Assessment of the zebrafish (Danio rerio) as a model for neurotoxicity in the screening of New Psychoactive Substances

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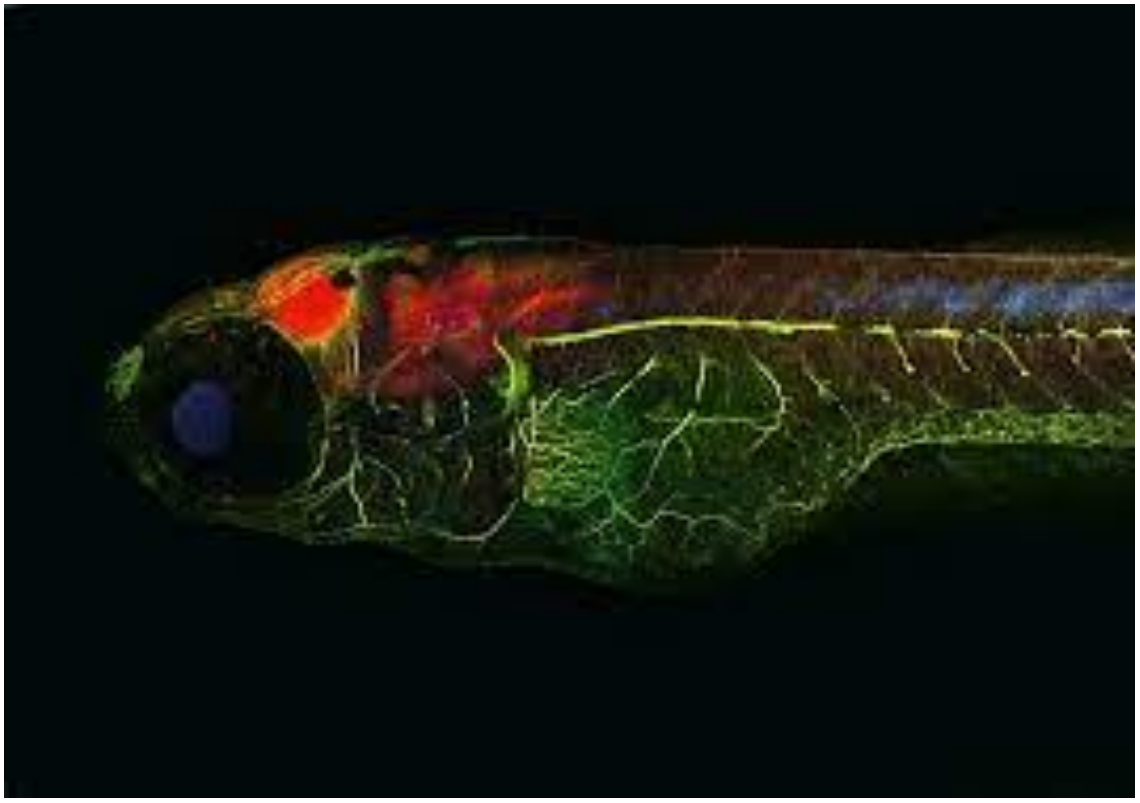

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Abstract

According to the world drugs report of 2021, 950 new psychoactive substances have been released illegally on the market in the last year. Due to the illegal status minimal research has been performed on the toxic and addictive properties. Consumers of these drugs are inexperienced in regard to specific dosing and administration, which results in unpredicted adverse effects. More information on the compounds can prevent accidental overdoses and lasting consequences for the consumers.

Factors that need to be taken into account in the risk assessment of recreational drug use include dosing, speed of entry, individual sensitivity, chronic/ repeated use, interaction with other compounds in the drug (contamination), interaction with other drugs (especially alcohol), and interaction with pre-existing pathologies. These are important endpoints which need to be evaluated in a potential model for a novel high-throughput screening approach.

This report proposes the zebrafish model as a screening approach. The small tropical fish has become popular in the field neuroscience because of the of rapid development, tractable genetics, ease in maintenance, and optical transparency in developing larvae. In addition, the major neurotransmitter systems abused by the recreative drug use have been identified with similar function in the zebrafish.

Due to the rise in popularity, the guidelines for behaviour assessment in the fish have become a more precise and quantifiable. These developments allow more elaborate and specific studies on the changes in behaviour due to drugs use, which can be compared to human and rodent patterns.

In conclusion, the larval and adult zebrafish are becoming key models in the field of neuroscience, representing a promising novel model for translational risk assessment on the drugs of the CNS.

Keywords: Neurotoxicity, Zebrafish, NPSs, Screening

Layman summary

Drugs are produced with the goal of curing people who are suffering from many different diseases. Some of these diseases are related to problems with the brain, for example people suffering from depression. Unfortunately, these drugs are abused by people who want to feel happy but have no permission from doctors. Because these drugs are used with no supervision from doctors, or for the reasons they were produced, the risk of negative effects on the health for the people who use these drugs increases. Because of the popularity of these drugs, more variants are produced to trigger the same or even stronger effect, which also increases the risk for negative effects. These newly and illegally produced drugs are not tested properly, which results in a dangerous situation for the people who take these drugs.

In our report we look at a new method of screening, which allows us to look for dangerous variants of the drugs fast. If these bad drugs are found, this information can then be shared with the users and therefore prevent negative health effects. This method needs to look at different factors such as; effects on behaviour, change of addiction increased risk through mixtures and contaminated pills

The zebrafish is a model used a lot in research on different diseases, because this fish contains similar organs as the human, for example a brain. We looked at important factors in the brain that are abused when people are taken illegal drugs and try to find similar structures in the brain of the zebrafish. In addition, it is important to look the behaviour of the fish and if it can be compared to human behaviour.

In conclusion, we found high similarities between the zebrafish model and humans. Also, the popularity of the zebrafish in research is growing fast, which results in a lot of new tools being developed. Making this model very promising in the fast screening of the new illegally made drugs.

Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CB	Cannabinoid Receptors
CNS	Central Nervous System
CsA	cyclosporine exacerbates
DA	Dopamine
DOP	Delta Opioid receptor
Dpf	Day Post Fertilization
GABA	γ-Aminobutyric acid
GRIN1	Glutamate Ionotropic Receptor NMDA Type Subunit 1
Hpf	Hours Post Fertilization
KOP	Kappa opioid receptor
L-DOPA	Dihydroxyphenylalanine
LSD	Lysergic acid diethylamide
mCPP	Meta-Chlorophenylpiperazine
MDMA	3,4-Methylenedioxymethamphetamine
MOP	Mu Opioid Receptor
NE	Norepinephrine
NMDA	N-methyl-D-aspartate receptor
NPS	New Psychoactive substances
NORt	Novel Object Recognition test
OPRM	Opioid Receptor Mu
OPRK	Opioid Receptor Delta
PPI	Pre-pulse inhibition
SERT	Serotonin transporter
TH	Tyrosine Hydroxylase
UNODC	United Nations Office of Drugs and Crime
WHO	World Health Organisation
5-HT	5-hydroxytryptamine
Δ9-THC	TetraHydroCannabinol

Table of content

Introduction

According to the World Health Organisation (WHO), around 5% of the adult population worldwide has used an illicit drug in the last year[1]. Since 1999, the number of deaths caused by a drug overdose has quadrupled and drug-related mortality remains one of the significant causes of death for young adults in Europe[2]–[4]. Many recreational users are unaware of the exact content and consequences of the drugs they obtained illegally, which leads to adverse effects and increases the risk of developing addiction and severe health problems[5]. Currently, every medical practitioner needs to have a basic understanding of the acute implications of illicit drugs, as direct and indirect drug-related features cause a significant burden for the health care system today. Additionally, drugs are related to increasing crime rates. In most countries, the use, possession, production, and dealership of popular recreational drugs are illegal, attracting a 'fast money' culture[6]. The influence of drugs on crime also includes crimes committed under the influence. Drugs can alter decision-making and change social behaviours, resulting in aggressive and impulsive choices[7], [8]. Lastly, substance abuse burdens society through the increased likelihood of unemployment and decreased chances of finding and holding down a job[9].

