdomen of each fish, as a means of preventing damage of internal organs. After injection, each fish was immediately transferred back to its warm water (about 28°C) tank for recovery. All drugs were dissolved in saline solution (0.9%) and were prepared fresh daily.

The drugs for social preference and fear response to predator tests were:

- **Oxytocin**, **vasopressin** ((Arg^{8})-Vasopressin) (AVP), **isotocin** ((Ser^{4},Ile^{8})-Oxytocin) (IT), and **vasotocin** ((Arg^{8})-Vasotocin) (AVT) (Bachem, Germany) dissolved in saline (0.9%) and injected i.m. in a range of doses between 0.001 and 40 ng/kg.

- DesGly-NH_2-d$(CH_2)_5$-[d-Tyr^{2},Thr^{4}]OVT) (**desglyDTyrOVT**) (kindly supplied by M. Manning, Toledo, USA); ((2S)-1-[[(2R,3S)-5-Chloro-3(2-chlorophenyl)-1-[(3,4-dimethoxyphenyl) sulfonyl]-2,3-dihydro-3-hydroxy-1H-indol-2-l]carbonyl]-2-pyrrolidine carboxamide) (**SR 49059**) (Sigma-Aldrich, St Louis, USA); (2S,4R)-1-[5-chloro-1-(2,4-dimethoxyphenyl) sulfonyl]-3-(2-methoxy phenyl)-2-oxo-2,3-dihydro-1 indol-3-yl]-4-hydroxy-N, N-dimethyl-2-pyrrolidine carboxamide (**SSR-149415**) (kindly provided by C. Serradeil-Le Gal, Sanofi-Aventis, France) were used as antagonists and injected i.m. 10 min before each agonist.

A wide range of doses (0.00001–20 ng/kg) for each antagonist was used. The solutions were stored at −20°C at a final concentration of 10^{-3} M.

The drugs used for memory tests were: **nicotine hydrogen tartrate salt** (Sigma–Aldrich, St. Louis, MO, USA). (0.02 mg/kg) given i.p, 20 min before the test; **scopolamine hydrobromide** (Sigma–Aldrich, St. Louis, MO, USA) (0.025 mg/kg) given i.p, 20 min before the test; **mecamylamine hydrochloride** (Sigma–Aldrich, St. Louis, MO, USA) (0.1 mg/kg) given i.p, 30 min before the test. Control groups received saline solution (0.9%) given im or ip.

Statistical analysis

All data were expressed as mean ± SEM. Pair-wise comparisons were assessed with Student's *t*-test. Different groups were assessed by one-way analysis of variance (ANOVA) for multiple comparisons followed by Tukey's post hoc test. Data obtained for the social preference and fear response to predator test were analyzed by linear or parabolic regression lines and ED50 values (with 95% confidence intervals) were calculated using the least-squares method of linear regression on the linear portion of the curves. For VORT data were expressed as discrimination index [(time spent exploring novel shape − time exploring familiar shape)/(time spent exploring novel shape + time exploring familiar shape)], as previously described in Pitsikas et al. (2001). The level of significance was taken as $P<0.05$. All statistical analyses were done using software Prism, version 6 (GraphPad, San Diego, CA, USA).

4.2. Mouse model

Male Swiss mice (Charles River, Calco, Como) 5–6 month old weighing 30 g (±3) were housed individually in polycarbonate cages with food and water freely available through wire lids. Cob-bedding was changed weekly, and the *vivarium* was 21 C with a 12 hours light cycle (lights on at 08:00). All the experimental procedures followed the guidelines established by the Italian Council on Animal Care and were approved by the Italian Government decree No. 28/2010. All efforts were made to minimize the number of subjects used and their suffering.

Behavioural Assay

Virtual object recognition (VORT) and Novel object recognition (NOR) test

Object recognition and *Virtual object recognition test* were conducted over a two-day period in an open plastic arena (38 cm × 30 cm × 18 cm). The apparatus was illuminated

by a fluorescent lamp placed centrally above it (75 W). The testing procedure was identical in both test.

The animals were first habituated to the test apparatus for 10 minutes on day 1 and then subjected to a familiarization trial (T_1) and a novel object recognition trial (T_2) on day 2. In VORT, T_1 consisted of a 10-min session during which two identical white geometrical shapes on a black background were shown on two 3.5-inch widescreen displays (iPod screens).

The objects moved in equal distances horizontally, vertically or diagonally (distance 320 px) at a constant speed of 120 px/s. The videos were created in Adobe Flash, with a frame size of 320 px × 480 px, frame rate of 30 fps and encoded with the apple native H.264 video codec. The stimuli were looped on a 3rd generation iPod Touch through iTunes for the duration of the experiment (320 px horizontal axis and 480 px vertical axis). The luminosity of the screens was constant across the two screens and testing sessions. In NOR, real objects were used. They consisted of white plastic cylinders and coloured plastic Lego stacks of different shapes. Each mouse was placed in the centre of the arena between the two shapes/objects for a maximum of 10 minutes or until it had completed 30 seconds of cumulative object exploration. Object/shape recognition was scored when the animal was within 1 cm of a shape/object with its nose towards the shape/object. For NOR, exploration was defined as follows: directing the nose to the object and/or touching the object with the nose. Sitting on, or leaning to, an object was not considered to be an exploratory behaviour. For NOR, care was taken to minimize the difference between the to-be discriminated objects in order to prevent a greater preference for one of the two objects. An experimenter blind to the treatment group manually recorded the exploration times to the shapes/objects in each animal. To reduce shape/object preference effect, the nature of the stimuli (familiar or new shape/object) was counterbalanced from mouse to mouse as previously suggested (Ennaceur & Delacour, 1988). Mice that did not explore any of the two shapes/objects for at least 30 seconds during T_1 were excluded from the data analysis. Animals were used more than once but specific shapes/objects were never repeated across the experiments. For static cue shapes were simple geometrical shapes (square, triangle, circle, cross, etc.) with equal surface areas (2.5 cm^2). During T_2 each mouse was placed

again in the same arena (retention session) in which one of the two identical familiar shapes was replaced with a novel one. For dynamic cue, the same above shapes could move in 8 directions separated by 45°/90°.

