Behavioural Brain Research 292 (2015) 537–546

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

Research report

Ketamine induces anxiolytic effects in adult zebrafish: A multivariate statistics approach

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HIGHLIGHTS

- Ketamine is an anesthetic agent with anxiolytic properties.
- Zebrafish is a promising animal model for pharmacological research.
- Anxiolytic effects of ketamine were investigated in zebrafish.
- Ketamine reduces anxiety-like behaviors, inducing a behavioral standardization.
- Multivariate statistics is a suitable method in behavioral data validation.

ARTICLE INFO

Article history:
Received 18 May 2015
Received in revised form 1 July 2015
Accepted 4 July 2015
Available online 15 July 2015

Keywords:
Zebrafish
Ketamine
Anxiety
Behavior analysis
Univariate statistics
Multivariate statistics

ABSTRACT

Ketamine inappropriate use has been associated with serious consequences for human health. Anesthetic properties of ketamine are well-known, but its side effects are poorly described, including the effects on anxiety. In this context, animal models are a safe way to conduct this neurobehavioral research and zebrafish (*Danio rerio*) is an interesting model which has several advantages. The validation and interpretation of results of behavioral assays requires a suitable statistical approach, and the use of multivariate statistical methods has been little explored, especially in zebrafish behavioral models. Here, we investigated the anxiolytic-induced effects of ketamine in adult zebrafish, using Light–Dark Test and proposing the Multivariate Statistics methods (PCA, HCA and SIMCA) to analyze the results. In addition, we compared the processing of data to the one carried out by analysis of variance (ANOVA) ketamine produced significant concentration of exposure-dependent anxiolytic effects, increasing time in white area and number of crossings and decreasing latency to first access to white area. Average entry duration behavior resulted in a slight decrease from control to treatment groups, with an observed concentration-dependent increase among the exposed groups. PCA results indicated that two principal components represent 88.74% of all the system information. HCA and PCA results showed a higher similarity among control and treatment groups exposed to lower concentrations of ketamine and among treatment groups exposed to concentrations of 40 and 60 mg L^{-1}. In SIMCA results, interclasses distances were concentration of exposure-dependent increased and misclassifications and interclasses residues results also support these findings. These findings confirm the anxiolytic potential of ketamine and zebrafish sensibility to this drug. In summary, our study confirms that zebrafish and multivariate statistics data validation are an appropriate and viable behavioral model for the study of psychoactive substances, providing a detailed and reliable analysis.

1. Introduction

Introduced as an anesthetic agent in 1965, ketamine (KT) is a noncompetitive *N*-methyl-D-aspartate (NMDA) receptor antagonist which affects glutamatergic activity blocking the ion influx on the channel controlled by NMDA [1–4]. KT also binds to α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate (AMPA) receptors, GABAA receptors, opioid receptors, cholinergic receptors, substance P receptors, dopamine D-2 receptors, 5-HT_2

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http://dx.doi.org/10.1016/j.bbr.2015.07.017
0166-4328/

receptors, and voltage sensitive Ca^{2+} and Na^{+} channels, at physiologically relevant concentrations [2]. Although it is widely used as a veterinary anesthetic, KT has been used since the 1970s as a club drug and, in recent years, the misuse of the KT has been extensively reported. The inappropriate use of KT may induce potent pharmacological effects and serious health problems, due to complex pharmacology of this drug. In general, NDMA receptor antagonists can cause severe deficiencies and behavioral disorders [5]. In some reported cases, high doses of ketamine administered for a prolonged period were responsible for inducing various side effects [6]. In this sense, the use of animal models is an important tool for prediction of toxicological effects of KT at non-therapeutic doses.

Although the use of rodents, cats and dogs in toxicological research is traditional and well-established, these mammalian models offer several disadvantages. High costs, large quantities of reagents and solvents, large spaces and laborious maintaining represent barriers to use mammalian models in research. In this sense, aquatic models are becoming popular in pharmacology and toxicology research, with several fish species showing efficacy as model systems for numerous human diseases [7,8]. The zebrafish (*Danio rerio*) has emerged as an interesting alternative to mammalian models for pharmacological and toxicological tests and today is the fish species most commonly used in laboratories [8–12]. Native to Southwest Asia, the zebrafish has high physiological and genetic homology to mammals, including humans, robust behavioral responses, external fertilization, rapid and short development and life cycle, transparency of embryos and larvae and ease of experimental manipulations requiring small spaces and low amounts of reagents and solvents [9,13–18]. Zebrafish also exhibits similarity with humans in neurochemistry and endocrine responses. Humans and zebrafish has the same time dynamics stress response and has cortisol as main stress hormone, unlike rodents [16]. Neurotransmitter receptors such as GABA, glutamate, dopamine, noradrenaline, serotonin, histamine, and acetylcholine (including expression patterns, binding and signaling), transporters and enzymes of synthesis and metabolism are similar to those observed in humans [19–21]. All these experimental advantages make the zebrafish model a great alternative in toxicological and pharmacological research.