Recreational drugs are illegal in most countries, resulting in users seeking newer options that offer advantages such as; being legal, less contaminated with adulterants, less expensive, or increased desirable effects. These alternative options have gotten multiple names, including; designer drugs, legal highs, bath salts, and new psychoactive substances (NPSs)[10], [11]. The most commonly used definition for NPS is; ''a new narcotic or psychotropic drug, in pure form or preparation, the United Nations drug conventions do not control that, but which may pose a public health threat comparable to that posed by substances listed in these conventions''[1]. Since these drugs are relatively new, limited research is conducted on the toxic and addictive properties. In combination of users being inexperienced regarding to dosing and administration, the exposure can lead to severe consequences and even lead to death[12].

Risk assessment on psychoactive substances used for medical treatment undergo a thorough investigation on possible toxicity. Unfortunately, the NPS used recreationally are not studied as carefully, primarily because of the illegal status. Another challenge of controlling the illegal substances consists of marketing as the compounds are sold as 'not for human consumption or described as 'bath salts' or 'plant fertilizer'[13]. In the majority of the countries, risk assessment on NPS's consists of a combination of medical records, including information on the mortality and acute intoxications, and data derived from in vivo and in vitro experiments, mainly focusing on the mechanism of action[14]. The high rate of new compounds appearing illegally on the market each year complicates the execution of qualitative screening. There is a high need for an assay that allows high throughput screening of the NPSs, detecting adverse acute, chronic and addictive properties. More information on the compounds can prevent accidental overdoses and lasting consequences for the users. In addition, fast screening could speed up the illegalization of compounds.

Hazard characterization

Psychoactive substances are medically used to treat various disorders, a few include anxiety, depression, schizophrenia, bipolarity, pain, and sleep malfunction. However, the psychoactive properties of these compounds have triggered the popularity of recreational users worldwide. Recreational use of psychoactive compounds increases the risk of adverse effects, which are a result of lacking control on normally monitored factors including dosing, contamination, the health status of user, lethal mixtures, and chemical content[16].

In order to create a model for the early screening of adverse effects, it is vital to incorporate assays that can detect acute, chronic, and addictive properties. In addition, the model needs to identify the desired effect linked to the psychoactive compounds. Information on the desired outcomes can better understand the reasons behind using, which could help specify the further investigation[17].

Desired effects

Psychoactive substances contain the ability to interfere with functional neurotransmission in the brain, which could result in changes in nerve cell communication and morphology. Given that behavior signifies the integration of the nervous system, it can be considered a sensitive indicator of possible interference of the normal process[15]. These changes can be positive since the targets are involved in the perception of reward, pain, and reality. According to the United Nations, the prevalence of recreative drug use in the USA in 2008 was 10,3% for marijuana, 4,8% for opioids, 2,1% for cocaine, 0,9% for MDMA, and 0,2% for heroin[16]. A pragmatic organization of these substances based on the effect could be; stimulant, sedative, and halogenating[15].

Stimulants are a group of compounds that enhance body activity, which is frequently abused due to their ability to induce euphoria, intensification of sensory experiences, sexual arousal, alertness, awareness, and energy[14]. These drugs are typically sold as pills or powders and interfere with the serotonin, dopamine, and noradrenaline levels in the synapsis system of the brain[17]. The classical stimulants include; cocaine, amphetamine, methamphetamine (also known as 'meth'), and ecstasy. The European drug report of 2021 published data on the average age of first use and frequency of drug use in Europe. In this report, the classical stimulant drugs of abuse were listed, the mean age (male and female) of the first use in Europe for cocaine was 23 years, amphetamine 21 years, methamphetamine 22 years, and MDMA 21 years. The frequency of use varied between 3.9 times per week for cocaine to 4.6 times per week for methamphetamine[18]. Commonly the NPS are sold as substitutes for the classical compounds, resulting in a variety of analogs that slightly differ from the original structures, examples include; 3-MMC, 4-MTA, 6-APB, mephedrone (MEOW), and MPA[19]. A number of the compounds associated with stimulant effects have also been shown to have halogenating properties, for example, the popular MDMA replacements, the 2C-serie[20].

Hallucinating substances have been well studied on their ability to alter the state of consciousness characterized by the dissolution of self-boundaries, distortions of perception, hallucinations, and the experience of union with the world[21]. The halogenating compounds can be divided into psychedelics and dissociative, which are based on the different mechanisms of action. Psychedelics are considered the 'classical hallucinogens', and primarily act as an agonist for the 5HT2A receptor[22]. The dissociative drugs, famous for the 'detachment of the world' feeling, are classified as selective non-competitive antagonists of the glutamatergic NDMA receptors[23]. The classical compounds include Ketamine and LSD, and the newer drugs detected on the market are the ketamine-like, methoxetamine, and the LSD- like, 25i-NBOMe[24], [25].

The last category consists of the sedative compounds, which are thoroughly studied for the substantially high abuse rates. A significant group of sedative drugs includes opioids, containing notorious compounds such as; heroin and morphine. These agents bind to the opioid receptors in the central and peripheral nervous system, which plays a vital role in regulating physiologic functions such as; pain relief, euphoria, and stress resilience[26]. The main difference between most opioids and the other substances mentioned is the method of entrance. Heroin and the increasingly popular NPS fentanyl are mainly used intravenously, which allows the drug to be admitted to the blood directly for 'a fast high' [27]. According to the European Drug Report of 2021, the average age of the first use of heroin is 23, with a high mean frequency of using six days per week[18]. Benzodiazepines are also categorized as sedative compounds, as they are positive allosteric modulators for the GABA receptors, which is the primary inhibitory neurotransmitter for the mammalian central nervous system[28]. Benzodiazepines are initially prescription drugs for people suffering from psychiatric disorders because of their anxiolytic, hypnotic, anticonvulsant, and muscle relaxant properties[29]. However, a growing concern is visible as new, more potent synthetic benzodiazepines appear on the market, which are mainly combined with opioids and alcohol[30].

Acute adverse effects

The goal of using drugs in a recreational environment is to trigger the desired psychoactive effect, which are described above. Parallel to the increasing popularity of drug use, a rise in the number of casualties related to drugs is observed in hospitals. Based on medical records and surveys, adverse effects occur from mild anxiety to severe psychosis and even death[31].

Frequently reported adverse effects of stimulant drugs consist of restlessness, irritability, violence, and a psychotic/paranoid state. In addition, more severe effects such as stroke and seizures have also been linked to the use of the classic and new psychoactive stimulants[17].

The more specific adverse short-term effects reported on hallucinogenic drugs included; disorientation, visual disturbances, and loss of coordination. Comparable to stimulants, hallucinogenic drugs are also associated with more severe effects such as; panic attacks, psychotic symptoms, seizures, paranoia, and memory loss[32]. Synthetic cannabinoids, which are famous for their relaxing hallucinating properties, have been involved with 65% of the drug-related emergencies of younger people (<20years), who experienced similar adverse effects of the patients misusing other hallucinating substances[14].

The last category, sedative drugs, creates a greater risk due to the method of administration, which is frequently performed intravenously. Direct admission to the bloodstream can cause suppression of respiration and induce coughing, which could lead to respiratory depression, coma, and hypertension[5], [17].

As fast detection methods for the more common drugs are developing, more information became available on drug trends. Surprisingly many patients are using multiple illegal drugs in single nights, with patients using up to 6 different illicit drugs on one evening. According to the World drug Report of 2021, an increase in deaths involving polydrug use is observed in different age groups, including teenagers and those over 50. In 76% of the drug-related deaths in the last year, opioids were involved[18]. In addition, based on the records from Emergency Departments in the USA, >60% of the drug-related admissions are caused by mixtures of psychoactive compounds[33]. Psychoactive compounds interfere with the neurotransmitters and receptors. If multiple drugs interfere with multiple mechanisms, the risk of adverse effects increases[14].

