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ORIGINAL INVESTIGATION

Role of neuronal nicotinic acetylcholine receptors (nAChRs) on learning and memory in zebrafish

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Abstract
Rationale Neuronal nicotinic acetylcholine receptors (nAChRs) play a modulatory role in cognition, and zebrafish provide a preclinical model to study learning and memory.
Objectives We investigated the effect of nicotine (NIC) and some new cytisine-derived partial agonists (CC4 and CC26) on spatial memory in zebrafish using a rapid assay on T-maze task. The role of α4/α6β2 and the α7 nAChRs in NIC-induced memory enhancement was evaluated using selective nAChR antagonists.
Results Low and high doses of NIC, cytisine (CYT), CC4 and CC26 respectively improved and worsened the mean running time, showing an inverted U dose–response function. The effective dose (ED50) ($\times 10^{-5}$ mg/kg) was 0.4 for CC4, 4.5 for CYT, 140 for NIC and 200 for CC26. NIC-induced cognitive enhancement was reduced by the selective nAChR subtype antagonists: methyllycaconitine (MLA) for α7, α-conotoxin (MII) for α6β2, dihydro-β-erythroidine (DhβE) for α4β2, the nonselective antagonist mecamylamine (MEC) and the muscarinic antagonist scopolamine (SCOP), with DhβE being more active than MLA or MII. All the partial agonists blocked the cognitive enhancement. The improvement with the maximal active dose of each partial agonist was blocked by low doses of DhβE (0.001 mg/kg) and MII (0.01 mg/kg). MLA reduced the effects of CC26 and CC4 at doses of 0.01 and 1 mg/kg, respectively, but did not antagonize CYT-induced memory improvement at any of the tested dose. No change in swimming activity was observed.
Conclusions Our findings demonstrate that zebrafish make a useful model for the rapid screening of the effect of new α4β2 nAChR compounds on spatial memory.

Keywords Spatial memory · T-maze · Neuronal nicotinic receptors · Cholinergic system · Zebrafish · Methyllycaconitine · α-Conotoxin · Dihydro-β-erythroidine · Mecamylamine · Scopolamine

Abbreviations

CYT	Cytisine
MII	α-Conotoxin
MLA	Methyllycaconitine
DhβE	Dihydro-β-erythroidine
MEC	Mecamylamine
SCOP	Scopolamine

Introduction

Cognition is a highly complex CNS function whose many components (e.g., memory, attention and executive processes) may be compromised by disease. The development of new therapies for improving cognitive function rightly attracts a lot of attention.

Zebrafish provide a preclinical model for behavioural studies of learning and memory. They have many innate characteristics that are advantageous in research, including their small physical size (~2.5 cm), high reproduction rates (100–300 embryos per mating or "clutch") and rapid cycle times (females can lay eggs

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every week), which make them highly cost-effective investigations (Zon 1999). As non-mammalian vertebrate, zebrafish are evolutionarily more distant from humans than rodent models, but evolutionarily closer to humans than other non-vertebrate models, such as yeast, worm or fruit fly. Although zebrafish are relative newcomers to studies of learning and memory (Sison et al. 2006), a number of studies have shown that they are capable of performing well in a range of learning tasks such as avoidance learning (Blank et al. 2009), olfactory conditioning (Braubach et al. 2009), shuttle box active appetitive conditioning (Pather and Gerlai 2009), place conditioning (Eddins et al. 2009), appetitive choice discrimination (Bilotta et al. 2005), visual discrimination learning (Colwill et al. 2005), active avoidance conditioning (Xu et al. 2007), alternation-based spatial memory task (Williams et al. 2002) and even automated learning paradigm (Hicks et al. 2006). More recently, Sison and Gerlai (2011) have designed an associative learning task that was deliberately made to resemble a classical radial arm maze in which the traditional cue (food reward) is replaced by the sight of conspecifics.

It has been found that the effects of nicotine (NIC) and other cognitive-enhancing drugs on zebrafish are similar to those obtained in rodents, monkeys and humans (Levin and Simon 1998; Levin and Rezvani 2002). NIC dissolved in the water tank causes a significant improvement in rapid spatial discrimination task as measured by delayed spatial alternation in the three-chamber task (Levin and Chen 2004; Levin et al. 2006a; Eddins et al. 2009; Levin 2011). NIC has an inverted U-shaped dose–response curve, with moderate doses improving cognitive function and high doses reducing it.

Neuronal nicotinic acetylcholine receptors (nAChRs) are ligand-gated cation channels that play a modulatory role in various components of rodent and human cognitive function, including learning, memory and attention (Kenney and Gould 2008; Placzek et al. 2009; Heishman et al. 2010). nAChRs are a heterogeneous family of homo- or heteropentameric receptors formed by the coassembly of α and β subunits. In mammalian brain, the two predominant subtypes are the α4β2 and α7, which have different pharmacological profiles; the α4β2 subtype has high affinity (nanomolar) for ACh and the antagonist dihydro-β-erythroidine (DhβE), and the α7 subtype has low affinity for ACh (millimolar) and high affinity for the antagonist methyllycaconitine (MLA) (reviewed in Gotti et al. 2006). Both the α4β2 and the α7 subtypes have received a great deal of attention as important drug targets for cognitive enhancement (Hahn et al. 2003; Bitner et al. 2007; Howe et al. 2010; Castner et al. 2011; Lendvai et al. 2013).

Zebrafish (*Danio rerio*) have been used to study the role of nAChRs in various behaviours including locomotor and stress responses, cognition and exploration (Levin 2011). Eight nAChR subunit cDNAs (α2, α3, α4, α6, α7, β2, β3 and β4) have been recently cloned (Zirger et al. 2003; Ackerman et al. 2009) which have a high degree of sequence identity and similarity to rats and human orthologues (Papke et al. 2012). This supports the use of zebrafish as a model in which to test the effect of nicotinic compounds.

