EFFECTS OF SELECTIVE α4β2 NICOTINIC ACETYLCHOLINE RECEPTOR (nAChR) LIGANDS ON THE BEHAVIOUR OF ADULT ZEBRAFISH (Danio rerio) IN THE NOVEL TANK DIVING TASK
(Efecto de ligandos selectivos del receptor nicotinico α4β2 (nAChR) sobre el comportamiento de peces cebra (Danio rerio) en el test de nado en un tanque nuevo)

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ABSTRACT

The same characteristics that have made zebrafish a powerful tool for developmental biology, in the past years have transformed it into an attractive model for drug discovery. It is easy to obtain large amounts of individuals for experimentation, they are cheap to maintain and they can absorb chemical substances from their tank water. The effects of nicotine on behaviour have been studied in several animal models, as well as in humans. In zebrafish, an anxiolytic effect of nicotine has been recently shown in a novel tank protocol. In the present study, we used this test on zebrafish to compare the behavioural effects of three drugs acting on nicotinic acetylcholine receptors: nicotine, cytisine and erysodine. Measurements of the swimming distance travelled and bottom dwelling were used to determine changes in the locomotor activity or dwelling behaviour. Our results agree with previous studies showing that nicotine has an anxiolytic effect in the novel tank test. Indeed, this effect is mediated, at least in part, by the α4β2 nAChR subtype, since erysodine decreased the effect of nicotine. In addition, the α4β2 agonist cytisine elicited nicotine-like behaviours, indicating that this effect is not restricted to nicotine but can be exerted by other selective nicotinic agonists.

Keywords: nicotinic receptors, nicotine, erysodine, cytisine, bottom dwelling behaviour, locomotor activity, zebrafish.

1 INTRODUCTION

The same features that have made zebrafish a powerful tool for developmental biology, have transformed it in recent years into an attractive model for drug discovery (Zon and Peterson, 2005; Rubinstein, 2006; King, 2009; Chakraborty and Hsu, 2009). Zebrafish exhibit a short development time and almost all organs are developed at 7 days post-fertilization; their fecundity makes it easy to obtain large numbers of individuals for experimentation, and they are cheap to maintain. In addition, they can absorb chemical substances from their tank water, and their genome is almost fully sequenced, which makes genetic manipulation more accessible. These characteristics have stimulated the use of zebrafish in medicinal chemistry to assay the effects of different compounds in whole animals (Goldsmith, 2004; Kaufman and White, 2009). Researchers have only recently discovered the potential of zebrafish for neurobehavioural studies (Levin et al., 2007; Bencan and Levin, 2008; Eddins et al., 2010). Thus, this animal has been used as a model in studies of memory (Levin and Chen, 2004), anxiety (Levin et al., 2007; Bencan et al., 2009), reinforcement properties of drugs of abuse (Ninkovic and Bally-Cuif, 2006), neuroprotection of dopaminergic neurons (McKinley et al., 2005) and movement disorders (Flinn et al., 2008).
Nicotine has been widely studied, especially concerning its role in tobacco addiction (Le Foll and Goldberg, 2006). However, several additional effects on the central nervous system have been described, affecting, e.g., locomotion and movement (Coolon and Cain, 2009; Li and Bevins, 2009) and depression (Paperwalla et al., 2004; Pedersen and Von Soest, 2009). The effects of nicotine on behaviour have been studied in several animal models (Le Foll and Goldberg, 2006; Coolon and Cain, 2009; Li and Bevins, 2009) as well as in humans (Paperwalla et al., 2004; Pedersen and Von Soest, 2009). In zebrafish, nicotine has anxiolytic properties, an effect that has been recently demonstrated in the novel tank protocol (Levin et al., 2007; Bencan et al., 2009; Egan et al., 2009). Additionally, different subunits of nicotinic acetylcholine receptors (nAChR) have been cloned and found to be expressed in zebrafish (Zirger et al., 2003; Ackerman et al., 2009), indicating that the effects observed for nicotine could be mediated by these receptors. Indeed, it has been shown that both α7 and α4β2 nAChR antagonists block the behavioural effects induced by nicotine (Bencan and Levin, 2008), further supporting the involvement of these receptor subtypes in nicotine’s anxiolytic effects.