As stated earlier, the new psychoactive compounds are mainly manufactured to increase desired effects compared to the classical compound. Consequences of the increased effects include a higher

risk of adverse effects, which makes acute neurological toxicity detection an important marker for a future screening model. As stated by the "founder of modern toxicology" Paracelsus, the dose makes the poison. This critical statement applies to a significant problem; overdosing[34]. Overdosing is a well-known term to describe a toxic or even lethal dose of a drug, which results in the yearly death of more than 9000 people in Europe alone[1]. The dose of medical drugs is well-established based on years of research which includes data from in vitro and in vivo studies followed by test trials in healthy humans[35]. In addition to the broad quality control, patients taking the drug are supervised by a licensed practitioner, limiting patient-specific toxicity. The illegal status of recreational drugs in most countries worldwide causes minimal funding for studies focusing on dosing and risk assessment. As seen in figure 1, the number of overdoses within the American population has nearly

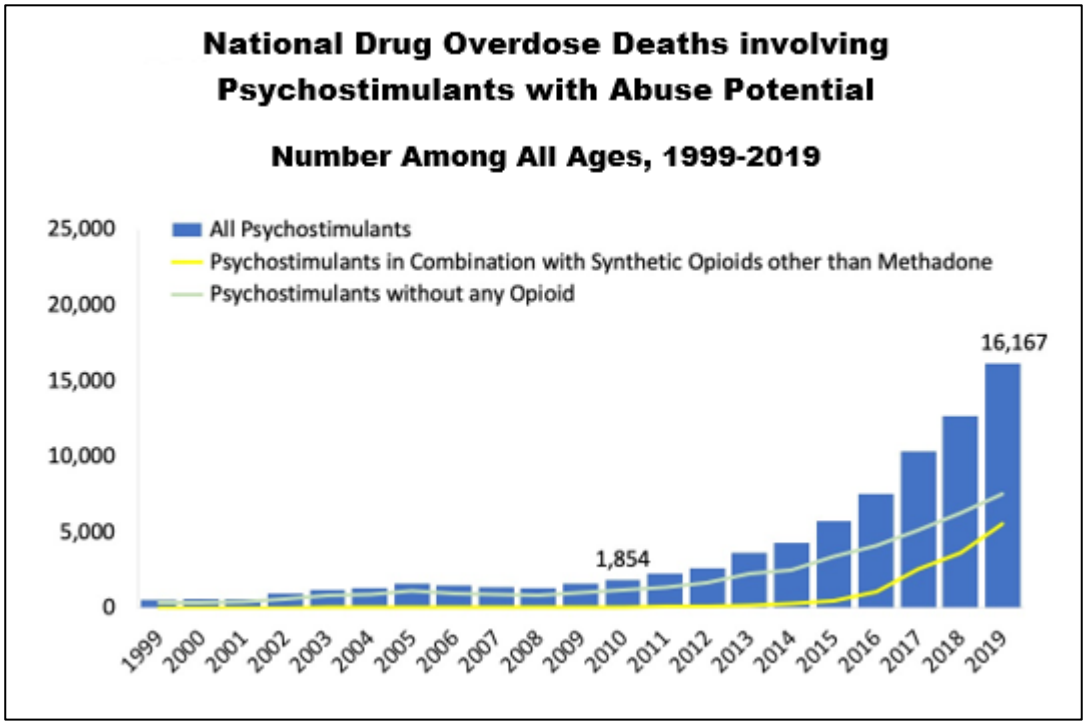

Figure 1 Graph stating the increasing numbers of mortality related to drugs overdoses in the USA from 1999-2019. Adapted from" Centers for Disease Control and Prevention, National Center for Health Statistics. Multiple Cause of Death 1999-2019" 12/2020.

been multiplied by a factor of 10 since 2010.

Another risk factor is the use of adulterants in illegally obtained substances. The exact composition of the "street drugs" is unknown to the consumer. Adulterants are added to the effective compound to increase the bulk, facilitate the administration route, reduce the chance of detection, enhance or mimic the pharmacological effect, or facilitate drug delivery[36]The combination of the different opponents in the drugs are not tested possible toxicity caused by the interaction of the different chemicals, which could result in additional adverse consequences.

Addiction

According to the American Psychiatric Association, the definition of drug addiction is defined as "compulsive administration of drugs of abuse despite negative consequences to the individual and a loss of control over the intake of such drugs" [37]. Addiction is further characterized by the dysregulation of the brain's reward system, linked to heritability between 40-80%, based on twin and adoption studies[38][39]. Other factors reportingly being involved in addiction development include; the age of first use, method of administration, psychiatric state of the user, and environmental

factors such as; social circles, addictive potency drugs, and substance access. The dysregulation of the natural reward system has been linked to a maladaptive neural plasticity response. Exposure to drugs in combination with the environmental factors are necessary for the detection of the addictive phenotype[40].

Repeated drug use usually leads to increased tolerance and dependence. However, the rate of these processes varies per drug and user. Addiction consists of mental and physical dependence, the opioids are well-known for the notorious physical withdrawal symptoms. By taking the dose of the drug triggering the desired effects can result in autonomic withdrawal syndrome, where patients suffer from thermoregulatory and gas intestinal disturbances. The discomfort of withdrawal symptoms have been studied intensely, and a variety of medication has been developed to limit the symptoms. However, multiple studies suggested that relief of withdrawal distress only adds to the motivation to continue self-administering drugs. Motivation to continue self-administration can be linked to the strength of the reinforcer (the drug), intravenous cocaine distribution in rats or monkey shows continues self-administration, even to the point of convulsions and deaths[41]. The physical discomfort users are willing to undergo, represents the strength of mental dependence.

A devastating characteristic many patients suffering from addiction experience is the high change of relapsing after a prolonged period of sobriety[42]. The tendency of relapse is caused by neurosynaptic plasticity, characterized by a decrease in the ability to change activity in response to stimuli by recognizing the structure, function, or connection in the nervous system[43], [44]. Treatment for these patients suffering from addiction has improved immensely due to significant advantages in the investigation of the reinforcement system in the brain and cognitive neuroscience[45]. Still, an estimated 35 million people worldwide suffer from addiction to various drugs, which was stated in the world drug report of 2019 by the United Nations Office of Drugs and Crime (UNODC)[46].

A significant contribution to the high number of patients suffering from addiction is the lack of knowledge on the substances obtained illegally. The NPS's are sold without any information on the addictive properties to young people still in development. These drugs enable alterations in developing brains, increasing the risk of profound and long-lasting consequences[17].

Models in the Neuroscience of drugs of abuse

The brain is a complex organ consisting of the cerebrum, cerebellum, and brainstem. These structures control most of the activities in the body by processing, integrating, and coordinating information received from the sensor organs. Neurons are the functional units in the brain and are responsible for communication facilitated by neurotransmitters, which are small endogenous chemicals that bind to neuroreceptors. An overview of neurotransmission is visualized in Figure 2, where neurotransmitters travel through the axon terminal via synaptic vesicles. Synaptic vesicles contain proteins essential for transport, involved in neurotransmitter uptake and trafficking, which are crucial for vesicle exocytosis, endocytosis, and recycling. The vesicles move towards the membrane, followed by fusion due to elevated Ca2+ levels in the presynaptic neuron. The opening of the Ca2+ channels is initiated by the action potential invading the nerve terminal[47]. After the release in the synaptic clefts, the neurotransmitters will bind to the specific neuroreceptors located in the dendrites of the postsynaptic neurons. The binding of neuroreceptors and neurotransmitters triggers a response of the cell, which depends on the type of receptor[48].