The static or moving shapes, showed to mice, were the same shown to zebrafish.

Drug and treatment

Each animal was tested for all inter-trial delays. The test order was counterbalanced across animals following a Latin square design. The following drugs were used: **nicotine hydrogen tartrate salt** (Sigma–Aldrich, St. Louis, MO, USA) (0.1 mg/kg), 5 min before the test; **scopolamine hydrobromide** (Sigma–Aldrich, St. Louis, MO, USA) (0.25 mg/kg), 20 min before the test; **mecamylamine hydrochloride** (Sigma–Aldrich, St. Louis, MO, USA) (1 mg/kg), 30 min before the test. Control groups received saline (0.9%). All the drugs were dissolved in saline and injected i.p. in a volume of 0.1/10 g. Drug solutions were prepared fresh daily.

Statistical analysis

Discrimination index [(time spent exploring novel shape/object − time exploring familiar shape/object)/(time spent exploring novel shape/object + time exploring familiar shape/object)] was calculated as previously described (Pitsikas et al., 2001). Data were expressed as mean ± SEM. Pair-wise comparisons were assessed with Student's *t*-test. Multiple group comparisons were performed using 1- or 2-way analysis of variance (ANOVA) followed by Tukey's or Bonferroni's procedure for mean comparisons. The significance level was taken as $P<0.05$. All statistical analyses were done using Prism 6 software (GraphPad, San Diego, CA).

Results

5. RESULTS

5.1. *Zebrafish*

Social preference and *fear response to predator* tests

Effects of neurohypophyseal hormones

Figure 1 and 2 show the effect of the four neuropeptides oxytocin (OT), isotocin (ISO), vasopressin (AVP) and vasotocin (AVT) on social preference and fear response to predator tests. All the peptides increased social preference and reduced fear to predator response in a dose-dependent manner interpolated by symmetrical parabolas [$R_{social\ preference}^2$=0.42, P<0.05 (OT- fig.1A), 0.39, P<0.05 (ISO- fig.1B), 0.78, P<0.05 (AVP- fig.2C), 0.67, P<0.0005 (AVT-fig. 2D); $R_{fear\ response}^2$ =0.47, P<0.005 (OT- fig.1A), 0.35, P<0.05 (ISO- fig.1B), 0.26, P<0.05 (AVP- fig.2C), 0.30, P<0.05 (AVT-fig. 2D)]. The ED50 (ng/kg) obtained for social preference and fear response to predator tests are reported in figure 3. On the basis of the calculated ratio, AVT was 32 and AVP 10 times more potent to elicit anxiolytic than social effect, while ISO and OT were equally potent to induce both the effects. ISO was about 20 times more potent than OT to elicit social preference and fear response to predator while AVT was 94 and 129 times more potent than AVP to elicit social and fear response, respectively.

Effect of selective antagonists for the human OT, V1a and V1b receptors

In figure 4 and 5, the effect of increasing doses of different antagonists, on social preference and fear response to predator respectively, induced by the neuropeptides, is shown. All the antagonists produced a dose-dependent inhibition increase of both social (figure 4) and anxiolytic (figure 5) effects induced by ISO, OT, AVT, and AVP. Such increase was interpolated by linear regression lines, statistically significant [$R_{social\ preference}^2$ = 0.98, P<0.05 (OT), = 0.88, P<0.01 (ISO), =0.99, P<0.005 (AVP), = 0.78, P<0.05 (AVT); $R_{fear\ response}^2$ = 0.93, P<0.05 (OT), = 0.96, P<0.02 (ISO), = 0.99, P<0.01 (AVP), = 0.91, P<0.04 (AVT)]. The ED_{50} (ng/kg·10^{-3}) and confidence limits for the different antagonists in blocking the social

and anxiolytic effect of the peptides are reported in figure 6. It appeared that all the antagonists were more active in blocking the anxiolytic than pro-social effect induced by all the peptides. Among the three compounds, SR49059 were found to be the most and SSR149415 the least selective compound.

Swimming activity

Figure 7 shows the swimming behaviour i.e. crossing lines by different group of zebrafish during 30 minutes of test, every 5 minutes for 30 seconds. Different neuropeptides and antagonists, given at the effective or maximal dose respectively, did not induce any change in swimming behavior ($F_{(7, 72)}$=0.93, P=0.50)

T-maze test

Nicotine improves spatial memory

The effect of nicotine is shown in figure 8. Nicotine had a significant main effect of dose ($F_{(4,45)}$ =10.75), P<0.0001) and post hoc analyses showed it had a significant pro-cognitive effect at a dose of 200 and 2000 x 10^{-5} mg/kg. Nicotine showed an inverted U-shaped dose-response function.

Nicotine-induced cognitive enhancement is reduced by nicotinic and muscarinic antagonists

Various nicotinic and muscarinic drugs were tested for their possible effects on nicotine-induced pro-cognitive effect. The results are shown in figure 9. There was a main effect of treatment ($F_{(11,108)}$=14.14, P<0.0001) when the antagonists were given alone (figure 9 A). As expected, scopolamine led to amnesic effects in terms of a reduced difference in running time in comparison with the control group receiving two injection of vehicle, whereas mecamylamine did not. The selective nicotinic antagonists, MLA and DHßE (0.01-0.1 mg/kg) also had amnesic effects, whereas MII had slight but not significant pro-cognitive effects. Scopolamine and mecamylamine significantly antagonized nicotine-induced

facilitation effect (figure 9B). All the selective antagonists significantly blocked nicotine-induced memory enhancement at all the tested doses with DHßE being more active than MLA or MII.