Cytisine is an alkaloid found in the seeds of Cytisus laburnum and many other plant species. It has been used for decades as an aid for smoking cessation (Tutka and Zatoński, 2006; Tutka, 2008), mainly in Eastern and Central Europe. Cytisine is a potent partial agonist of the α4β2 nAChR subtype, which is believed to be essential in the reinforcing effects of nicotine associated with smoking addiction (Champtiaux et al., 2003). Its behavioural effects have been tested in mice, but never before in zebrafish. Erysodine, on the other hand, is an alkaloid found in the seeds of Erythrina species whose pharmacology has been poorly studied. It has been shown earlier that, when compared with its better known analogue dihydro-β-erythroidine, erysodine displays higher affinity for rat brain nicotinic sites labelled with [3H]cytisine or [3H]nicotine and better selectivity versus receptors labelled with [125I]α-bungarotoxin (α7), and also neuromuscular-type nAChR in Torpedo electroplax (Decker et al., 1995). In fact, erysodine is a potent competitive antagonist at (human and rat) α4β2 nAChR, exhibiting high selectivity over the α7 subtype (Decker et al., 1995; Iturriaga-Vásquez et al., 2010). Its effects in vivo have only been tested in rodents (Decker et al., 1995; Mansbach et al., 2000; Iturriaga-Vásquez et al., 2010), mainly as related to inhibiting the effects of nicotine. The present study is the first to test both cytisine and erysodine in a zebrafish model.

The novel tank diving test has been previously used by other groups (Levin et al., 2007; Bencan et al., 2008; Bencan et al., 2009, Egan et al., 2009) as a model for anxiety studies in zebrafish. It is conceptually similar to the rodent open field test, because it takes advantage of the instinctive behaviour of both zebrafish and rats to seek refuge when exposed to an unfamiliar environment (Levin et al., 2007). In the case of the novel tank diving test, the fish dives to the bottom of the tank and remains there until it presumably feels safe enough to explore the rest of the tank (Egan et al., 2009). The time during which the zebrafish remains at and the time elapsed in the upper part of the tank, as well as erratic movements or freezing, have been established as anxiety indices (Egan et al., 2009). It is considered that the zebrafish is anxious when it shows a longer latency to enter the upper part of the tank, or when the time spent at the top is reduced.

With the aim of further exploring the role of α4β2 nAChR on the anxiolytic effect of nicotine and to evaluate if this action might be extended to other nicotinic agonists, in the present study we used this novel tank diving test on zebrafish to compare the behavioural effects of three drugs acting on nicotinic acetylcholine receptors: nicotine, cytisine and erysodine. Measurements of total distance travelled and bottom dwelling were used to determine changes in the locomotor activity or dwelling behaviour. Our results agree with previous studies that consider nicotine as an anxiolytic in the novel tank test (Levin et al., 2007; Bencan et al., 2008). Indeed, this effect seems to be mediated by activation of the α4β2 nAChR subtype since erysodine decreased the effect of nicotine in a competitive manner. In addition, cytisine -in agreement with its α4β2 agonist properties- showed nicotine-like behaviour in the novel tank test.

2) MATERIALS AND METHODS

All experiments were carried out in accordance with the National Institutes of Health (NIH) guidelines regarding the care and use of animals for experimental procedures. In addition, the procedures employed conform to the indications of the Bioethics Committee of the Facultad de Ciencias, Universidad de Chile, which oversees all aspects related to animal welfare in research.

2.1) Animals

Adult zebrafish (Danio rerio), both male and female, 6-12 months of age from the Tübingen strain were used; all animals were born and raised in our facility. They were kept at 25-28 °C on a 14:10-h light/dark cycle (lights on: 8 a.m.; off: 10 p.m.). The experiments to assess drug effects were performed during the light phase between 12:00 a.m. and 3:00 p.m. Deionised water with sea salt (Instant Ocean 1.2 g/20 L of water) was used in all tanks, which were maintained with constant filtration and aeration. Zebrafish were fed daily with flake fish food and Artemia salina nauplii. The animals were all drug-naïve and each fish was...
used only once. No distinction was made between male and female subjects. 10 animals per condition were used. Following trials, the fish were euthanized by an overdose of anaesthetic (0.05 mg/ml tricaine).