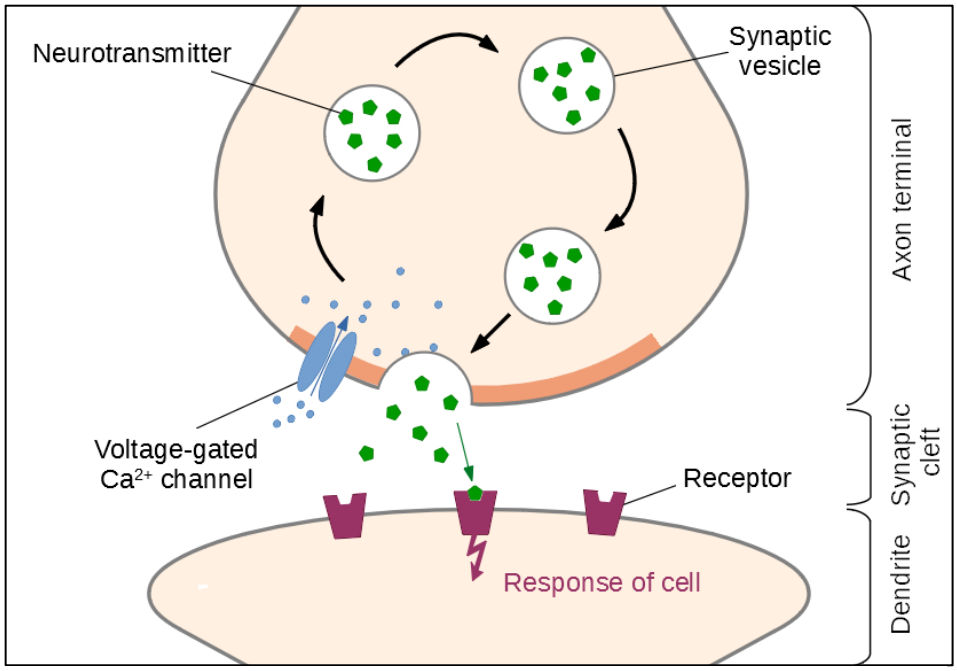

Figure 2 Schematic overview of the basic function of a chemical synapse (C. Schütte, 2004. et al)

Psychoactive substances trigger a response through the interference of the process described above, exploiting mechanisms such as antagonism, agonism, and blockage of neurotransmitter reuptake. Agonistic psychoactive drugs bind synaptic receptors, which increases the effects of the neurotransmitter. Antagonistic drugs also binds to the receptors but has an inhibiting effect on the neurotransmitter. According to the study conducted by *Dalangin*, the classical neurotransmitters has to meet the following criteria; presence in the neuron, stored in synaptic vesicles, released in Ca2+ - dependent matter, and possess a mechanism for its removal from the synapse[49]. The removal from the synapse is often through recycling, which entails the neurotransmitter reuptake by a neuro-transporter located in the membrane of the presynaptic neuron. Blockage of the reuptake of the neurotransmitter from the synaptic cleft results in an increased extracellular neurotransmitter concentrations and increased neurotransmission[50].

The identification and function of neurotransmitters abused by illicit drugs remain important in predicting possible adverse effects caused by these substances.

Introduction of the models

In neurotoxicology, the main focus is on the changes in function and structure of the nervous system as a result of chemical exposure and the interpretation of consequences and adversity of those changes. As explained above, the central nervous system is one of the most complex organs in the body, with specialized metabolic and physiological features that convey vulnerabilities to toxic compounds on multiple sites in different ways[45]. Throughout the years, the demand for a model for neurotoxicology has increased. In this report, three different models in neurotoxicology are compared and discussed, featuring; in vitro, rodent, and the zebrafish.

In vitro

The prospects of science include animal-free experimental set-ups. Currently, many studies consider the 3R's in their research. The 3R's entail; Reduction, Replacement and Refinement, in order to limit the amount of animals needed for each study as much as possible[51]. Therefore many research groups shift the focus on enhancing current in vitro experiments. The in vitro model has proven to be a crucial source of information in the field of neurology, allowing specific investigation on individual functions of complex brain structures. In addition, in vitro assays allow testing on human cell lines in a tight controlled chemical and physical environment, limiting translation variation and being closest to human testing as possible[52]. Notably, in vitro experiments have been proven helpful in the study of drugs of abuse. The popularity of drugs originates from the effects triggered in the brain, which are linked to specific neuroreceptors. Many receptors related to drug abuse have been identified and thoroughly studied in order to further understand the involvement in addictive and toxic properties. In vitro models allow investigation on the function of single receptors and the identification of drugs that could interrupt the regular activity. Currently, a variety of in vitro assays exist that can measure the effects on crucial mechanisms in de brain, including cell viability/ death, energy metabolism, glucose uptake, oxidative stress, calcium homeostasis, electrical activity, neurotransmission, axonal transport, receptor and channel activation, enzyme activity, and neuronal-glial interaction[53]. Psychoactive compounds have been linked to altering the normal process of the monoamine receptors, which can be measured with a fluorescence-based high-throughput assay or with single-cell imaging techniques[54], [55].

However, currently in vitro modelling is not enhanced enough to recreate the complexity of in vivo models, resulting in the constant use of laboratory animals. A crucial limitation of the in vitro system remains the inability to test behavioural endpoints. These tests have an advantage over pathologic measurements, as behavioural assays allow repeated evaluation of a single animal over time to determine the characteristics of a neurotoxic injury[56]. In vitro models are generally used to study the mechanisms of toxicity instead of the detection and for now play a complementary role to in vivo testing[53].

The Rodent

The brain is a fundamental organ that is connected to all the systems in the body, yet due to the vast complexity, much is still unknown. In order to study the effects of this multi-layered organ, whole animal experiments are still a necessary. The rodent, which includes the rat and mouse, is currently still the most popular model in biomedical sciences because of humans' genetic similarities. In addition, high resemblances to the human have been observed in the biological and behavioural characteristics. Behaviour is an essential endpoint in investigating the adverse effects of drugs. Therefore the use of animals in behaviour studies is continuously growing, with currently more studies employing the mouse over the rat model. This increase in mice studies can be explained by

the fast developments in genetic manipulation tools, which allow the modelling of various disorders[57]. Decades of research in neuroscience have taken place using the rodent model, which created a great source of information on behaviour and genetic characteristics[58]. Critical events in brain maturation, including neurogenesis, synaptogenesis, gliogenesis, oligodendrocyte maturation, and age-dependent behaviours, are consistent between humans and rodents[59]. However, it should be recognized that the translational value of the rodent model to the human remains far from perfect[58]. The function and structure of the brain cannot be completely investigated only based on information generated from animal models. The primary challenge remains to find the closest en most efficient method to further explore this complex organ[60].

The zebrafish

In recent years the zebrafish (*Danio rerio*) has become an increasingly popular model in neuroscience. The zebrafish is a small tropical fish initially from northern India and adjacent countries[61]. The general strengths of the zebrafish in biological research consist of rapid development, tractable genetics, ease in maintenance, and optical transparency in developing larvae[62]–[64]. Through the years, multiple studies have indicated the excellent potential of the zebrafish in linking genes with disorders of the CNS. The nucleotide sequence of the genes in the zebrafish is homologous (exceeding 70%) to the corresponding human genes, including similar functions [65]. However, genetic manipulation tools for the zebrafish are not as advanced in comparison to the mouse model. Unfortunately, the zebrafish brain is less complex than the mammalian brain, lacking the trisynaptic circuitry involved in complex learning[66]. Models for well-established disorders related to chronic drug use are not complete, limiting investigating and screening properties for the chronic effects of NPS.

Nevertheless, developments in large-scale mutagenesis and gene mapping, transgenesis, protein overexpression or knockdown, and chemical screens create opportunities to further investigate molecules in the brain and confirm their role in adverse function in humans and zebrafish. At five days post fertilization (dpf), the zebrafish displays a complete neurotransmitter system in the brain, which can be visualized[67]. These neurotransmitters are part of a relatively simple nervous system that differentiates to coordinate larval movement[68], a comprehensive collection describing multiple behaviours has been developed for further use of the model in neurotoxicology[69].