Swimming activity

Nicotine was given at doses effective to improve memory (figure 10). Non-selective and selective antagonists were given at doses able to reduce nicotine-induced improvement. ANOVA did not show any significant treatment effect ($F_{(6,64)}$ =0.37, P=0.88, n.s.).

Visual Object Recognition **test (VORT)**

Effect of different delays in VORT

In figure 11 A and B, the effects of different delays on Virtual Object Recognition test (VORT), concerning the discrimination index (A) and the exploration time respectively (B), are shown. One-way ANOVA showed (figure 11 A) a significant difference ($F_{(3,36)}$=75.5, P<0.0001) among groups. The post-hoc analysis indicated that index evaluated at 96 h decreased than the other intervals. Concerning the exploration time (figure 11 B), one-way ANOVA revealed a significant difference among the T2 exploration time ($F_{(9,90)}$= 18.11, P<0.0001). The post-hoc analysis showed that the time spent to explore the new shape was higher than the exploration time of familiar shape in all the delays but not at 96 hours. Moreover, post hoc analysis did not show any significant differences in the T1 exploration time.

Highly discriminated and not discriminated shapes in VORT

Figure 12 shows which shapes were easily discriminated (figure 12 C, left) and which other shapes were not (figure 12 C, right). The results (figure 12 A) indicated that the mean discrimination indexes were significantly different (t=5.47, P< 0.0001). In figure 12 B, the ANOVA indicated that using highly discriminated shapes, more time was spent exploring the novel shape than the familiar one ($F_{(3,36)}$=3.53, P=0.001). In contrast, a similar mean

exploration time for the familiar and the novel shapes for not discriminated shapes was obtained.

Effects of cholinergic drugs on VORT

In figures 13 and 14, the effects of different treatments on VORT parameters are shown in presence of highly discriminated or not discriminated shapes. In figure 13 A, data analysis showed that there was a significant increase in the mean discrimination index when nicotine was given to zebrafish in presence of not discriminated shapes (t=3.55, P=0.0019). Conversely (figure 13 B), one-way ANOVA indicated a significant difference in the discrimination index among groups ($F_{(3,36)}$ =18.17, *P*<0.0001). Post hoc tests revealed that, when discriminated shapes were presented to zebrafish, cholinergic antagonists decreased discrimination index in comparison with vehicle, while treatment with nicotine did not further improve the parameters.

In figure 14 A and B, the exploration times after treatments are shown. One-way ANOVA revealed (figure 14 A) a significant difference in exploration times when not discriminated shapes were presented only when nicotine was injected ($F_{(3,36)}$=3.14, P=0.0033). Post hoc tests revealed that, during T_2 phase, the exploration time of novel shape was higher than the familiar one. Figure 14 B shows that when discriminated shapes were presented, one-way ANOVA revealed a significant difference in the exploration time of zebrafish treated with vehicle and nicotine ($F_{(3,36)}$=2.63, P=0.0008). Tukey's test indicated that zebrafish spent more time exploring novel than familiar shape in these groups.

Discrimination of different movements applied to discriminated shapes in VORT

In figure 15, the effect of movement applied to discriminated shapes on VORT is reported. When a novel movement was applied during T2 to the same or different shapes (figure 15 C) presented during T1, a similar discrimination index (figure 15 A) was obtained (t=0.96 n.s.), indicating that zebrafish were able to distinguish different kinds of movements (vertical, horizontal, oblique). ANOVA showed that the exploration time (figure 15 B) was

significantly higher in T2 than T1 between the two conditions when a new movement was introduced (same shapes: $F_{(3,36)=}$ 2.88, P=0.04; different shapes: $F_{(3,36)}$= 2.37, P=0.07).

Discrimination of different movements applied to not discriminated shapes in VORT

In figure 16, the effect of movement applied to not discriminated shapes on VORT is reported. The discrimination index (figure 16 A) increased significantly when presented moving shapes ($F_{(2,27)}$= 129.6, P<0.0001). Post hoc comparison revealed that the application of movement significantly increased discrimination index if the movement was different from T1 or it was the same. No difference was found in exploration time of stationary shapes ($F_{(3,36)}$=1.54, P=0.20 n.s.) (figure 16 B). In contrast, there was a difference among groups in the exploration time when either different ($F_{(3,36)}$=8.13, P<0.0001) or the same ($F_{(3,36)}$=4.56, P=0.007) movement were applied to shapes. Post hoc test revealed that during T2 there was a significant increase in the exploration time of novel shapes compared to the familiar ones, independently whether the movement was changed or not (figure 16 C).

5.2. Mice

VORT

Effect of different delays on NOR and VORT

In figures 17 and 18, the effects of different delays on Novel Object Recognition (NOR) and Virtual Object Recognition test (VORT), concerning the discrimination index and the exploration time respectively, are shown. Two-way ANOVA revealed no difference in mean discrimination index between 2D and 3D objects ($F_{(1,72)}$=2.34, P=0.13 n.s.). A significant difference in delay time (from 5 minutes to 96 hours) was found ($F_{(3,72)}$ =15.34, P<0.0001). Post hoc analysis indicated that the mean discrimination index evaluated at 96 h for both

NOR and VORT was significantly lower than that evaluated at the remaining time intervals (figure 17 A and B). There was no object x time interaction effect ($F_{(3,72)}$=0.39, P=0.75 n.s.). In figure 18, no difference between retention interval on the level of exploration in T1 was observed ($F_{(3,36)}$ =5.22, P=0.5 n.s.). One way ANOVA revealed a difference in exploration time in both NOR ($F_{(7,72)}$ = 4.91, P<0.0001) (figure 18 A) and VORT ($F_{(7,72)}$=10.87, P<0.0001) (figure 18 B). Post hoc analysis showed that the time spent exploring the novel object/shape was significantly higher than familiar one from 5 min to 24 h delay but not at 96 h delay in both NOR and VORT.