The aim of this study was to investigate the effect of NIC and new mammalian α4β2 nicotinic partial agonists (Sala et al. 2013) on learning and memory in zebrafish using a simple, rapid and inexpensive T-maze task. Then, by means of selective antagonists, we investigated the role of nAChR subtypes on NIC effects.

Materials and methods

Animals

Adult short-finned wild-type (WT) zebrafish (*D. rerio*) (0.4–1 g) of heterogeneous genetic background were obtained from a local aquarium supply store (Aquarium Center, Milan, Italy). Zebrafish were 6–12 months of age and were 3–4 cm long. In all experiments, the sex ratio of zebrafish was approximately 50–50 %. Males and females were identified as previously reported (Braida et al. 2012). Fish were kept at approximately 28.5 °C on a 14:10-h light/dark cycle. Behavioural testing took place during the light phase between 0900 and 1400 hours. Tank water consisted of deionised water and sea salts (0.6 g/10 L of water; Instant Ocean, Aquarium Systems, Sarrebourg, France). Approximately 30 adult fish were maintained in 96 L home tanks (75 cm long, 32 cm wide and 40 cm high) provided with constant filtration and aeration. Animals were acclimated for at least 2 weeks before the experiments. Fish were fed daily with brine shrimp and flake fish food (tropical fish food, Consorzio G5, Italy). All the fish were drug naive, and each fish was used only once. Ten fish per group were used. The experimental protocol was approved by the Italian Governmental Decree No. 29/2010. All efforts were made to minimise the number of animals used and their discomfort.

T-maze

The transparent Plexiglas T-maze (filled with tank water at a level of 10 cm) was used according to Yu et al. (2006) with slight modifications. The apparatus (Fig. 1) included a starting zone (30 cm×10 cm) separated from the rest of the maze by a transparent removable door. Behind the partition, there was a long (50 cm×10 cm) arm and two short (20 cm×10 cm) arms, which led to the removable deep water chambers (30 cm×30 cm). One of two chambers, used as reservoir, contained artificial grass, shells, stones and coloured marbles that offered a favourable habitat for the fish. To prevent viewing of the two chambers, two removable opaque partitions (4.5 cm×30 cm)

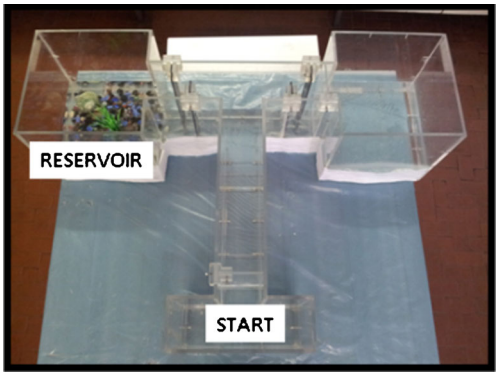

Fig. 1 Illustration of the T-maze apparatus for testing spatial learning in zebrafish

were put, in a staggered way, at the beginning of each short arm. To minimise procedural novelty stress, the fish first underwent two habituation trials of 1 h every day for 3 days, which also served to reduce handling stress. During these trials, the fish (in groups of 16) were allowed to freely explore the entire maze. To minimise acute social isolation stress, zebrafish groups were only gradually reduced in size during the experiment according to Sison and Gerlai (2010) starting with 16 fish per group on day 1 to 8 fish per group on day 2, 4 fish per group on day 3 and individual fish testing starting from day 4. Each subject received two training trials of exposure in the T-maze, at an interval of 24 h. During each trial, each fish was placed in the start box for 5 min with its door closed. Then, the start box door was raised and then lowered after the fish had exited. Ten minutes was allowed to reach the reservoir or the other chamber. Fifty percent of the fish within each group had the reservoir to the left, and the other 50 % to the right. Thus, the location of the reservoir was the same for each subject. Fish had half the reservoir to the left hand half to the right. The running time taken to reach the reservoir and stay for at least 20 s was recorded by an experimenter blind of pharmacological treatments. After 20 s, the fish returned to their home tank. The fish were then given a second session 24 h later. In a further group, fish were given the second trial at 3 h later. The difference between the running time taken to reach the reservoir and stay for at least 20 s between the first and the second trial was calculated as a measure of memory of the spatial location of reward.

Swimming behaviour

Each subject was placed in a transparent observation chamber (20 cm long × 10 cm wide × 15 cm tall) containing home tank water filled at a level of 12 cm. The floor of the chamber was divided into ten equal-sized 2 × 10-cm rectangles. Using a time sampling procedure, swimming activity was monitored by counting the number of lines crossed in a 30-s observation period every 5 min, for a total of six observation bins over 30 min (Swain et al. 2004). The mean of the six observation bins was calculated.

Treatment

Zebrafish body weight was measured as previously described (Braida et al. 2007, 2012). Briefly, fish were removed from their tank using a net and placed in a container containing tank water, positioned on a digital balance. Fish weight was determined as the weight of the container plus the fish minus the weight of the container before the fish was added. The mean of three measurements was recorded. Each fish was injected i.p. Each volume, depending on the fish's weight (2 μl/g), was given using the Hamilton syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland). For i.p. injection, fish were anaesthetised with ice as previously described (Kinkel et al. 2010) and placed in a supine position. The injection was made in the abdominal cavity. No more than the tip of the needle was inserted into the abdomen of each fish, as a means of preventing damage of internal organs. After injection, each fish was immediately transferred back to its warm water (about 28 °C) tank for recovery.