NPS are drugs that impact not only on a neurological level but have also been shown to disrupt multiple systems in an organism. In vitro assays are not developed enough to study the exposure effects on a multiple organ level. The zebrafish is a complete organism with strikingly similar toxicity profiles to mammals. This makes the zebrafish a promising model for the rapid detection of unsafe NPS found on the market[70].

The aim of this report is to evaluate the zebrafish model and the eccentric value in comparison with the current models in use for the risk assessment of NPS's . Table 1 describes multiple experimental setups focused on investigating the effects of seven classical drugs and one NPS, creating a comparison between the three models.

Table 1 Overview of classical drugs and new psychoactive compounds and the effects observed in the zebrafish model, rodent model and in vitro models.

Drug	Zebrafish model	Rodent model	In Vitro model
Ketamine	Increase in anxiety like behaviour and decrease in avoidance behaviour (6dpf)[71] Disorientation and hallucinogenic-like state (adult)[72]	Increase in aggressive behaviour, dysfunction of sensorimotor gating[73]	Decrease of differentiation form monocytes into immature dendritic cells through agonistic mechanism on the NDMA receptor[74].
Cocaine	Induces hypoactivity at high concentration exposure (6dpf)[75]	Enhanced processing of the motivational value[76]	Cocaine inhibits cell firing, by blocking dopamine uptake in dopaminergic neurones of the rat [77]
MDMA (ecstasy)	Reduced anxiety like behaviour (adult)[78]	"serotonin syndrome" [79] [80] Increased social behaviour and decreased aggressive behaviour[81]	Inhibiting the serotonin (5-HT) transporter, and also inducing 5-HT and NE release[82]
d-Amphetamine	Biphasic dose-response pattern (6dpf) [75]	Inducing stereotyped behaviour and locomotor hyperactivity [83]	Recognition of d-Amphetamine by x-ray crystal structures of dopamine transporter[84]
LSD	Disorientation and hallucinogenic-like state (adult)[85]	Decreased neophobic and investigatory behaviour (adult rats) [86]. Disruption pre-pulse inhibition(PPI), indicating hallucinating effects[87]	Agonistic effects on the 5-HT2A receptor in the cortex of rats[22].
Δ^9-THC	Biphasic effect on the locomotor activity[88]	Biphasic effect on the motor activity[89][90]	Decrease of dopamine uptake into crude synaptosome preparations[91] and degenerative changes in cultured cortical neurons though CB1 activation[92]
Fentanyl (NPS)	Hypoactive behavioural (5dpf), high acute toxicity ZET test, reduced locomotor activity [93]	Reduced behavioural activity, decreased sleep-wake state fragmentation, and eliminated REM sleep. Which is consisted with	Affinity and potency in binding to hMOR cells, [95]

		sedation[94].	
Meta-Chlorophenylpiperazine (mCPP) (NPS)	Hypoactive behaviour[96]	Dose-dependent hypolocomotion through sub-cutaneous injection[97]	Antagonistic effect on the 5-HT_{1A} receptor in the CNS of the rat [98]

Notably, the table highlights the contribution of in vitro experiments, where a specific investigation is performed on single brain structures. This information can contribute to more specific endpoints in the whole animal experiments, resulting in less animals needed[52].

Neurotransmitters implicated by drugs of abuse

The total number of neurotransmitters remains unknown, over a 100 have been established. Current knowledge suggest the involvement of glutamate, GABA, serotonin, dopamine, opioid and cannabinoid in the effects observed after illicit drug exposure. These drugs and receptors can further divided in the amino-acid, monoamine and other neurotransmitters.

Amino-acid neurotransmitters

Amino acids are organic compounds consisting of an amino- and carboxyl group, which functions as the fundamental building blocks for proteins. In the brain amino-acids are crucial as the main excitatory and inhibitory neurotransmitters; Glutamate and GABA[49].

Glutamate

Identification of glutamate as a neurotransmitter has taken a long time because of its abundance in the brain and involvement in multiple metabolic pathways. The neurotransmitter became a hot research topic after recognizing the excitatory action, which resulted in cloning several families of the glutamate receptors through molecular cloning. The receptors were later classified as N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), kainite, and metabotropic. Almost all the cells present in the brain express at least one of the glutamate receptors. Glutamate has been associated with neurodevelopment, learning and memory, general cognition, neurodegenerative disorders, and pathologic conditions[99].

The N-methyl-D-aspartate receptors (NMDARs) are a subclass of ionotropic glutamate receptors located in the central nervous system at the presynaptic terminal and postsynaptic membrane. These receptors play an essential role in the regulation of neuronal communication and synaptic function in the CNS[100]. The NDMA receptor has been linked to the dissociative anaesthetic; ketamine, which has an antagonistic effect, as seen in table 1. In a study conducted in adult zebrafish, the effects of ketamine on the expression of early proto-oncogene *c-fos* gene were compared in the rodent en fish model. The *c-fos* gene has been suggested to be involved in hallucinogen effects in the rodent model. The zebrafish presented similar effects to the rodent model, including; elevated *c-fos* levels, decreased cortisol, and changes in behaviour [101]. According to the data published by *Talise E. Müller*, the nucleotide similarity of the gene (GRIN1) for the glutamate ionotropic receptor NMDA between humans and the two analogs of the zebrafish is 78.81% (*grin1a*) and 92.86% (*grin1b*). Nucleotide sequence similarity between rodents and human is 87.19%[102].

Glutamate can be visualized in the zebrafish using the situ hybridization for vesicular glutamate transporters. Also, three subtypes of the ionotropic receptors NDMA have been found in zebrafish

embryos beginning at 20-24hpf. The expression of the metabotropic glutamate receptors in the zebrafish reveals similar patterns as observed in the rodent models, with expression in larval en adult phase in the olfactory bulb, optic tectum, hypothalamus, cerebellum, and retina[65].

γ-Aminobutyric Acid

γ-Aminobutyric acid, abbreviated as GABA, has been identified as the primary inhibitory neurotransmitter in the adult central nervous system. GABA functions as a signalling molecule to generate changes in signal transduction in the presynaptic and postsynaptic neurons. Glutamate is transformed into GABA through the decarboxylation process by glutamate decarboxylase. GABA is recycled via the GABAergic synapses with a mechanism analogous to the glutamate-glutamate cycle[6]. Based on current information, three subtypes of the GABA receptor have been identified in the mammalian brain, the ionotropic GABAA and GABAC, and the metabotropic GABAB[49], [103].

As early as 16hpf, can the start of the development of the GABA-immunoreactive cells be found in the telencephalic of the zebrafish embryo. At 3dpf, the zebrafish brain displays high similarities of the GABAergic neuron distribution compared to the mammalian brain. During development into adulthood, GABA expression remains similar to the larvae phase, however, it is more broadly distributed[65].

In 1955, the first benzodiazepine was discovered, Librium. The benzodiazepines are a class of anti-anxiety medication well studied for the GABA agonistic properties. Currently benzodiazepines are often detected as one of the compounds in mixtures taken by drug-related patients admitted to the hospital[30]. In a study on diazepine exposure in zebrafish, two forms of inhibitory effects on the locomotion were observed. At lower concentration, the larvae presented motor reduction, the higher concentrations resulting in immobility and high sedative- behaviour. These findings are consistent with the diazepine-induced behaviours observed in the adult zebrafish and rodent model[104]. In addition to the benzodiazepines, reports of effects on the GABA-receptor function and voltage-gated calcium channels by MDMA and amphetamine have been made[14].

Unfortunately, benzodiazepines became a significant substance of abuse, with increasing trends of combining the substance with classical drugs such as cocaine, heroin, and morphine. The increasing popularity of the drug caused the creation of more potent NPS variations[24].

Monoamine neurotransmitters

The monoamines consist of a particular chemical structure; one amino group connected to an aromatic ring by a two carbon chain. Depending on the on the precursor, the monoamine transmitters can be classified into two categories; the catecholamines (dopamine) and the indolamines (serotonin)[105].