Highly discriminated and not discriminated shapes in VORT

Figure 19 shows which shapes were easily discriminated (figure 19 C, left) and which were not (figure 19 C, right). The results (figure 19 A) indicated that the mean discrimination indexes were significantly different ($t_{(38)}$ = 3.47, P= 0.0009). One way ANOVA analysis indicated that (figure 19 B), using highly discriminated shapes, more time was spent exploring the novel shape than the familiar shape ($F_{(3,76)}$ = 8.38, P=0.0002). In contrast, a similar mean exploration time was obtained for the familiar and the novel shapes ($F_{(3,76)}$=2.76, P=0.05) when not discriminated shapes were presented.

Effects of cholinergic drugs using discriminated and not discriminated shapes in VORT

As shown in figure 20 A, there was a significant increase in the mean discrimination index when nicotine was given to mice in the presence of not discriminated shapes (t_{18}=2.13, P=0.04). Conversely, (figure 20 B) there was a treatment effect when scopolamine or mecamylamine or nicotine were given to mice in the presence of highly discriminated shapes ($F_{(3,36)=}$100.9, P=0.0001). Post hoc analysis revealed that cholinergic antagonists decreased the discrimination index (P<0.0001) while treatment with nicotine did not further improve this parameter. When mice were presented not discriminated shapes (figure 21 A), one way ANOVA revealed no significant difference in the exploration time in

the saline treated group but a significant treatment effect in the nicotine group ($F_{(3,36)}$=47.97, P<0.0001). The exploration time with the familiar and novel shapes after treatment with cholinergic antagonists and nicotine is shown in figure 21 B. One way ANOVA revealed significant differences in the level of exploration time when mice were presented highly discriminated shapes after treatment with saline ($F_{(3,36)}$= 9.91, P<0.0001) and nicotine ($F_{(3,36)}$= 10.52, P<0.0001). Post hoc analysis revealed a significant difference in the exploration time during T2 between the novel and the familiar shape in both saline and nicotine group.

Discrimination of different movements applied to discriminated shapes in VORT

In figure 22, the effect of different movements applied to one shape on VORT is reported. When a novel movement was applied during T2 to the same or different shapes (figure 22 C) presented during T1, a similar discrimination index (figure 22 A) was obtained (t=0.22 n.s.) indicating that mice were able to distinguish different kinds of movements (vertical, horizontal, oblique). One way ANOVA indicated a significant difference in the T2 exploration times (figure 22 B). Post hoc analysis revealed that the exploration time was always higher in T2 than T1 between the two conditions when a new movement was introduced (same shapes: ($F_{(3,36)}$=3.44, P=0.02); different shapes: ($F_{(3,36)}$= 3.79; P=0.01).

Discrimination of different movements applied to not discriminated shapes in VORT

As reported in figure 23 A, one way ANOVA shows that the discrimination index increased significantly when presented moving shapes *(*$F_{(2,27)}$=7.75; P=0.002). Post hoc comparison revealed that the application of movement significantly increased discrimination index either if the movement was different from T_1 or it was the same. In figure 23 B, ANOVA analysis indicated that no difference was found in exploration time of stationary shapes. No difference was found in exploration time of stationary shapes ($F_{(3,36)}$ =0.41, P=0.74, n.s.). In contrast, there was a difference among groups in the exploration time when

either different ($F_{(3,36)}$=13.74; P<0.0001) or the same ($F_{(3,36)}$=17.16, P<0.0001) movement were applied to shapes. Post hoc test revealed that during T_2 there was a significant increase in the exploration time of novel shapes compared to the familiar ones, independently whether the movement was changed or not (figure 23 C).

Discussion

6. DISCUSSION

It is known that, in zebrafish experiments, different drugs have always been dissolved in tank water. In our study we decided to inject the compounds i.m. in social preference and in fear response to predator tests and i.p. in T-maze and in Virtual Object Recognition tests. These two kinds of injection controlled the amount of drug each fish received more precisely, using a Hamilton syringe. However recently, in T-maze studies i.p. injections were preferred than i.m. treatments to prevent damages on caudal musculature, compromising the motor ability. For i.p. injection, we chose cold water rather than chemical anaesthesia in zebrafish in order to limit the effect of stress as much as possible: cold-anaesthesia does not increase blood glucose levels, which is an index of stress in teleost fish (Kinkel et al., 2010). Moreover, the injection was made in the abdominal cavity and no more than the tip of the needle was inserted into the abdomen of each fish, as mean of preventing damages of internal organs.

Social and anxiety-related behaviour

Our results demonstrated for the first time that the neurohypophyseal oxytocin (OT) and arginine- vasopressin (AVP) peptides and their teleost fish homologs isotocin (ISO) and vasotocin (AVT) increased social behaviour and reduced fear response to predator in zebrafish, indicating a neuromodulatory role in these complex behaviours.

In the shoaling preference task, WT zebrafish increased the time spent in proximity of the usually neglected *Nacre* mutant following treatment with OT and AVP and their respective non-mammalian homologue ISO and AVT. Similarly, in the fear response to predator test, the exposition to each neuropeptides reduced the anxiety, allowing the single zebrafish to swim bravely close to the predator. Our data are consistent with Dębiec (2005) that demonstrated how these neuropeptides could regulate different emotions and behaviours as social memory, stress, anxiety and fear. Specifically, the four neuropeptides

increased sociability and reduced fear to predator in a dose-dependent manner as interpolated by symmetrical parabolas. OT and ISO elicited pro-social and anxiolytic effects in the same dose range, with ISO resulting approximately 20 times more active than OT. Both AVP and AVT induced anxiolytic effect at doses lower than those needed to induce a pro-social effect. AVT was 40 times more active than AVP to elicit a pro-social effect and 130 times more potent than AVP to induce an anxiolytic effect. The results showed that low doses of each peptide appeared more effective than high doses, suggesting an inverted-U dose–response function typical of many peptides. These findings agree with Thompson and Walton (2004) who found in goldfish that sociality and anxiety levels could be associated with neuromodulatory action of endogenous ISO/AVT and possibly with a balance of both peptides. We can exclude that inhibitory effects, observed at high doses, were influenced by changes in general locomotor activity. The total number of crossed lines in the swimming activity test was not modified by the maximal doses of the neuropeptides. Therefore, we hypothesized that low concentration of neuropeptides probably couldn't activate a sufficient amount of receptor able to elicit either pro-social or anxiolitic effect. On the contrary, high doses of neuropeptides inhibited both the pro-social and anxiolytic behaviour and only specific doses increased the social approach. A possible pharmacological interaction of OT or ISO with the AVP/AVT receptors, as demonstrated by Blanchard et al. (2005) in hamster can not be excluded. The results obtained with the AVP and AVT treatment had a different trend in the two behavioural tests. For both neuropeptides, low doses probably activated a not relevant amount of V1a receptor to induce an aggregative or anxiolytic effect while high doses induced an aggressive approach rather social effect.

