Study of 3-(4'-Nitrobenzamido)coumarin using an Alzheimer's Disease Model

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Abstract

3-(4’-Nitrobenzamido)coumarin (**MJM255**), a potent *in vitro* acetylcholinesterase (AChE) inhibitor, was selected as an *in vivo* candidate for the discovery of new therapeutic solutions for Alzheimer’s disease. Computational (*in silico*) studies showing the theoretical physicochemical properties indicate desirable a pharmacokinetic profile for this molecule to cross the blood-brain barrier (BBB). An *in vivo* study, using the object recognition test (ORT) mice model, was carried out. This compound exhibited a similar effect as eserine, a well-known AChE inhibitor able to cross the BBB.

Introduction

Alzheimer’s disease (AD) is among the most prevalent neurodegenerative disorders (Winblad et al. 2019). It is the most frequent cause of dementia worldwide, and no effective treatment has been yet found for this disease. Neuronal death and loss of cholinergic synapses are amongst the most important consequences of the pathology and are responsible for its characteristic decrease of cognitive function and behavioral symptoms. They may be related to several pathological disfunctions in brain function (Sharma et al. 2020). Inflammation, protein misfolding, energy metabolism and enzymatic activity dysfunctions are some of the occurrences already described as potential features of this disease (Kamal et al. 2014). Amyloid beta plaques formation and neurofibrillary tangles of the aggregated protein tau are present in patients’ brains (Yamada et al. 2020). Nevertheless, neurotransmitter modification is still the approach of the available drugs: cholinesterase inhibitors and an antagonist of the *N*-methyl-D-aspartate receptor (NMDA) (Hu et al. 2020). The pharmacotherapy of AD has been, therefore, mainly focused on increasing the cholinergic function in the brain (LiverTox 2012) because of the destruction of cholinergic neurons in the main cholinergic pathways, as septo-hippocampal pathway and the pathway from the nucleus basalis magnocellullaris (Meynert) to the neocortex, that leads to a decrease in the levels of this neurotransmitter (Ribeiro et al. 2016). Inhibitors of acetylcholinesterase (AChE), that prevent the acetylcholine (ACh) metabolism, proved to improve the symptoms of Alzheimer’s patients (Sharma 2019). As so, four different AChE inhibitors have been approved for AD in the United States during the last three decades: tacrine (Cognex, 1993, withdrawn from the market due to its hepatotoxic effects), donepezil (Aricept, 1996), galantamine (Razadyne, 2001) and rivastigmine (Exelon, 2002).

Given the prevalence and grade of life-changing of this disease, developing effective therapies for AD has become urgent. Different methods have been used to induce AD-like symptoms in rodent in order to screen many therapeutic drugs. Scopolamine (SCO) is among the drugs with ability to cross the BBB and proven antimuscarinic properties which has been used to induce AD-like cognitive impairment in rodents (Moklas et al. 2019). Object recognition test (ORT) is nowadays amongst the most commonly used behavioral tests for evaluating different parameters of learning and memory in mice (Freret et al. 2013; Lueptow 2017).

Our research group has been studying 3-amidocoumarins as potential multitarget candidates for the development of drugs against AD (Uriarte et al. 2012; Viña et al. 2015; Santana et al. 2021). Among these

molecules, 3-(4'-nitrobenzamido)coumarin (**MJM255**) proved to be the best AChE inhibitor so far. Therefore, this molecule was selected for this *in vivo* study, following the ORT in mice treated with SCO. The main results are reported, and their impact is highlighted in this manuscript.

Materials And Methods

Chemistry

Procedure for the preparation of MJM255. The 3-aminocoumarin (commercially available, 1.0 mmol) was dissolved in dichloromethane (9 mL). Pyridine (1.1 mmol) was then added, and the mixture was cooled to 0 °C. The 4-nitrobenzoyl chloride (1.1 mmol) was added dropwise at this temperature, and the mixture was stirred overnight at room temperature. The batch was evaporated and purified by column chromatography (hexane/ethyl acetate, 9:1) to give the desired **MJM255** (Uriarte et al. 2012).