Serotonin

Serotonin, or 5-hydroxytryptamine (5-HT), is an indolamines monoamine, one of the most ancient signalling molecules as it was already found in single-celled eukaryotes paramecium. 5-HT is synthesized starting with the amino acid tryptophan, which is transformed into 5-HT through the enzymes tryptophan hydroxylase and decarboxylase. After the release of 5-HT in the synaptic cleft, the neurotransmitter travels towards the postsynaptic, where 5-HT can bind to g-protein receptors, which initiates second messenger cascades[106]. Not surprisingly, the long evolutionary history and ability to bind to g-protein receptors resulted in a vital role of serotonin in multiple physiological processes, including cardiovascular, developmental, gastrointestinal, endocrinal, sensory perception, and behaviours such as; aggression, appetite, sex, sleep, mood, cognition and memory[106]. Currently, fifteen 5-HT receptors have been identified, which are distributed among seven distinct families. Differences between these families are primarily based on the amino-acid sequence and structural properties. The zebrafish only expresses four 5-HT receptors, *htr1aa, htr1ab, htr1bd*, and *htr2c*, analogs for the HRT1A, HTR2A, and HTR2C genes in the human. Sequence similarity is between 67.31% and 70.88% for the comparison between humans and zebrafish, which is lower than observed between rodents and humans, lying between 81.58% and 87.12%[102].

Three compounds listed in table 1, show specific interference with the 5-HT receptors. First, MDMA induces a euphoric effect by inhibiting the 5-HT receptor and serotonin receptor (SERT) and increasing the 5-HT and norepinephrine (NE) release[82]. In a study conducted in the zebrafish by *Norton*, gene expression of five crucial 5-HTergic pathways were recognized as similar in the brain of vertebrates. These findings, increase the value of the zebrafish model in drug screening, as serotonin is a pathway commonly abused by psychoactive compounds[107]. The second compound that targets the 5-HT2A receptor specifically, is the popular synthetic hallucinogen LSD (lysergic acid diethylamide). LSD induces both hallucinating and decreased exploratory effects in both the rodent and zebrafish models, supporting experiences observed by humans[85]. The last compound listed in table 1 is the NPS mCPP, which has become a popular drug of abuse and is often observed to be taken with MDMA. mCPP has shown interaction with the 5-HTc receptor in rodent. The zebrafish displayed strong similarities in behaviour in comparison with the rodent model, suggesting that alterations in the 5-HT receptor occurred after mCPP exposure[96].

Dopamine

Dopamine is a monoamine catecholamine neurotransmitter synthesized by removing a carboxyl group from the precursor chemical dihydroxyphenylalanine (L-DOPA), which is converted from tyrosine by tyrosine hydroxylase (TH) in the brain and kidneys. The neurotransmitter has been identified as a crucial factor in memory, attention, impulse control, locomotion, sleep, learning, cognition, and decision making[45]. The established dopamine receptors are located in the central nervous system and consist of 5 metabotropic, G protein-coupled receptors[108]. As listed in table 1, dopaminergic pathways are linked to various drugs, as dopamine plays a vital role in the human reward system [64].

Dopamine has been linked numerous times with addiction, especially cocaine, which has a reinforcing and rewarding effect that often leads to abuse and addiction. Addiction is primarily mediated by inhibiting DA uptake through the blocking of the dopamine binding site, resulting in increased presynaptic dopamine levels, in which the consumer will experience euphoric effects[45], [109], [110].

Like the mammal model, dopamine is a vital catecholamine neuroreceptor involved in learning, memory, motor control, food intake, and motivation in the zebrafish. The first dopaminergic neuron development is detected around 18hpf, followed by rapid development of dopaminergic clusters during the larval phase[65]. Three analogs have been identified for the human dopamine receptor D1 and D2; drd1, drd2b, and drb2c, presenting sequence similarities between 70.99% and 78.43%. The rodent model has a sequence similarity of 74.56% for DRD1 and 87.92% for DRD2[102].

Peptide neurotransmitters

Peptides are involved in the communication in the body between the organs. Peptides consist of amino-acids, and can function as hormones and neurotransmitters[111].

Opioids

The opioid receptor, located in the peripheral nervous system, is the target of the highly abused category of drugs; opioids[26]. The opioid system plays a role in regulating pain, emotions, sociality, and reward, which explains the suggested association with anxiety, depression, schizophrenia and addiction.

The mu-, kappa- and delta-opioid receptors (MOP, KOP, and DOP) are considered the classical receptors in humans and are well-studied in patients suffering from substance abuse[112]. The analogs for the functional genes of the classical receptors have been identified in the zebrafish and the rodent model. The OPRM1 (opioid receptor mu1) is expressed as *oprm1* in the zebrafish with a nucleotide sequence similarity of 77.68% compared to 84.70% between rodents and humans. The second gene, OPRKA1, which translates for opioid receptor kappa1, is identified as analogue *oprk1* with a similarity of 76.42% between humans and zebrafish compared to 73.73% between de humans and rodents. The last receptor described is the opioid receptor delta1 (OPRK1), which is linked to two analogs *oprd1* and *oprd1b*. The nucleotide sequence similarity between the human zebrafish is 73.79% (*oprd1*) and 76.99% (*oprd1b*) compared to 86.70% between human and rodent[102].

Fentanyl is currently a hot topic of interest in the USA. This compound is added to classical drugs to increase potency, resulting in extremely fast-growing numbers of people suffering from addiction. As seen in table 1, this drug shows an affinity for the MOP receptor, which plays a vital role in the central and peripheral nervous system to elicit analgesia[95], [113]. These opioid receptors have been well established and investigated for similarity in the rodent model. However, the high potential of the zebrafish model allows more research to be done on the translation to the mammalian model[114].

Other neurotransmitters

Cannabinoid

Lastly, the cannabinoids are found in cannabis preparations and bind to the same receptor as Δ9-tetrahydrocannabinol (Δ9-THC), the cannabinoid receptor. The cannabinoid receptor has been popular target for a variety of medical drugs used for various properties such as; nausea and vomiting during chemotherapy, stimulate appetite in adults, and relieve neuropathic pain. The receptor consists of 2 subtypes, CB1 and CB2, and both are G protein-coupled[115]. Activation of the CB1 receptor leads to the inhibition of adenylyl cyclase, activation of mitogen-activated protein kinases, inhibition of specific voltage-gated calcium channels, and the activation of G-protein-linked inwardly rectifying potassium channels. The activation of these pathways leads to neural suppression and inhibition of neurotransmission[116]. After the discovery of CB1, CB2 have been identified as a regulator of immune responses, mainly expressed by cells of the hematopoietic system. CB1 in currently known known for being responsible of the psychoactive effects in the brain[117].

The genes encoding for the cannabinoids receptors have also been identified in the zebrafish, *crn1*, and *crn2*. The nucleotide sequence similarity found between human CNR1 and zebrafish is 75.53% compared to the rodent similarity of 82.22%. The second receptor presented a similarity of 100% between human (CRN2) and zebrafish compared to a similarity of 79.21% for the rodent and human[102].

Drugs of abuse and behaviour

Behaviour can be considered a sensitive indicator of possible interference in the normal process of neurotransmission. The golden standard in behavior studies has been the rodent model. However, in this report, the focus is on the possibilities of the zebrafish model. Depending on the endpoints of a specific study, a model can be chosen based on the complexity of the behaviours. As seen in figure 3, various models are being used in biomedical sciences. The zebrafish is scaled somewhere in the middle based on throughput and complexity of behaviours[66].