Drugs

The drugs used were NIC bi-tartrate (20–20.000 × 10^{-5} mg/kg) (Sigma-Aldrich, St. Louis, MO, USA), cytisine (CYT) (1–10,000 × 10^{-5} mg/kg), 1,2-bis(cytisin-12-yl)ethane (CC4) (0.1–1,000 × 10^{-5} mg/kg) (synthesised as described by Carbonnelle et al. 2003) and 1,4-bis(cytisin-12-yl)-2-butyne (CC26) (10–100,000 × 10^{-5} mg/kg) (synthesised by Prof. F. Sparatore, Genova, Italy) (Fig. 2). As CC26 is a new cytisine derivative, we preliminarily tested its affinity for the mammalian α3β4, α4β2 and α7 subtypes using the same method as that described in Sala et al. (2013). The affinity (Ki) values were 2,700 nM (23 %) for the human α3β4 subtype,

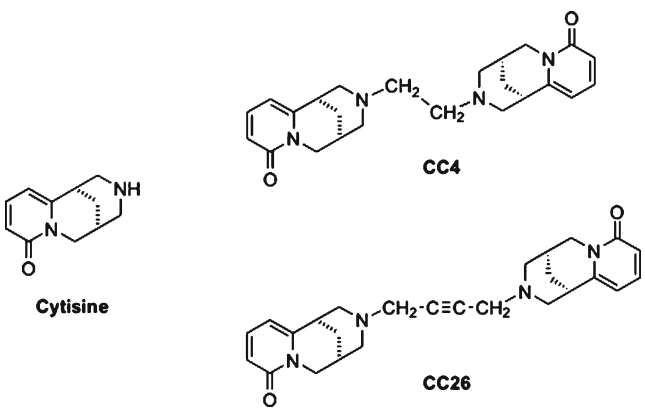

Fig. 2 Chemical structure of cytisine and its derivatives CC4 and CC26

11.6 nM (22 %) for the rat α4β2 subtype and 2,390 nM (42 %) for the rat α7 subtype, respectively.

NIC and nicotinic partial agonists were administered i.p. 10 min before the first probe trial or before the swimming behaviour evaluation.

For antagonism studies, scopolamine (SCOP) (0.025 mg/kg), mecamylamine (MEC) (0.01 and 0.1 mg/kg), CYT (0.01 mg/kg), CC4 (0.01 mg/kg) and CC26 (1 mg/kg) were given i.p. 10 min before the maximal active dose of NIC (0.02 mg/kg). To further characterise the involvement of nicotinic receptor subtype, selective antagonists, MLA (0.01–1 mg/kg), α-conotoxin (MII) (0.001–0.1 mg/kg) and DhβE (0.001–0.1 mg/kg) (Sigma-Aldrich, St. Louis, MO, USA) were given 10 min before saline or the maximal active dose of NIC, CYT, CC4 and CC26. As no information are present in the literature reporting injection in zebrafish of cholinergic compounds, the doses of drugs were chosen based on previous pilot studies performed in our laboratory. The range of doses of nAChRs partial agonists was initially chosen on the basis of that of NIC. All the drugs were dissolved in sterile saline to minimise infections. All the injections were made with sterile syringes and needles. Vehicle group received one injection of saline (2 μl/g). For antagonism studies, vehicle group received two injections of saline. All the fish were drug naive, and each fish was used only once. Ten fish per treatment were used. The doses of the drugs were calculated as salt. All drugs were prepared fresh daily.

Statistical analysis

The data were expressed as mean ± standard error of the mean (SEM). Different groups were assessed by one-way analysis of variance (ANOVA) for multiple comparisons followed by Tukey's post hoc test. Running times obtained with different nicotinic compounds were analysed by linear regression lines. Effective dose (ED)50 values (using the least squares method of linear regression on the linear portion of the curves) and 95 % confidence intervals were also calculated. All statistical analyses were done using the software Prism, version 6 (GraphPad, San Diego, CA, USA).

Results

Cognitive ability of zebrafish in the T-maze task

Untreated fish initially took an average of about 270±57 s to find the reservoir (baseline), but this was reduced by about 75 % in most fish in the second trial performed after 3 h (65±23 s); however, after 24 h, the time had increased to 220±32 s (Fig. 3). Treatment with saline did not change the cognitive performance measured as running time (312±53 s at 0 h, 52±30 s after 3 h and 268±40 s after 24 h). ANOVA revealed a difference among groups when considering the difference between pre-training running time (at 0 h) and post-training running time (at 3 or 24 h) ($F_{(3, 36)}=24.42, p=0.0001$). There was a difference in running time between the performance at 3 and 24 h both in basal and saline groups, while no difference was shown between basal and saline groups at any time (Fig. 3).

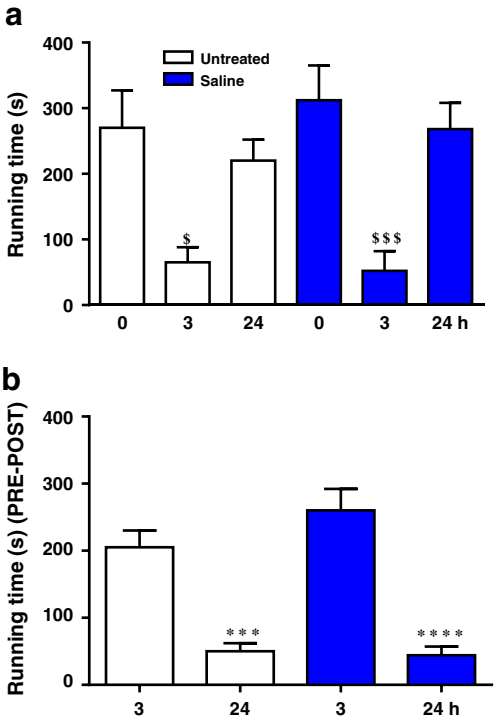

Fig. 3 Cognitive ability of zebrafish in terms of running time (second) to reach the reservoir in a T-maze in untreated and saline treated i.p. fish. Saline treatment did not change the cognitive ability in terms of difference of pre-training running time (*PRE*) (at 0 h) minus post-training running time (*POST*) (at 3 or 24 h). Each *value* represents the mean ± SEM of ten observations per group. $^{\$}p<0.05$; $^{\$\$\$}p<0.001$, compared to corresponding 0 and 24 h group; $^{***}p<0.0001$; $^{****}p<0.00001$, compared to corresponding 3-h group