Even though OT and AVP that are structurally very similar, (differing by only two amino acids in most mammals) (Wallis, 2012), do not posses physical /chemical properties that allow their passage across the blood-brain barrier (BBB) (Barros et al., 2008). This does not exclude the possibility that passive transport of minute but effective amounts might occur if high doses of peptides are injected peripherally (Ermisch et al., 1985). At least in mice, central administration of L-371257, an OT antagonist, which does not cross the BBB, was

able to fully reverse the anxiolytic effects of OT administered peripherally (Ring et al., 2006), arguing that the anxiolytic-like effects of OT are mediated specifically through OT receptors within CNS. As there is no evidence for relevant differences between the endothelial tight junction-based BBB of zebrafish and that of higher vertebrates (Jeong et al., 2008), it seems likely that the observed changes in social and anxiolytic effect of the peptides in zebrafish were centrally mediated.

In this experimental setting, zebrafish of both sexes were used as male and female showed the same shoaling preference according to Engeszer et al. (2004). Howerer, we used only adult zebrafish, having a length over 15 mm (Engeszer et al., 2007) since shoaling develops when visual system is completely mature (Buske & Gerlai, 2010). In addition, stimulus and subject fish were separated by a transparent barrier allowing the transmission of visual cues while attenuating any potential non-visual cues (olfactory cues) used in the identification of conspecific (McLennan & Ryan, 1997).

Response to predator, as an unconditioned behaviour set up by Bass and Gerlai (2008*)* with some modifications, has been used as anxiety test. A single zebrafish stimulus test was used to evaluate the effects of the peptides in the fear response to predator test, differently from Bass and Gerlai (2008) who used a group of five test fish. In fact, we better characterized the pharmacological effect of the peptides in individual fish even if shoal cohesion could not be measured. Furthermore, a possible confounding effect due to predator inspection, a behaviour more related to activity than anxiety was avoided. For the first time, *Astronotus ocellatus* was used as stimulus predator. This is an allopatric (different geographical origin from zebrafish) predator belonging to *Cichlid family*. Allopatric predators exhibit the largest number of attacks compared to other stimulus fish (Bass and Gerlai, 2008). *A. ocellatus* induced fear as observed with an allopatric fish (*Compressed Cichlid*) by Bass and Gerlai (2008). It is well documented that fear may occur in several ways in fish (Domenici et al. 2007). Fish may freeze, hide or alternatively choose active escape, depending on environmental circumstances.

We were unable to source receptor antagonists specific for ISO/AVT receptor subtypes in zebrafish (Filby et al., 2010). Thus, we decided to use peptidic analogues with high

receptor selectivity for the human OT/AVP receptors. The different AVP/OT receptors cloned in mammals (OT, V1a, V1b, and V2 receptors) are closely related, as their overall homology varies from 40% to 85%, the most conserved regions being the transmembrane α-helices and the first extracellular loop. The OT/AVP receptors are also highly conserved in evolution. In particular, one ISO and one AVT receptor have been cloned and pharmacologically investigated in *Catostomus commersoni* (Mahlmann et al., 1994; Hausmann et al., 1995) and *Cyprinodon nevadensis amargosae*. There is also evidence of two V1a-subtype (V1a1 and V1a2) receptors and one V2-type receptor in *Actinopterygian fish* (Lema, 2010). These three AVT receptors exhibit distinct tissue pattern expression and are differently regulated in the hypothalamus and gill in response to hyperosmotic challenge. In recent years, a gene expression study, carried out in zebrafish, evidenced one gene encoding for an AVT receptor (avplr1b arginin vasopressin-like receptor 1beta, corresponding to a V1a subtype), and one gene encoding for an ISO receptor (Filby et al., 2010; Lema, 2010). Even if a complete pharmacological characterization of teleost fish receptors is still lacking, it has been shown that AVT and ISO receptors may act through a PLC/IP3 intracellular signaling pathway as the mammalian OT receptor/V1a/V1b receptor subtypes (Warne, 2001). Finally, a V2-type receptor, acting by modulating intracellular cAMP levels in isolated renal tubules from rainbow trout, has been reported (Perrott et al., 1993).

To test whether the effects observed on shoaling and fear to predator response induced by the neuropeptides were mediated by specific ISO/AVT receptor subtypes, we used the most selective OT and AVP receptor antagonists on human receptor subtypes. In particular, we employed the previously characterized highly selective V1a and V1b receptor antagonists SR49059 and SSR149415 (Serradeil-Le Gal et al., 2002) and a peptidic analogous highly selective in the rat, desglyDTyrOVT (desGly) (95-fold more potent versus the OT receptor than V1a receptor - Manning et al., 1995). This antagonist has been found extremely selective also on human receptors where it binds with high affinity only to the OT receptors. Our findings indicated that the three antagonists were all more effective in blocking the anxiolytic than the social effect. In particular, desGly and SR 49059 blocked

the OT- and AVP-induced effects on social preference and fear response respectively. The less selective antagonist appeared to be SSR 149415.

These findings argue for a maintained selectivity, also in zebrafish, of desGly for an OT-like receptor and of SR49059 for V1a-like receptor.