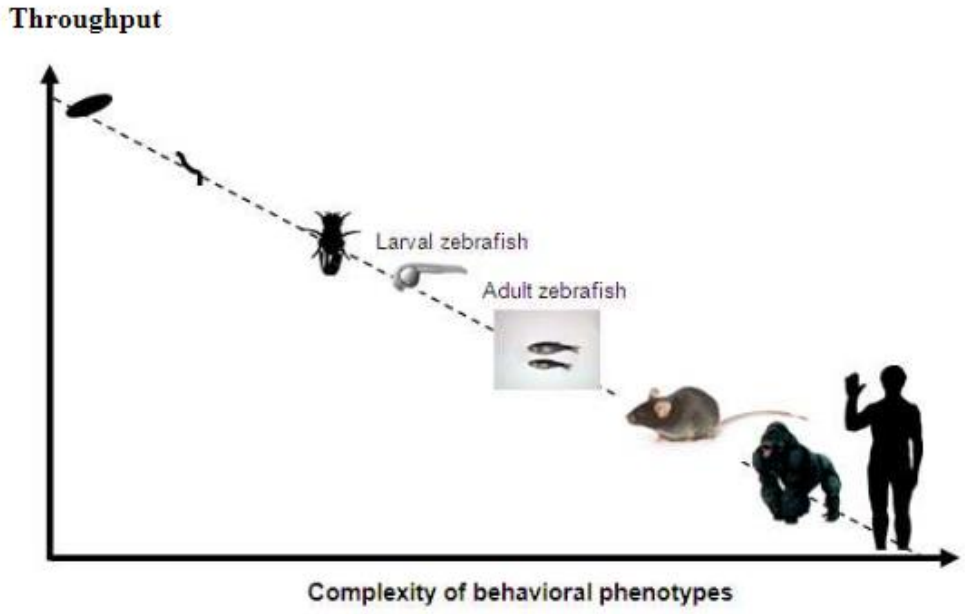

Figure 3 Scale overview of the models in biomedical sciences. Adapted from "The Developing Utility of Zebrafish in Modelling Neurobehavioral Disorders,", A. Stewart, F. Kadri, J. Dileo, K. Min Chung, J. Cachat, and A. Kalueff. International Journal of Comparative Psychology, 2010.

Based on the described effects of these drugs, the model needs the ability to measure alteration in behaviour involved in stress, aggression, memory, cognition, energy, and awareness.

Stress and anxiety

Stress and anxiety can cause behavioural, biochemical, and immunological changes in response to various stressors. High amounts of stress and anxiety can therefore cause increased vulnerability of the organism[118].

The open-field test has been well established in identifying anxiety in rodents, where exploratory behavior and general activity are measured as endpoints. The rodents have a natural aversion to a bright open space, however, they also have a drive to explore. Decreased anxiety can be linked to increased exploratory behaviour. A conceptionally similar test for the zebrafish consists of the novel tank test, which evokes a robust anxiety response as they dive to the bottom of the tank until they become acclimated to the environment. As in mice, zebrafish have the tendency to move towards walls and corners[66]. Improvements in the automated video-tracking techniques provide a more standardized unbiased approach for the quantification of zebrafish behavior[69].

Another popular experiment to study the effect on anxiety in rodents is the light/dark box based on the aversion to open illuminated areas. Previous research has proven that anxiogenic drugs can have the opposite effect, and decrease anxiety causing increased exploratory effects. As described by *Adam Stewart,* zebrafish demonstrate similar utility of the light-dark situation in the modelling of anxiety. The exploration-based experiments in the zebrafish display similar evolutionarily conserved factors are involved compared to the rodent model[119], [120].

Aggression

Aggression is an essential state for fitness and survival in organisms. However, drugs have proven to contain the ability to induce aggression by altering neurotransmitters such as; DA, NE, GABA, and 5-HT. Notably, similar neurotransmitters and brain areas have been linked to aggressive behavior in other vertebrates, which highlights the translatability of the zebrafish model[65]. Commonly, drugs are involved in impulsive aggression, which proposes a great threat for society. Users are not capable of reasoning and proper decision making, creating great risk for themselves and people surrounding them[121]. Therefore, aggressive behaviour needs to be detected in the screening model.

Like the rodent, a zebrafish is an excellent approach for detection of triggers involved in aggression. The novel genetic and neurobiological tools, further helped to understand the stereotypical body postures that the fish use to display aggressive behavior. Other behavior traits expressed by the zebrafish in an aggressive state include; extending fins to circling, chasing and attempts to bite, increased frequency of chases, and attempts to strike the opponent[65]. Aggression can be measured in the laboratory by monitoring two fish in a tank or recording the interaction of one fish with a mirror (mirror induced aggression)[120].

Memory and cognition

Memory is the capacity to receive and store information, which is crucial to all forms of learning. Cognition is the process of acquiring knowledge and understanding through thought, senses, and experience. The most straightforward method of examining memory and cognition is habituation behavior, which attenuates innate behaviours as test subjects become accustomed to new environments[56].

Only recently, the consumption that fish behavior was mainly instinctively driven has been disproven. The zebrafish has been proven to have the capability of forming spatial memories and cognitive maps. The Novel Object Recognition test (NORt) enables the assessment of the recognition memory. In the research conducted by *Matteo Bruzzone*, the NORt was performed on zebrafish larvae in an early stage of development. This study suggested the presence of recognition memory. However, non-cognitive factors may hinder the NORt application in the zebrafish because of the early stage of development[122]. Additional studies have been suggesting the ability to habituate to various stimuli, such as conditioned place preference, light/dark locomotion, and the startle reflex[65], [66], [120].

Another method of testing the effect on cognition is the ability of hatched larvae to catch any potential food, also referred to as prey capture. This complex locomotor behavior can be measured as early as four dpf, by placing tiny air bubbles in the test area. The response of the zebrafish to the air bubbles provides information on recognition, approach, decision making, and capturing[120].

Energy and awareness

One of the most popular characteristic of a ‘’partydrug’’’ is the enhanced energy levels, resulting in the ability of prolonged partying. These compounds can suppress the natural urge to sleep, which is followed by a recovery pattern in a healthy individual. However, chronic use can lead to an abnormal recovery pattern resulting in chronic lack of sleep. Many studies have been dedicated to proving the importance of quality sleep, having direct effects on energy, motivation, concentration and metabolism[123].

The zebrafish has a similar sleep pattern as the human, as the larvae showed a higher sleep percentage than the adults. Through video-tracking systems, activity during a 24-hour cycle can be measured and used a high-throughput screening of compounds. A simple but effective method of evaluating the awareness of a test subject is with the startle response. Test subjects are exposed to sudden, abrupt, and robust stimuli, consisting of sound or unexpected touching. The response provides information on the status of the integration of sensory and motor stimuli. The visual startle response in the zebrafish develops at day three post-fertilization, measured with the visual motor response test. On day 5, the larvae represent the acoustic startle response, in which response is measured to sound frequencies higher than 200Hz[33].

The hallucinogens are characterised by sensory experiences that appear real but are created in the mind. These effects can be divided in different stages based on the strength of the hallucination, varying from mild, also referred to as the comforting stage, to severe. Severe hallucinations, the controlling phase, can cause extreme anxiety and induce panic attacks in the user. Users lose attachment to reality, resulting in uncontrolled behaviour and consequences. In the zebrafish, hallucinating properties have been investigated by the earlier mentioned light and dark test. Parallel to the rodent model, LSD induced distorted perception which was observed the less entries in the light part in the light and dark test. These findings suggest an common behavioural profile of hallucinogens in different species[85].

Discussion

Throughout this report, the zebrafish has been compared to existing models in the screening of NPS's. The biggest limitation of the current approach, which mainly consists of risk assessment of neurotoxicity in the rodent model, remains the low number of substances that can be tested each year. In January 2020, the emerge of 950 NPS´s were reported to the UNDOC[18]. Therefore, a high throughput screening method for NPS is crucial.

Important factors involved in toxicological characterization include dosing, method of administration, individual sensitivity, chronic/ repeated use, interaction with other compounds in the drug (contamination) and interaction with other drugs (especially alcohol). These are essential factors that need to be incorporated in a potential novel model for a complete risk assessment[14].

The main goal of screening psychoactive compounds is to minimize to biological consequences of recreative drug use in current society. Therefore, the most important characteristic of a potential screening approach is the high translational value towards the human. As described, the rodent model is currently mainly in use, this model not only helped further understand the human biology, genetics and diseases but is also involved in the investigation of lineage-specific evolution[124]. However, the rodent model can test only a limited number of compounds each year. The zebrafish has been evaluated in this paper as a potential novel high throughput screening for the NPS's.