NIC and partial agonists improve spatial memory

The effect of NIC and the nicotinic partial agonists is shown in Fig. 4. NIC (Fig. 4a) had a significant main effect of dose ($F_{(4, 45)}=10.75$, $p<0.0001$), and post hoc analyses showed that it had a significant pro-cognitive effect at a dose of 200 and $2{,}000\times10^{-5}$ mg/kg. The partial agonists also had a significant main effect of dose (CYT, $F_{(6, 63)}=8.81$, $p<0.0001$; CC4, $F_{(5, 54)}=4.75$, $p=0.002$; CC26, $F_{(5, 54)}=18.04$, $p<0.0001$) (Fig. 4b–d). Tukey's test revealed that CYT was maximally active at a dose of$\times10^{-5}$ mg/kg; CC4 appeared to be the most active compound as it was effective starting from 1×10^{-5} mg/kg. CC26 was the least active as it was effective only at a dose of $1{,}000\times10^{-5}$ mg/kg. All of the compounds showed an inverted

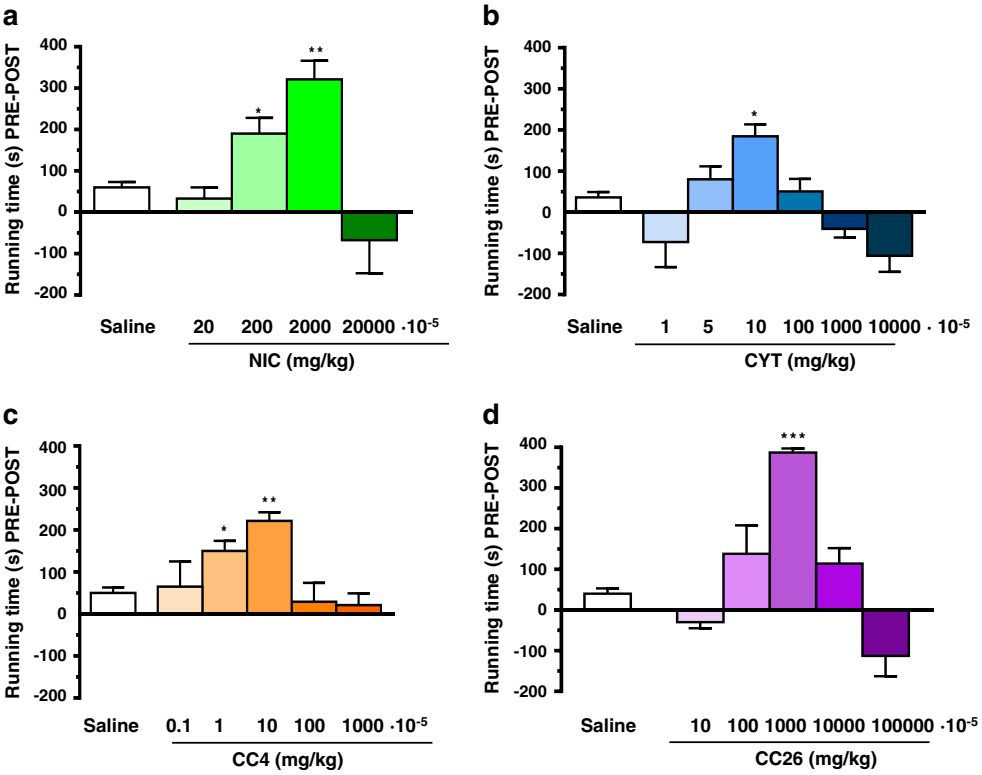

Fig. 4 Cognitive performance of zebrafish in terms of running time difference (second) of pre-training running time (*PRE*) (at 0 h) minus post-training running time (*POST*) (at 24 h) following nicotinic drugs treatment. Nicotine (*NIC*) (**a**), cytisine (*CYT*) (**b**), *CC4* (**c**) and CC26 (**d**) increased spatial memory performance following an inverted U-shaped dose–response curve. Each *value* represents the mean ± SEM of ten observations per group. *$p<0.05$; **$p<0.01$; ***$p<0.001$, compared to corresponding saline group

U-shaped dose–response function. The ED50 ($\times 10^{-5}$ mg/kg) obtained for the mean running time were as follows: CC4, 0.4 (0.3–0.5); CYT, 4.5 (3.5–5.5); NIC, 140 (138.7–141.3); and CC26, 200 (170–230), i.e. CC4 > CYT > NIC > CC26.

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

Various nicotinic and muscarinic drugs were tested for their possible effects on NIC-induced pro-cognitive effect. The results are shown in Fig. 5. There was a main effect of treatment ($F_{(11,\ 108)}=14.14$, $p<0.0001$) when the antagonists were given alone (Fig. 5a). As expected, SCOP led to amnesic effects in terms of a reduced difference in running time in comparison with the control group receiving two injections of saline, whereas MEC did not. The selective nicotinic antagonists, MLA and DhβE (0.01–0.1 mg/kg) also had amnesic effects, whereas MII had slight but not significant pro-cognitive effects. SCOP and MEC significantly antagonized NIC-induced facilitation effect (Fig. 5b). All the selective antagonists significantly blocked NIC-induced memory enhancement at all the tested doses with DhβE being more active than MLA or MII.