2.2) Drugs and pharmacological manipulation

Nicotine ditartrate was purchased from Sigma-Aldrich. Cytisine and erysodine were isolated from Calia secundiflora and Erythrina falcata respectively, as previously described (Houlihan et al., 2001; Iturriaga-Vásquez et al., 2010). Briefly, the crude alkaloid fractions were obtained from chloroform extracts and purified by column chromatography (Games et al., 1974). Structures were confirmed using one- and two-dimensional $^1$H and $^{13}$C NMR analyses. All drugs were dissolved in freshly deionised water with sea salt. Drugs were administered by immersing the zebrafish in a beaker with different concentrations of each drug: 50, 100 and 200 mg/L for nicotine and cytisine; 50 and 100 mg/L for erysodine. Drug concentrations were calculated based on the weight of the salt form (nicotine) or free base (cytisine and erysodine). The zebrafish were immersed in the drug solution for 3 min. After that, the animals were placed in a holding tank (containing deionised water with sea salt) without drug for 5 min, which served as a delay between the administration of the different drugs and the start of the trials according to previous reports (Levin et al., 2007). Controls were exposed to deionised water with sea salt from the housing tank without any drug. Then the zebrafish were moved to the test tank, in which they were tested for 5 min. In tests where multiple drugs were used, these were administered at the same time. There was no drug exposure either in the test tank or in the housing tank.

2.3) Test apparatus and procedure

The test tank was a glass trapezoid, 22.9 cm long at the bottom, 27.9 cm long at the top and 15.2 cm high, with the diagonal side 15.9 cm long, 6.4 cm wide at the top and 5.1 cm wide at the bottom, filled with 1.5 L of salt water. The test tank was located 50 cm from a Sony DCR-3R45 camera used to record the trials, which lasted 5 min. The tank was backlit with a white acrylic sheet to provide contrast for the image analysis program. Swimming behaviour was analyzed with the Noldus Image Analysis Software (Wageningen, The Netherlands). Time spent in each third of the tank (top, middle or bottom), and total travelled distance were measured. These variables were chosen based on previous reports using the tank diving paradigm for zebrafish (Levin et al., 2007; Kaufman et al., 2009).

2.4) Statistical analysis

Results are presented as means ± SEM. Data were analyzed with one-way analysis of variance followed by Tukey’s multiple comparison test. Statistical significance was set at 0.05.

3) RESULTS

Swimming activity of untreated control zebrafish was relatively low during the first minute, but it increased as the experiment progressed. Thus, control animals initially remained at the bottom of the novel tank and their exploratory activity increased progressively during the test, but still remaining largely at the bottom and in the middle third of the tank. Figure 1 illustrates this by showing representative traces of characteristic behaviour of control and drug-treated animals.

![Figura 1.]

Representative traces of characteristic behaviour of control, erysodine and nicotine treated animals.
Nicotine treatment significantly reduced bottom dwelling time, from $242\pm 43$ sec in control conditions to $69\pm 30$ sec in treated group with nicotine $200$ mg/L (defined as time spent in the bottom third), as shown in Figure 2A. Nicotine-treated zebrafish also stayed at the bottom of the tank at the beginning of the experiments, but their exploratory activity increased so that they mostly occupied the upper portion of the tank after an initial adaptive period (Fig. 1). The effect of nicotine was dose-dependent, and a statistically significant effect on bottom dwelling was found at $50$ mg/L and higher doses. In addition, nicotine significantly increased locomotor activity (Fig. 2B), in a dose-dependent fashion, from $541\pm 93$ cm. in control conditions to $827\pm 120$ cm. in treated group with nicotine $200$ mg/L.

**4) DISCUSSION**

Increased locomotor activity caused by nicotine has been described in many mammalian species including rat and mouse (Redolat et al., 2009; Abin-Carriquiry et al., 2010). Previous studies have reported controversial effects of nicotine on locomotion in zebrafish, showing either decreases (Levin et al., 2007) or increases (Bencan and Levin, 2008) of swimming activity. Our results agree with the notion that locomotor activity is increased by nicotine in zebrafish and that this effect appears to be dose-dependent. Drug-free zebrafish spend substantial time at the bottom of the novel tank when they are first introduced and then decrease their time at the bottom, beginning exploration of other levels of the test tank. This diving response is a very common reaction in prey fishes associated with stress behaviour and it is thought that it serves to avoid the action of predators (Egan et al., 2009).

Confirming the previously reported anxiolytic effect (Levin et al., 2007; Bencan and Levin, 2008), nicotine induced a significant decrease of the time spent at the bottom of the novel tank, which was accompanied by an increase of exploratory activity in the upper third of the tank (not shown).