The role of V1a/V1b and OT receptor on pro-social behaviour has been demonstrated in several vertebrate species. Veenema (2012) demonstrated in mice that the early social environment might alter social behaviours via changes in the oxytocin and/or vasopressin systems. To test this hypothesis, and to gain mechanistic insights, rodent models mimicking either a deprived (e.g. maternal separation) or enriched (e.g. neonatal handling) early social environment have been utilized. The results showed that differences in the quality of the early social environment were associated with brain region-specific alterations both in oxytocin / vasopressin expression and OT / V1a receptor binding. Early social environment-induced changed in OT and AVP systems were associated with changes in several forms of social behaviour, including maternal care, aggression, play-fighting, and social recognition.

The action of OT and AVP on OT, V1a and V1b receptors have been shown to be especially important in the regulation of affiliative behaviour (pair-bonding and maternal behaviour), social cognition (social memory), and social approach (social preference or social avoidance) (Lukas & Neumann, 2013). Mice lacking AVP1a receptor showed reduced anxiety and impaired social behaviour (Egashira et al., 2007). A role of AVP1a subtype receptor on pro-social behaviour has been in recent years demonstrated in mice lacking OT receptor (Sala et al. 2011). In zebrafish, very little is known about involvement of ISO and AVT receptors on social behaviour. On the other hand, it is known that in fish, male blue head wrasses, *Thalassoma bifasciatum*, exogenous AVT increased courtship but treatment with a V1a receptor antagonist had the opposite effect (Semsar & Godwin, 2004). Another work (Toyoda et al., 2003) showed that administration of a V1a receptor antagonist, [d(CH2)5[1], Tyr(Me)2, Arg8-vasopressin] suppressed the expression of the courtship behaviour induced by AVT in *Cynops pyrrhogaster,* a common asian newt, indicating that AVT acted via a V1a subtype receptor to induce courtship behaviour and pheromone

release. Targeted disruption of the V1b receptor markedly reduced male–male territorial aggression and maternal aggression (Wersinger et al., 2002), which may be considered social forms of aggression. Reduced anxiety was shown in mice treated with the selective AVP1b subtype receptor antagonist SSR149415 (Griebel et al. 2002; Blanchard et al. 2005; Stemmelin et al. 2005).

In conclusion, our findings show, for the first time, that ISO/AVT modulate in zebrafish social behaviour, unrelated to sex and anxiety, and that these effects are mediated by at least two different receptors. Interestingly, OT and AVP were active as ISO/AVT to modulate the same effects. Even if specific antagonists for ISO/AVT receptors are not available, despite the evolutionary distance, receptor similarities could make zebrafish receptors, once cloned and fully characterized, useful tools to investigate the pharmacological profiles of OT/AVP compounds. The zebrafish model is a great use to test the potential of novel drugs in their ability to promote sociability and relieve anxiety for the pathogenesis of the complex behavioural disorders that constitute psychiatric diseases. For patients with impaired social functioning, the use of intranasal OT or psycho-therapy is a promising treatment approach. Such psycho-pharmacotherapy, however, should be individually adapted according to the patient's endogenous OT system activity. Not only low, but also hyper-activity of the OT system, as seen in William's syndrome (Dai et al., 2012), results in social dysfunctions. Consequently, a well-balanced activity of the endogenous OT system, driven genetically or by environmental especially early life factors, is essential for healthy social interactions. Thus, increasing brain OT availability, if impaired, may contribute to the endogenous OT neurotransmission, thereby promoting affiliative behaviour, social cognition and social preference.

Spatial memory

Spatial memory is importantly involved in different neurological diseases. Our results, obtained using zebrafish with an associative and modified spatial learning T maze task together with nicotine and some selective and non-selective nAChR antagonists, indicate

that nAChRs play a role in zebrafish cognition. The ability of zebrafish to attain good performance at associative learning tasks has been clearly documented using a three-chamber delayed spatial alternation task (Levin & Chen, 2004), a Y-maze (Cognato et al., 2012), and a T-maze (Darland & Dowling, 2001).

Recently, a lot of studies have used this model in alternative to the classical rodents, because zebrafish has more advantages: cheap, manageable, small, treatable in short time, it requires no longer training and above all, evolutionarily it shows analogies with vertebrates (Williams et al., 2002).

Even though T-maze test is mostly used in rodents to study learning and memory (Moy et al., 2007; Sala et al., 2013), recently, this task has been adapted to zebrafish. For example, Colwill et al. (2005) studied the learning and memory on a T-maze in zebrafish, using the ability to discriminate different colours. Saili et al. (2012) developed a T-maze test in which zebrafish had to associate a negative shock with a wrong choice. In our study, we modified the T-maze original procedure of Darland and Dowling (2001) not using reward but only visual stimuli and by increasing the time between the two sessions to 24 h. Using this interval, acquisition was sufficient to provide a basis for determining the enhancing effects of nicotine. In this task, zebrafish should remember the T-maze arm in which there was the attractive stimulus, based on a previous experience. We chose as target an enriched environment (*reservoir*) with coloured stones, shells and plants. The colours of stones have been chosen in agreement with Avdesh et al. study (2012); they showed that zebrafish could discriminate different colours for the spatial orientation in their habitat, preferring primarily red, green, yellow and much less blue.

Our findings show that spatial learning can be studied very quickly in individual zebrafish: two trials were enough to reduce the running time to reach a good performance, whereas other studies have required 7 to 28 sessions (Williams et al. 2002; Levin & Chen 2004). Low i.p. doses of nicotine induced cognitive enhancement whereas high doses impaired memory, thus indicating an inverted U-shaped dose-response function. This trend has been previously observed in zebrafish exposed to nicotine solution (Levin & Chen 2004; Eddins et al., 2009), as well as in rats, monkeys, and humans (Levin et al. 2006b).