Pharmacology

Inhibition of human cholinesterases

Using human recombinant acetylcholinesterase (*h*AChE) and human serum butyrylcholinesterase (*h*BuChE), the Ellman method (Feather-Stone et al. 1961) was followed, according to the experimental details previously described by us (Viña et al. 2015; Viña et al. 2014; Matos et al. 2021).

Object recognition test (ORT)

Animals. Swiss male mice with a weight of 25 ± 5 g were used. The stabling, handling and the different experimental techniques, were carried out in accordance with the Directive 2010/63/EU of the European Parliament and of the Council of September 22, on the protection of animals used for scientific purposes, and the Royal Decree 53/2013 of February 1, which establishes the basic rules applicable to the protection of animals used in experimentation and other scientific purposes, including teaching and the Guide for the care and use of animals Laboratory developed by AAALAC International (Association for Assessment and Accreditation of Laboratory Animal Care International, International Association for the Evaluation and Accreditation of Laboratory Animal Care). The experiments were carried out following the procedure informed with Registration Code 15007AE/09/INV MED 02/NER02/JAFG13, authorized by the Consellería do Medio Rural – Autonomous Government of Galicia (Xunta de Galicia). The mice were housed in standard Makrolon cages without any restriction on access to food and water, except during the experiment. Prior to conducting the experiments, the animals were acclimatized, for a minimum of 72 h, to the environmental conditions in a silent, thermostatted chamber (22 ± 1 ºC), with a 12-h light/dark cycle (08:00–20:00 h) and with humidity controlled (45–65%). The experiments were always carried out every day at the same hour in order to avoid alterations due to the circadian cycles. The mice were used only once to avoid alterations in the response due to tolerance or learning phenomena.

Equipment. Memory evaluation in mice was performed using the Object Recognition Test (ORT). For the experiment, the animals were placed in a black square box (1x1x0.30 m^3), subdivided into 4 independent

areas (0.50x0.50x0.30 m^3) where each animal was placed independently. The evaluation of the ORT was carried out from a room adjacent to the one containing the animal, using a video registration system. The behavior of the animals was captured with an analog camcorder (Sony DXC-107A, Sony Corporation, Japan) suspended on the ceiling. The camera is connected to an adapter (Sony CMA D2) that sends the signal to a monitor (Sony PVM-14M2E) and to two digitizing cards: i) an internal one located in a PCI slot of the computer (Picolo frame graber, Euresys, Liege, Belgium); ii) an external one with USB connection (DVC-USB, Dazzle, USA). The direct signal of the Picolo card is used by the video-computerized animal observation system (EthoVision V. 3.1.16, Noldus Information Technology, Wageningen, The Netherlands). The EthoVision software locates the center of the animal, stores the data and allows further analysis.

Protocol. To evaluate the possible *in vivo* ChE inhibitory activity of **MJM255**, it was administered at the dose of 25 mg/kg, in a suspension of 1% sodium carboxymethyl cellulose (CMCNa), to both mice without pretreatment, and mice pretreated with scopolamine (SCO), used as a pharmacological AD model.

Eserine (ESR) was used as the reference drug, at a dose of 0.25 mg/kg. **MJM255** and ESR were administered intraperitoneally (ip) in a volume/weight ratio of 10 mL/kg. The groups of animals used in the ORT were:

- Mice without pretreatment, administered 30 minutes before the start of the experiment physiological solution/saline (sodium chloride, NaCl 0.9%), via ip.
- Mice treated 30 minutes before the start of the experiment with a dose of 1 mg/kg of SCO.

Regardless of the treatment group, 15 minutes before starting the experiment, the animals were administered the CMCNa (1% w/v) ip, the reference drug (ESR, 0.25 mg/kg, ip) or **MJM255** (25 mg/kg, ip).