Like nicotine, cytisine, a well known selective $\alpha4\beta2$ nAChR agonist (Houlihan et al., 2001), elicited a decrease in bottom dwelling and an increase of the total distance travelled. The similar behaviours elicited by nicotine and cytisine further support the idea that nicotine’s effects are mediated, at least in part, by the $\alpha4\beta2$ nAChR (Bencan and Levin, 2008), and also show that the selective activation of this receptor subtype induces anxiolytic effects. Indeed, our results indicate that anxiolytic activity associated to the activation of nAChR is not restricted to nicotine and highlight the suitability of zebrafish as a model for the search of novel compounds with anti-anxiety properties. It should be noted that, although cytisine had been shown to be more potent than nicotine in binding affinity and functional assays using mammalian $\alpha4\beta2$ nAChR (Wonnacott and Barik, 2007), our results indicated that nicotine was more potent than cytisine regarding the anxiolytic effects in zebrafish. This suggests that the action of nicotine might involve an additional mechanism (or mechanisms). In this context, Bencan and Levin (2008) have shown that $\alpha7$ nAChR are also involved in the
nicotine-induced anxiolytic effect in zebrafish. Therefore, the lower activity of cytisine in this paradigm might be related to its poor affinity for the latter receptor subtype.

In the same line, we found that the competitive and selective antagonist of the α4β2 nAChR erysodine, potently antagonized the effects of nicotine. Moreover, erysodine had a strong effect per se, decreasing swimming activity, although it did not affect the time spent in the bottom third of the tank. This suggests that locomotor activity in zebrafish might be under tonic cholinergic control mediated by α4β2 nAChR. It is noteworthy that previous studies have shown that another selective α4β2 nAChR antagonist, dihydro-β-erythroidine, had no effect on swimming activity (Bencan and Levin, 2008). This discrepancy might be due to the differing potency and selectivity of both compounds at α4β2 nAChR (Decker et al., 1995), and underlines the need to further characterize the subtle differences between these two nicotinic antagonists.

Erysodine produced an important decrease in travelled distance, that became even more evident at doses higher than 100 mg/L (data not shown), at which the zebrafish stayed completely still during almost the entire trial. This behaviour has been described by others as ‘freezing’ (Blaser et al., 2010). Therefore, our results suggest that the novel tank diving test might be not only suitable for measuring anxiolytic effects but also for determining anxiogenic actions of novel compounds. The effect of erysodine was completely reversed by doses greater than 200 mg/L of nicotine. We thus confirmed the competitive character of erysodine’s ability to block nicotine-evoked activity.

In summary, we have confirmed that the anxiolytic effects of nicotine in zebrafish are mediated, at least partially, by α4β2 nAChR, and that this action is not restricted to nicotine but can be exerted by other selective nicotinic agonists. Finally, the zebrafish novel tank model promises to be very useful for the pharmacological evaluation of new ligands, as shown here for subtype-selective nicotinic receptor agonists and antagonists.

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Las mismas características que han hecho del pez cebra una poderosa herramienta para el desarrollo de la biología, en los últimos años se ha transformado en una atractiva herramienta para el descubrimiento de drogas. Por su facilidad de obtener una gran cantidad de individuos para experimentación, por su bajo costo de mantención y capacidad de absorber sustancias desde el agua de su tanque. Los efectos de la nicotina en comportamiento han sido estudiados en varios modelos animales, como también en humanos. En el pez cebra, un efecto ansiolíptico de la nicotina ha sido recientemente mostrado en el protocolo del tanque nuevo. En el presente estudio, usamos este test en pez cebra para comparar los efectos de comportamiento de tres drogas que actúan sobre los receptores nicotinicos de acetilcolina: nicotina, erisodina y citisina. Medidas de la distancia recorrida en el nado y permanencia en el fondo de la pecera fueron usadas para determinar los cambios en la actividad motora o el comportamiento en el nado. Nuestros resultados concuerdan con estudios previos que muestran que nicotina tiene un efecto ansiolítico en el test de nado en el tanque nuevo, este efecto es mediado, al menos en parte, por el subtipo α4β2 de los receptores nicotínicos, ya que erisodina disminuye el efecto de la nicotina. Además, el agonista de los receptores α4β2, citisina ejerce un efecto tipo nicotina, indicando que este efecto no es restrictivo para la nicotina y que puede ser ejercido por otros selectivos agonistas de los receptores nicotínicos.