The behavior spectrum of zebrafish is complex and allows the development of a range of behavioral parameters [14]. In the scototaxis (light–dark preference) behavioral model, anxiolytic effects of pharmacological agents are rapidly assessed and quantified [22,23]. As adult zebrafish has an inherent preference for dark environments [22,24,25], the Light–Dark Box Test can be used to study the specific behavioral anxiolytic-like responses. This test is based on conflict between the aversion of the animal to bright area and preference for protected areas and the innate tendency to explore novel environments [22]. This test is a well-established behavioral model of anxiety which permits animal to explore compartments that vary in size, color (white/black) or illumination (bright/dim) [26,27]. In this study, we used a model which compartments receives the same amount of light, but it varies in colour (white/black). In this test, anxiolytic effects are associated with increased time in the white compartment and increased swimming activity, which is measured by the total number of crossings between both compartments [8,11,13,22,27]. For example, Gebauer et al. observed that benzodiazepines—which presents potent anxiolytic effects—and ethanol increased the time in light compartment [13]. Some studies have been performed to investigate the behavioral effects of KT in zebrafish. In anxiety states, the acute effects of KT were described by Riehl et al. [8]. According to the evaluated behavioral paradigms, KT has an anxiolytic profile, similarly to observed in rodents exposed to this drug [8,28].

In behavioral assays, several parameters (variables) are determined for each animal, and a wide range of data is generated. Analysis, validation and interpretation of all data require a statistical approach in order to describe an experimental system. Univariate analysis is a statistical approach which each variable is examined individually in a data set. This method requires a large number of tests for the optimization model and does not consider the interactions between variables which describe the system [29,30]. In this sense, Multivariate Analysis is more suitable for validation of behavioral data. Multivariate Analysis is the measurement of several variables for each sample and provides information about interaction of them with a small number of experiments [29,31,32].

Different methods can be used for pattern recognition. The aim is to evaluate the data and determine the similarity among them. Principal component analysis (PCA) is a method that extracts the patterns in the original data matrix and develops a new model with a new set of variables (called Principal Component, PC or Factors). These new variables concentrates most of the system information in a few variables, reducing the dimension of the system, without losing significant information [33,34]. Hierarchical cluster analysis (HCA) is an exploratory method which identifies clusters and natural patterns of the samples and determines the similarity of the data according to the distance between points (samples or variables) in a two-dimensional space [31,35]. The Soft Independent Modelling of Class Analogy (SIMCA) method determines an optimal number of principal components independently for each class [33,36]. PCA and SIMCA methods enable the identification of anomalous behavioral results based on all set of quantified variables (behavioral endpoints), defined as outliers. Outliers are extreme values which exerts a disproportionate influence on the analysis, distorting the statistics and lead to non general results [37]. The criterion for multivariate outliers is Mahalanobis distance, evaluated as χ^2 with degrees of freedom equal to the number of variables [38].

Although the pharmacological properties of KT for anesthesia are well-characterized, side effects of this drug, such as anxiolysis, remain poorly understood. In this sense, the analysis and validation of results of behavioral tests in zebrafish requires a powerful and robust statistical method. The aim of this study was the validation of the KT anxiolytic-induced behavior results using Multivariate Statistics methods, which has been little explored in zebrafish behavioral research.

2. Methods

2.1. Animal and housing

A total of 124 adult wild type short-fin zebrafish (6–8 months/3.6–4.0 cm long/50% male and 50% female) were obtained from a local commercial distributor (Aquatica Ribeirao®) and housed in groups of 30 fish in a 30 L tank. The tanks are filled with water dechlorinated previously treated with liquid filter biological material Sera® Bio nitrivec and water conditioner Sera® aquatan (to remove chlorine, chloramines and heavy metals). The maintenance of water quality was carried out by mechanical, biological and chemical filtration (using activated carbon) constant. The water was maintained at 26 °C ± 2 °C, with pH 7.5, and the illumination was provided by fluorescent light lamps with lights turned on at 08:00 h and off at 18:00 h according to the standards of zebrafish care [39]. Feeding was carried out twice a day with an automatic feeder. All animals were kept in quarantine before the beginning of the experiments for acclimatization to the laboratory environment and to identify sick specimens. Animal experiments were approved by Ethics Committee on Animal Use (CEUA) of University of Sao Paulo (Protocol 14.1.750.53.6) and adhered to National and Institutional guidelines and regulations.