Moreover, it is known that nicotine improves cognitive functions in zebrafish as a result of the nAChR modulation of dopamine release and/or metabolism (Levin et al. 2006a). Accordingly, Eddins et al. (2010) have shown that nicotine exposure increases learning rates and the levels of the dopamine metabolite dihydroxyphenylacetic acid (DOPAC) and that both effects were blocked by the antagonist mecamylamine.

In order to better characterize the nAChR subtypes involved in nicotine-induced cognitive facilitation, various selective and non-selective antagonists were administered in combination with nicotine. As expected, scopolamine and mecamylamine completely antagonized the memory enhancing effect of nicotine, but the nAChR subtype- selective antagonists were also all effective in reducing the effect. It is worth noting that the memory blocking effect on nicotine stimulatory effect induced by the non-selective and selective antagonists was obtained using doses that do not affect cognition. We can exclude that the improvement in memory induced by nicotine was influenced by changes in general activity because the total number of crossed lines in the swimming activity test was not modified by the maximal effective doses of drugs.

Then, we used selective antagonists to evaluate the specific subunits of nAChRs involved in these cognitive mechanisms. Our data showed that the α4β2 selective antagonist DHβE was more effective than MLA or MII, thus indicating a major role of the α4β2 subtype in the learning and memory effects of nicotine. These results are in agreement with antagonistic studies designed to determine the reversibility of the effects of nicotine (Svoboda et al., 2002; Levin et al., 2006a; Bencan & Levin, 2008; Eddins et al. 2009). In fact, our data further confirm the functional similarity in pharmacological response between mammal and zebrafish nAChRs. Mecamylamine (a non selective nAChR antagonist that acts on mammalian $\alpha4\beta2$, $\alpha3\beta4$, $\alpha3\beta2$, and $\alpha7$ subtypes) (Papke et al., 2001) blocks a broad range of nicotine-mediated effects in zebrafish, including motor sensitization, improved learning, and reduced anxiety (Levin et al., 2006b, 2007; Eddins et al., 2009; Petzold et al., 2009). The nicotine-induced anxiolytic effect in zebrafish is also reversed by the subtype- specific nAChR antagonists DHβE, MLA and MII (Bencan & Levin,

2008). In summary, our data support the zebrafish system as a means of rapid screening of the effect of new $\alpha4\beta2$ nAChR compounds on spatial memory.

Selective attenton and visual discrimination in mice and zebrafish

We developed a new Virtual Object Recognition test, VORT, which results a valid task to study selective attention and memory. This test permitted us to optimize stimuli used in the classical Novel Object Recognition (NOR) task. VORT is a novel variant of a well-estabilished spontaneous object recognition task used to assess recognition memory for 3D object (Berlyne 1950).

Firstly, we showed that when a visual stimulus was adopted in mice, they were able to discriminate virtual objects (geometric shapes) by exhibiting a good memory performance that is similar to that obtained with NOR. Accordingly, we found that mice submitted to NOR or to VORT showed a comparable discrimination index, even if the nature of the sensory information available to the mice was strongly different. In fact, in the NOR the quantity of information is greater than in the VORT because exploration of real objects stimulates multiple senses (smell, touch) while virtual objects evoke visual senses. In a previous study (Forwood et al., 2007), a reduced exploration level was found if rats discriminated between two shapes or between two 3D objects in a rewarded Y-maze. This reduced performance could be due to the complexity of the visual stimuli (shapes with differing fill patterns requiring discrimination of high resolution detail). Even though we used a dfferent animal species, a similar mean total time exploration between the two tests was found during exploration choices, confirming the validity of our method. Extra-information (smell, touch and exploration with whiskers) were absent when virtual shapes were presented, since the Ipods were inserted into a plastic transparent container and mice were unable to touch or smell the screens. From data obtained we can say that mice were able to discriminate between different shapes, showing a robust recognition across different delays at a level not significantly different from 3D objects even though a progressive decrease of total exploration time after 5 min delay was observed. When a

visual stimulus is presented alone, it may require more attention for processing this one (Lee et al., 2012). According to Forwood et al. (2007), a significant decay of memory occurs within 60 min in rats while we found that no significant loss in performance until at least a 24h delay, using simple geometric figures. The performance pattern in NOR studies appears to depend on the choice of objects. Objects that might induce natural preferences, masking the detection and exploration of an object as a result of its novelty, should be avoided. In our comparable NOR test, we tried to equate the pairings of objects in order to avoid any induced preferences biases. Therefore, in VORT it was possible to select pairs of shapes that mice found easier to discriminate: objects that induced a sufficient amount of exploratory behaviour or identical objects that were not differentially preferred during familiarization phase (T1) and finally objects that could be easily discriminated by the animals. We used 10 different shapes (abstract objects controlled for colour, texture, size and other low-level visual cues such as curvature vs linearity).

In other studies, authors (Bussey et al., 2001, 2012) also showed virtual shapes to rodents, C57BL6 mice, using white geometric shapes on a black background; unfortunately, these protocols required mice to be trained on a complex task that took more than 10 sessions for each animals to reach a criterion. Another task was developed for learning to rats the association between a touch on screen (corresponding a particular visual stimulus) with a reward (Bussey et al., 1997). The results of all these works showed that rodents could discriminate visual stimuli but these tests required long and complex trainings. VORT test needs to a short familiarization trial and it requires no training and so it appears more simple, fast and less expensive.

In a second step, we used also the zebrafish in VORT, to study selective attention in this new and emerging model. It is known that this animal has an higher visual acuity than rodents (Tappeiner et al., 2012) and a lot of authors used zebrafish, exploiting its visual capability to distinguish different colours (Advesh et al., 2012) and different cues associated to a food-rewards (Colwill et al., 2005). Similarly to mice, zebrafish, submitted to VORT in different delay times, showed a significant memory decay only after 96 h from T1, maintaining higher total exploration time in the different trials than mice. In contrast

to rodents, which we evaluated in 5 min, 120 min, 24 h and 96 h delays, zebrafish was tested in the same time intervals, excepted for 120 min which was replaced with an interval of 3 h. Preliminary results showed bad performances in 120 min delay for zebrafish, allowing us to hypothesize high stress levels as the cause. In fact, it is known that zebrafish are social animals which live all together in their habitat, making shoaling, for food, breeding, defending from the aggressors (Krause & Ruxton, 2002). For all these reasons, zebrafish choose carefully their co-specifics and this capability shows not only that zebrafish have a good visual acuity, discriminating colours and streaks of fish but also that they increases their stress levels when are alone and frightened (as observed during the test) (Engeszer et al., 2007; Bergner et al., 2009).