Partial agonists block the NIC-induced cognitive enhancement

To investigate the ability of partial agonists to block NIC-induced cognitive enhancement, CC4, CYT and CC26 were tested at doses that did not affect cognition per se and given before the maximal active dose of NIC (Fig. 6). There was a significant main effect of treatment ($F_{(4,\ 45)}=3.55$, $p=0.017$), and post hoc analyses revealed that the effect of NIC was significantly blocked by all the partial agonists.

Involvement of different nAChR subtypes in the cognitive improvement induced by partial agonists

There was a treatment effect when CYT, CC4 and CC26 were combined with different nAChR subtype-specific antagonists (CYT, $F_{(9,\ 90)}=11.85$, $p<0.0001$; CC4, $F_{(9,\ 90)}=44.58$, $p=0.0001$; CC26, $F_{(8,\ 81)}=39.90$, $p<0.0001$) (Fig. 7). Post hoc analysis revealed that MII (0.1 mg/kg) antagonized CYT-induced memory improvement, whereas MLA did not at any the tested doses (Fig. 7a). The lowest dose of DhβE significantly antagonized memory improvement, but higher doses were progressively less active. CC4-induced cognitive improvement was blocked by the highest dose of MLA and by the low doses of MII and DhβE (Fig. 7b). CC26-induced facilitation was blocked by the lowest dose of MLA and by both the doses of DhβE and MII (Fig. 7c).

Cholinergic drugs do not affect swimming activity

NIC, CC4, CYT and CC26 were given at doses effective to improve memory and at doses able to block NIC effect (Fig. 8).

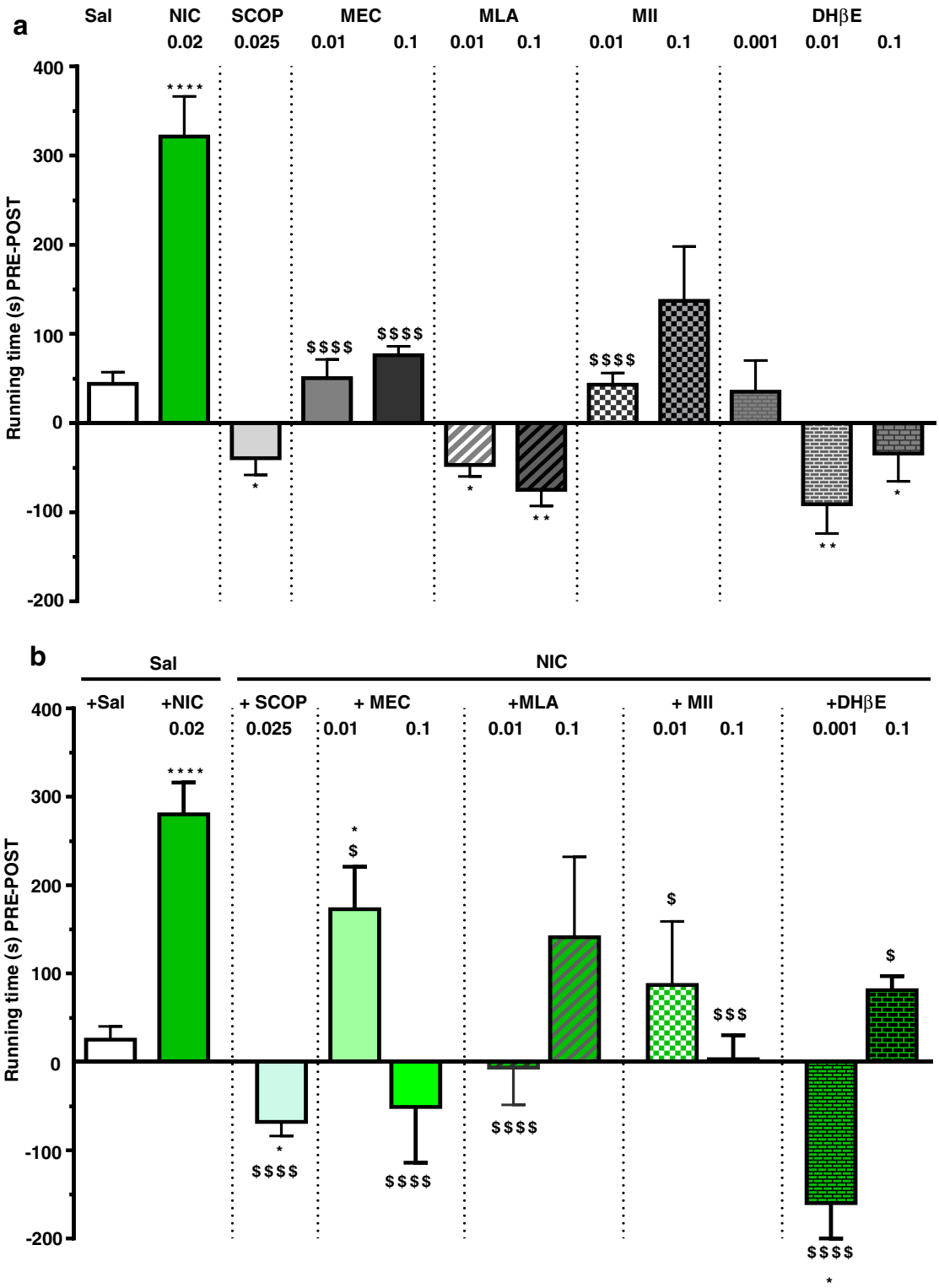

Fig. 5 Effect of different nonselective or selective nAChRs subtype receptor antagonists given alone or 10 min before nicotine (*NIC*) in terms of running time difference (second) of pre-training running time (*PRE*) (at 0 h) and post-training running time (*POST*) (at 24 h) in a T-maze. **a** Scopolamine (*SCOP*), methyllycaconitine (*MLA*) and dihydro-β-erythroidine (*DHβE*) reduced per se the performance, while mecamylamine (*MEC*) and α-conotoxin (*MII*) had no effect. **b** When combined with NIC, all the antagonists significantly reduced NIC-induced pro-cognitive effect. The doses are expressed as milligram per kilogram. Each *value* represents the mean ± SEM of ten observations per group. *Sal* saline. $^{*}p<0.05$; $^{**}p<0.01$; $^{****}p<0.0001$, compared to corresponding saline group; $^{\$}p<0.05$; $^{\$\$\$}p<0.001$; $^{\$\$\$\$}p<0.0001$, compared to corresponding NIC