The zebrafish model displays high similarities to the human brain, as they consist of a fore-, mid- and hind brain, including diencephalon, telencephalon, cerebellum, and a peripheral nervous system with motor and sensory components[67]. However, in the zebrafish, the telencephalon only consists of a rudimentary cortex, resulting in more 'simplified' behaviours than humans. Nevertheless, the zebrafish still displays integrated neural functions, including memory, conditioned responses, and social behavior. In addition, the nucleotide sequence of the genes in the zebrafish is homologous to the corresponding human genes (exceeding 70%), including a frequent similar function[65].

The main pathways abused in recreative drug use include glutaminergic, GABAergic, dopaminergic, serotoninergic, opioid, and cannabinoid. The analogs of the majority of the genes involved in these pathways have been identified in the rodent and zebrafish model[38]. The similarities of the nucleotide sequence of the corresponding genes between the animal models and humans were listed in the article published by *T.E. Müller* in 2020. Notably, the genes encoding for fundamental receptors presented close similarities between rodents and zebrafish, which is promising for the zebrafish[102].

Even though the zebrafish presents promising potential regarding the translational value, it is worth nothing; no model can completely mimic the complexity of the human brain. In research, we strive to find the model with the ability to screen compounds with the highest quantity and quality. The zebrafish model needs fewer funds, time, and resources for the experimental setup. This approach would increase the amount of substances that can be subjected to toxicological characterization, which is necessary as the numbers of NPSs keeps growing each year[1].

The translational value of the zebrafish is promising, allowing further exploration of the applications of this model for different factors influencing the adverse consequences of recreative drug use.

Many of the adverse effects of recreative drug use are linked to changes in behaviour, making this an essential endpoint in the screening model. As stated earlier, biomedical science is moving towards animal-free research. However, in vitro assays are not enhanced enough to model behavior and chronic toxicity[70]. The EU directive 2010/63 EU on the protection of animals used for scientific purposes states that, ''the earliest life-stage of the test subject are not considered protected''. Independent feeding is considered the framework of dealing with an animal experiment, meaning

the zebrafish can be used with limited constriction until five dpf[125]. Behavioural assays, including startle response measurements, prey capture, sleep monitoring, and light-dark locomotion, can all be applied before the larvae passes the five dpf. These assays provide information on awareness, anxiety, aggression, energy, cognition, and memory[120]. However, the larvae still do not exhibit the rich behavior of adult zebrafish, lacking the fully developed endocrine systems and some neural circuits and projections. Also, the protective mechanism are not fully developed in the larvae, making the zebrafish in early development more sensitive to external exposures[66].

A severe consequence of recreative drug use is the development of addiction, a complex neurobehavioral disorder. These levels of disorders are genetically complex and validated biological markers for characterizing and distinguishing are still lacking[67]. The most important characteristics of addiction include reward seeking behaviour, sensitivity towards rewarding effects, and tendency to relapse after prolonged abstinence. Recent studies have further investigated the effects of punishment on reward seeking behaviour and the frequency of relapse in the zebrafish. The findings included changes in gene expression, consistent with the development of dependence. The currently identified genes and neural pathways involved in chronic drugs use, are well conserved between mammals and fish[65]. Furthermore, withdrawal plays a devastating role for the patients suffering from addiction, this endpoint should be measurable in the model. An method was proposed by *Adam Stewart,* indicating that increased cortisol levels observed in zebrafish experiencing withdrawal were consistent with glucocorticoid dysregulation in rats and human with withdrawal syndrome[66]. In addition, the zebrafish model is developing quickly with improved guides on translational behavior and enhanced genetic techniques, including a variety of mutants. An example is the NAD^- -mutant (no addiction), this mutant does not display addiction after chronic amphetamine exposure. This model can help further identify genes involved in addiction development to these compounds[67]. Modelling addiction in the larvae stage remains challenging[67].

Individual sensitivity has also been listed as one of the factors influencing adverse effects after drug use. Users vary on notorious important factors, important factors include gender, age, size, tolerance, medical background and psychiatric status[45]. Woman have been proven to be more sensitive to psychoactive compounds, as a result of different hormones, body composition and metabolism. Risk assessment needs to be based on both male and female exposures, the female zebrafish have shown to be more sensitive to a chronic alcohol exposure compared to the male fish[126]. However, information on differences in sensitivity to other compounds is lacking. Next is tolerance, which means a decrease in drugs sensitivity resulting higher in doses needed to obtain the same effects. The zebrafish has been extensively used for the modelling of alcohol tolerance, it is proven that chronic exposure to ethanol led to reduced responses in adult zebrafish[127].

Another contributor to the effects of drugs is the route of administration. The most common methods of administration include injecting, smoking, inhaling, snorting and swallowing[5]. The different routes have an effect on the absorption, bioavailability and metabolism of the compound, which has a great influence on the time between exposure and observed effects. In the mammals most of the compounds are metabolized in the liver by the CYP family, with CYP3A as the most important of these enzymes[63]. CYP3A orthologous have been identified in the zebrafish and expression was observed in the liver and intestine in the larvae and adult phase. In the study conducted by *Yi Mi Park,* an comparison of the metabolites after exposure to synthetic cannabinoids in human urine, HepaRG cells, and microinjected zebrafish was performed. A similarity of 70% was observed for the metabolites between the human urine and the zebrafish which were microinjected in the caudal vein. In conclusion, the zebrafish was presented as an excellent in vivo model for the further investigation of drug metabolites and the corresponding effects in the brain and behaviour[128]. However, a study conducted in 2018 showed significant differences in the

absorption of the NPS mCPP in the zebrafish in comparison to the rodent and human. Absorption from the media into the larvae was measured as 40 times higher compared to absorption in the plasma of humans after oral administration, which could be explained by the less barriers present in the zebrafish. In addition, differences were observed in the uptake of mCPP in the effective tissue. Despite these significant differences, similar behaviour and further kinetics were observed in response to internally measured mCPP concentrations in the zebrafish, rodent and human as seen in Table 1[96].

Unfortunately the differences in absorption and lack of information on the conversion of dose between fish and human, poses a problem for toxic dose predictions, especially for the most common rout of exposure; oral ingestion. However, as the zebrafish becomes more popular in biomedical research, the amount of studies focussing on dose conversion increases. In 2020, a study determined the concentrations of chemotherapy bases on a formula which included data on the concentrations in the fish water, concentration of chemotherapy administered to humans, volume of human blood, and the equivalent dose. The values calculated were further established by matching to previous collected data from safety studies. Findings were in good agreement with observations in clinical studies[129]. Even though more studies are investigating the conversion of dosing between human and zebrafish, it is still far from the more established guidelines for the rodent model[130].

Lastly, as seen in figure 1, drugs are often used in combination with other drugs. Hospital records often show the presence of polydrug in the systems of people who were admitted to the intensive care, in drug related cases[131]. Although polydrug use is very common, the understanding of the effects of multiple substances and the analysis of various drug-drugs interactions, received little attention in previous and current research[96]. The effects of drug-drug interaction have been studied using the zebrafish, for medically used substances. In a study focused on the effects of ketamine and cyclosporine exacerbates (CsA), zebrafish embryos were exposed (24hpf) to the compounds in a mixture and separately. Exposure to the mixture led to increased lethality in comparison to exposure to only ketamine or CsA[132]. Information on drug-drugs interaction could provide a safer environment for users and prevents deaths of unexperienced users.

Conclusion

As the behavioural and genetic information and tools for the zebrafish continue to develop quickly, the model creates a great platform for the investigation of neurological disorders and the contribution of psychoactive substance abuse. In this report a small section of the possibilities of the zebrafish have been highlighted, with fast improvements of the genetic tools, more functional genes being identified each year. The larval and adult zebrafish are becoming key models in the field of neuroscience, representing a useful novel model for translational risk assessment on the drugs of the CNS.

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