So, comparing the VORT results between mice and zebrafish, we obtained not only good performances in both models but also similar means of exploration time, demostrating the VORT validation also in zebrafish. Nothing is known about the discrimination criterions used by mice and zebrafish, however, we only observed that in some cases, shapes with different features as edges (triangle or square) or roundness (circle) were easily discriminated than a couple of similar shapes.

Then, we tested the validity of VORT investigating the effect of amnesic and pro-amnesic cholinergic compounds in mice and zebrafish. It is known that cortical acetylcholine release has been implicated in novelty-induced arousal, attention, the encoding of novel stimuli and memory consolidation (Acquas et al., 1998; Sarter et al., 2000). In NOR, scopolamine and mecamylamine have been shown to impair memory performances in Swiss mice when acutely administrated (Dodart et al., 1997; Obinu et al., 2002). The doses of scopolamine and mecamylamine, used by us, were similar confirming the validity of the test. Also in zebrafish, it is known that these two antagonists were able to slow the learning and to inhibit the memory consolidation, blocking the nicotine pro-mnesic action (Eddins et al., 2009; Richetti et al., 2011). On the other hand, it is known that nicotine improved memory in mice in a range of doses similar to that used in NOR (Puma et al., 1999). Similar to mammals, also in zebrafish (as reported previously in the spatial memory discussion) nicotine is involved in cognitive enhancement (Levin & Chen,

2004). This result confirms further the anatomical and physiological similarities between zebrafish models and human. It is possible that nicotine increased attention and enabled animals to distinguish very similar visual stimuli whose discrimination under normal circumstances is not easy. It is not surprising that no further improvement was observed in the discrimination index with highly discriminable shapes after nicotine treatment both in mice and in zebrafish. Accordingly, it has been reported that the lack of an effect with nicotine in mice may be due to a "*ceiling effect*" that does not allow further improvement in performance (Young et al., 2012). In zebrafish, we could hypothesize that nicotine didn't increase the discrimination capability due to its anxiety effects in according to Dunnet et al. (1990) that showed the involvement of nicotinic receptors in the stress responses, preventing good behavioural performances.

The last step has been shown that both mice and zebrafish were able to identify objects with characteristics motion. The objects were identical to those used in VORT to investigate the potential interaction between the static object properties and the sensitivity to motion. Mice and zebrafish were able to discriminate the direction of motion (vertical, horizontal or oblique) independetly from the shape information. For the first time, our data indicate that both animal models are not only able to distinguish different 2D moving shapes but they are also better at discriminating shapes that were difficult to be discriminated when stationary, distinguishing different real movements. By showing that applying the same movement in both T1 and T2 the discrimination index increased, our results support the idea that motion is a powerful cue that makes the task more valuable for studying attention. This indicates that motion is a strong attentional cue causing a shift of visual attention towards the moving shapes. There is an evidence that motion may contribute to object recognition in humans (Foster & Gilson, 2002), in patients affected by neurodegenerative diseases such as Alzheimer's (Poissonnet et al., 2012) and Parkinson's disease (Meppelink et al., 2009) or in Williams syndrome (Farran et al., 2013), a rare genetic disorder in which visuo-spatial functioning is impaired relative to verbal abilities (Jarrold et al., 1998). In fact, it is known that in humans, the most conspicous and consistent feature of neuronal diseases is memory impairments. Investigations of human

memory are typically based on self-reports by subjects recalling or recognizing information in an experimental setting, for instance lists of word. Visual discrimination tasks that vary in the degree to which they likely access structural and semantic knowledge may provide insight into potential visual perceptual or object recognition deficits in neuronal diseases that may impact picture naming. Shape-based object recognition is achieved when an observer apprehends an object's spatial contours sufficiently to match it to a known exemplar.

The use of virtual 2D shapes has numerous advantages over the real 3D objects: unlimited control over the shape of objects, their position, speeds and movement trajectories over the screen. It is possible to change the size of the objects and their mutual spatial relations in ways that would be impossible with physical objects. Moreover, the automation of the task could minimize the variability due to manual scoring and make the test less time–consuming than NOR. This new VORT paradigms is based on simple virtual shapes and offers the possibility to obtain rapid information on the amnesic or pro-mnesic potential of new drugs, screening firstly in zebrafish and then in mice. In contrast to most of the classic learning and memory paradigms, it does not require long periods of training, food and water diet, or aversive stimuli. Such a method is clearly useful for drugs and lesion studies in wild type animals and for the behavioural characterization of transgenic and gene-knock down models. Our results obtained in mice with VORT are comparable to those obtained with classical NOR, confirming the test validity in this model.

Finally, in this project, we demostrated that zebrafish was a good animal model alternative to classic rodents. Taken togheter, our findings showed for the first time the pro-social and anxiolytic properties of OT/AVP system mediated by different receptors in zebrafish and open a new avenue of research for the development of new drugs to treat anxiety–related diseases or abnormal social behaviours including autism and schizophrenia. Moreover, we confirmed the important role of cholinergic system in the processes of acquisition and memory consolidation in zebrafish similarly to mammals and we developped an innovative VORT task for the study of selective attention, never previously

studied before both in mice and in zebrafish. In future studies, this test, by use of the zebrafish model, will give rapid informations on attentional memory for the discovery and the screening of new compounds for the treatment of neurodegenerative diseases.

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Figures