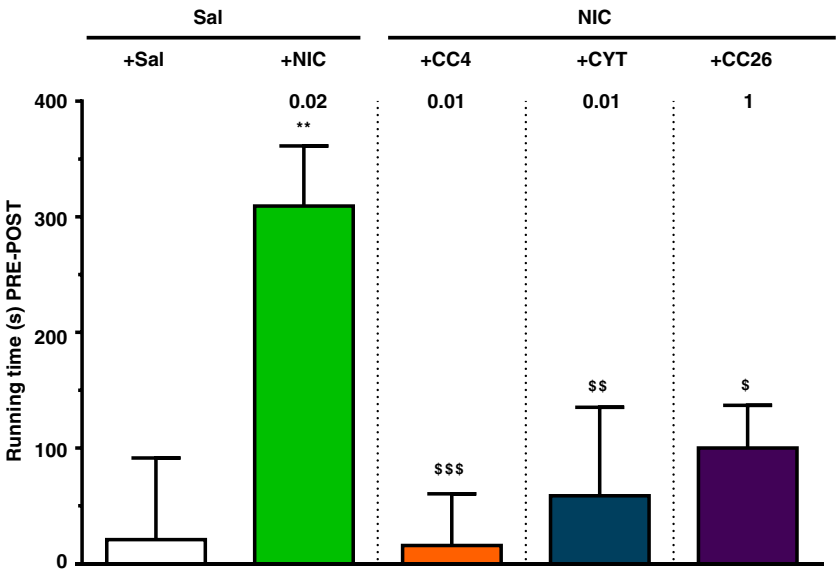

Fig. 6 Different partial agonists on nicotine (*NIC*)-induced cognitive improvement in a T-maze task in terms of running time difference (s) between pre-training running time (*PRE*) and post-training running time (*POST*). NIC performance was significantly reduced by pretreatment (10 min before) with CC4, cytisine (*CYT*) and CC26 at doses which per se did not affect cognitive ability. The doses are expressed as milligram per kilogram. Each *value* represents the mean ± SEM of ten observations per group. *Sal* saline. $^{**}p<0.01$, compared to saline group. $^{\$}p<0.05$; $^{\$\$}p<0.02$; $^{\$\$\$}p<0.001$, compared to corresponding NIC group

Nonselective and selective antagonists were given at doses able to reduce NIC-induced improvement. ANOVA did not show any significant treatment effect ($F_{(12,\ 117)}=1.15$, n.s.)

Discussion

Our data obtained using zebrafish, an associative spatial learning task, and some selective and nonselective nAChR agonists/partial agonists or antagonists indicate that nAChRs play a role in zebrafish cognition. The ability of zebrafish to attain good performance at associative learning tasks has been clearly documented using a three-chamber-delayed spatial alternation task (Levin and Chen 2004), a Y-maze (Cognato Gde et al. 2012) and a T-maze (Darland and Dowling 2001). However, NIC and the other drugs have always been dissolved in tank water, whereas we injected it i.p. in order to control the amount of drug each fish received more precisely. We chose cold water rather than chemical anaesthesia in order to limit the effect of stress as much as possible; cold anaesthesia does not increase blood glucose levels, which is an index of stress in teleost fish (Kinkel et al. 2010). Notably, only 10 % of lethality was observed after i.p. injection. We also modified the original procedure of Darland and Dowling (2001) by increasing the time between the two sessions to 24 h, an interval in which acquisition was sufficient to provide a basis for determining the enhancing effects of NIC and the other drugs. Our findings show that spatial learning can be studied very quickly in individual zebrafish; two trials were enough to reduce the running time to reach a good performance, whereas other studies have required 7 to 28 sessions (Williams et al. 2002; Levin and Chen 2004).

Low i.p. doses of NIC induced cognitive enhancement, whereas high doses impaired memory, thus indicating an inverted U-shaped dose–response function. This trend has been previously observed in zebrafish exposed to NIC solution (Levin and Chen 2004; Eddins et al. 2009) as well as in rats, monkeys and humans (Levin et al. 2006b).

In order to better characterise the nAChR subtypes involved in NIC-induced cognitive facilitation, various selective and nonselective antagonists were administered in combination with NIC. As expected, SCOP and MEC completely antagonized the memory-enhancing effect of NIC, but the nAChR subtype-selective antagonists were also all effective in reducing the effect. It is worth noting that the memory-blocking effect on NIC stimulatory effect induced by the nonselective and selective antagonists was obtained using doses that do not affect cognition.

We can exclude the possibility that the improvement in memory induced by different cholinergic agonists was influenced by changes in general activity because the total number of crossed lines in the swimming activity test was not modified by the maximal effective doses of drugs.

Our data show that the α4β2-selective antagonist DhβE was more effective than MLA or MII, thus indicating a major role of the α4β2 subtype in the learning and memory effects of NIC. The greater potency of α4β2-selective antagonist is in agreement with the functional data of Papke et al. (2012) who

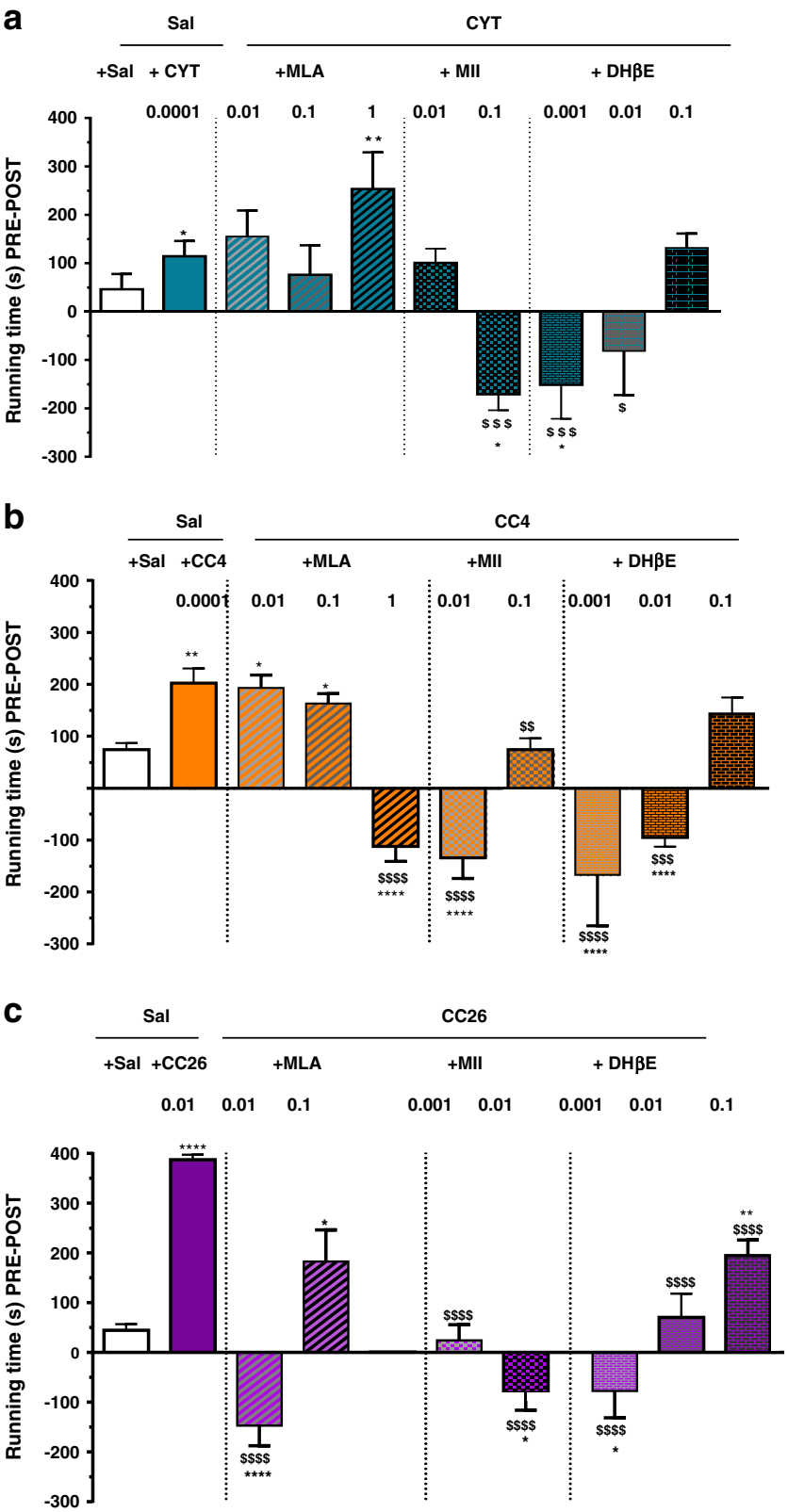

Fig. 7 Effect of selective nAChRs subtype receptor antagonists given 10 min before different partial agonists, in terms of running time difference (s) between pre-training running time (*PRE*) and post-training running time (*POST*) in a T-maze task. **a** Cytisine (*CYT*)-induced cognitive improvement was blocked by both α-conotoxin (*MII*) and dihydro-β-erythroidine(*DHβE*), but not by methyllycaconitine (*MLA*). **b** CC4- and **c** CC26-induced cognitive abilities were reduced by all the selective nAChRs subtype receptor antagonists. The doses are expressed as milligram per kilogram. Each *value* represents the mean ± SEM of ten observations per group. *Sal* saline. $^{*}p<0.05$; $^{**}p<0.01$; $^{****}p<0.0001$, compared to corresponding Sal group. $^{\$}p<0.05$; $^{\$\$}p<0.01$; $^{\$\$\$}p<0.001$; $^{\$\$\$\$}p<0.0001$, compared to the corresponding partial agonist alone

found that NIC had a more potent effect on the oocytes-expressed zebrafish α4β2 subtype than on the α7 nAChR subtype; the NIC concentration that provoked an electrophysiological response midway between baseline and maximum (effective concentration EC50) was 6±2 μM for the α4β2 subtype and 27±6 μM for the α7 nAChR subtype.

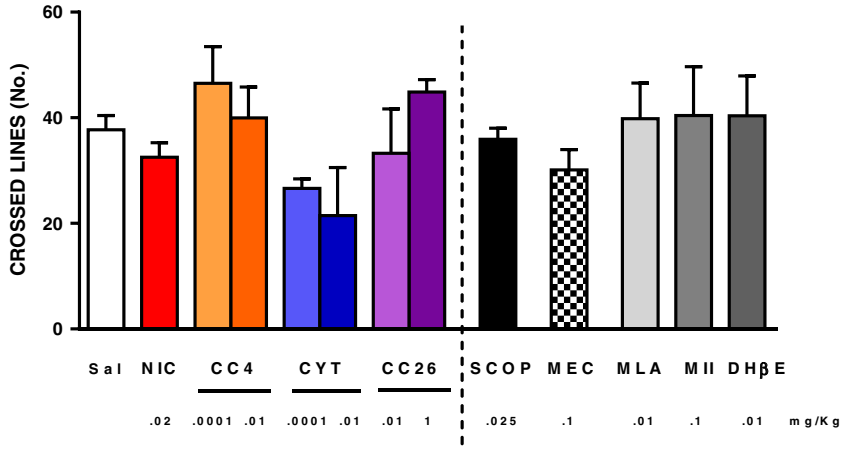

Fig. 8 Results from swimming activity test. Treatment with saline (*Sal*), nicotine (*NIC*), the different partial agonists and antagonists did not induce any change in swimming activity in zebrafish. Animals were individually recorded by counting the number of line crossings in a 30-s observation period every 5 min over 30 min. The doses are expressed as milligram per kilogram. Each *value* represents the mean ± SEM of ten observations per group. *CYT* cytisine, *SCOP* scopolamine, *MEC* mecamylamine, *MLA* methyllycaconitine, *MII* α-conotoxin, DHβE dihydro-β-erythroidine

In agreement with previous antagonist studies designed to determine the reversibility of the effects of NIC (Svoboda et al. 2002; Levin et al. 2006a; Bencan and Levin 2008; Eddins et al. 2009), our data further confirm the functional similarity in pharmacological response between mammal and zebrafish nAChRs. MEC (a nonselective nAChR antagonist that acts on mammalian α4β2, α3β4, α3β2 and α7 subtypes) (Papke et al. 2001) blocks a broad range of NIC-mediated effects in zebrafish, including motor sensitization, improved learning and reduced anxiety (Levin et al. 2006b, 2007; Eddins et al. 2009; Petzold et al. 2009). The NIC-induced anxiolytic effect in zebrafish is also reversed by the subtype-specific nAChR antagonists DhβE, MLA and MII (Bencan and Levin 2008).

The α4β2-specific partial agonists CYT, CC4 and CC26 per se improved learning and memory with similar dose–response curves to those found using another selective α4β2 partial agonist, varenicline. The s.c. administration of varenicline to rodents induces dose-dependent (0.1–3.2 mg/kg) cognitive-enhancing effects in terms of the time spent exploring a novel object and an increased capacity to attenuate impaired performance under challenging distractor conditions (Rollema et al. 2009; Howe et al. 2010). In addition, varenicline improves the cognitive deficits associated with smoking cessation (Patterson et al. 2009).

On the basis of its calculated ED50, CC4 appeared to be the most active α4β2 nAChR partial agonist possibly because it has better brain penetration and a longer half-life than CYT (Riganti et al. 2005). The important role of the α4β2 subtype in learning and memory is also indicated by studies of animal models (Buccafusco et al. 1995; Gatto et al. 2004) and humans (Potter et al. 1999; Wilens et al. 1999; Dunbar et al. 2007; Wilens and Decker 2007; Rushforth et al. 2010) in which α4β2 nAChR subtype-selective agonists (ABT-418, ABT-089, isopronicline and metanicotine) improved memory.

It has been suggested that NIC improves cognitive function in zebrafish as a result of the nAChR modulation of dopamine release and/or metabolism (Levin et al. 2006a). Accordingly, Eddins et al. (2010) have shown that nicotine exposure increases learning rates and the levels of the dopamine metabolite dihydroxyphenylacetic acid (DOPAC) and that both effects were blocked by the antagonist MEC.

We have recently shown that CC4 and CYT reduce behaviour associated with NIC addiction in rats (Sala et al. 2013) by inhibiting NIC-induced dopamine release. It is thus possible that, when given together with nicotine, CC4 may reduce the NIC-induced release of dopamine that plays an important role in cognition. Further studies will clarify the role of dopaminergic system in zebrafish cognition.

We found that the memory enhancement induced by all the partial agonists was significantly antagonized by the α4 and α6β2 nAChR subtype-selective antagonists DhβE and MII. Furthermore, the memory improvement induced by CC4 and CC26 was reduced by the co-administration of MLA, a mammalian α7-selective antagonist. The results obtained using CYT were rather surprising because, although CYT is a full agonist of mammalian and zebrafish α7 subtypes, MLA was ineffective in blocking CYT-induced memory facilitation under our experimental conditions. However, these findings are in line with the functional electrophysiological data reported by Papke et al. (2012), who found that CYT had less efficacious but more potent effects on α4β2 than on the α7 subtype; the EC50 of CYT was 0.47±0.09 μM for α4β2 subtype and 15±3 μM the for α7 subtype.

A limitation of our study is that nothing is known about the absorption, distribution and metabolism of the drugs we used. There is also a complete lack of data about how these drugs reach the brain and which of the cerebral areas are involved.

Recent studies have demonstrated that the α7 nAChR subtype plays an important role in cognition (Wallace and Porter 2011); α7 nAChR agonists improve the performance of rodents, monkeys and humans in many cognitive tasks, and the injection of α7 nAChR antagonists in the hippocampus impairs working memory in rodents. Moreover, α7 nAChR knockout mice show impaired choice accuracy in the radial maze (Levin et al. 2009) task. Our results obtained with CYT, a selective mammalian and zebrafish α7 nAChR agonist, do not confirm the involvement of α7 nAChR subtype in the spatial memory in zebrafish, thus indicating that further investigations are needed in order to cross-validate CYT in the zebra fish system.

Our data support the zebrafish system as a means of rapid screening of the effect of new α4β2 nAChR compounds on spatial memory.

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Conflict of interest All authors declare that they have no conflicts of interest in this study.

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