The objects chosen for the ORT test were prisms (OBJ-1 and OBJ-2) and pyramids (OBJ-3). The objects were located 10 cm from the edges closest to the wall of the divisions of the area and 30 cm from each other, in two different places called Zone A and Zone B. A distance around each zone of 4 cm was defined.

The experiments were carried out in two different phases (P1 and P2), both lasting 10 minutes and 10 minutes apart. During phase P1, a prism was placed in both Zone A and Zone B (OBJ-1 in Zone A and OBJ-2 in Zone B). During P2 phase, OBJ-1 was maintained in Zone A and OBJ-2 in Zone B was replaced by OBJ-3 (pyramid).

During the first 10 minutes of the assay (phase P1), the animals were placed in the corresponding arenas. After that time, the animals were removed from the experimental area and housed again in the corresponding cage. During the 10 minutes, rest period between P1 and P2, both arenas and objects (repeated and new) were cleaned with 15% ethanol water to avoid interference due to odor. The animals were then placed back in the box for another 10 minutes (phase P2) (Scheme 1).

In each assay, three parameters were evaluated: Latency period before the first approach (s), Frequency of approximation (nº) and Exploration time (s).

Expression of the results, statistical analysis and graphic representation. The results are expressed as the mean ± the standard error of the mean (S.E.M.) of at least 8 animals (n = 8). Statistical analysis and graphical representation were performed with the GraphPad Prism® program (v 6.0, San Diego, USA). Statistically significant differences were determined by the one-way ANOVA test (treatment) followed by the Dunnet test or by the two-way ANOVA test (treatment time) followed by the Bonferroni test.

Results And Discussion

Chemistry

MJM255 was synthetized starting from the 3-aminocoumarin and the 4-nitrobenzoyl chloride, by an acylation reaction, as described in Scheme 2, using pyridine in dichloromethane, from 0 °C to room temperature, overnight. The obtained mixture was then purified by flash chromatography, using a mixture of *n*-hexane and ethyl acetate (9:1), to afford **MJM255** (Uriarte et al. 2012).

Enzymatic activity inhibition (*h*AChE and *h*BuChE)

MJM255 (Uriarte et al. 2012), tacrine and eserine (ESR), the last two used as reference controls, were evaluated as inhibitors of human cholinesterases (*h*AChE and *h*BuChE) following the Ellman method (Feather-Stone et al. 1961; Viña et al. 2014; Matos et al. 2021). The results are organized in Table 1.

MJM255 is less active on *h*AChE than both tacrine and ESR, the reference controls. However, this compound is selective against *h*AChE, and it proved to be the best 3-amidocoumarin described so far by our group (Uriarte et al. 2012; Viña et al. 2015). Therefore, this molecule was selected for the *in vivo* test to try to understand its real potential as a promising lead compound.

In silico ADME properties

Crossing the BBB is essential for a drug to act in the central nervous system (CNS). Therefore, theoretical physicochemical properties of **MJM255**, tacrine and ESR were calculated. These physicochemical properties are a good predictor of ADME pharmacokinetics parameters (absorption, distribution, metabolism and excretion) of a molecule, as well as its capacity to cross through cellular membranes. Molinspiration cheminformatics software (Molinspiration Cheminformatics 2020) was used to calculate the octanol/water partition coefficient (LogP), the polar surface area (TPSA), the number of atoms and molecular weight (MW), the number of H-bond acceptors (ON) and H-bond donors (OHNH), as well as the volume (V) and the number of rotatable links (rotb). Additionally, we have used the program CBLigand-BBB predictor to evaluate the capacity of **MJM255** to cross the BBB and access to the CNS (Blood-Brain Barrier Predictor–CBLigand 2020). All the results, together with the prediction of the violations of Lipinski rules (n viol), are reported in Table 2.

Theoretically, **MJM255** possesses the desirable physicochemical properties for a good bioavailability as no violations of the Lipinski rule of five were detected. In addition, theoretically **MJM255** presents the same capacity to cross the BBB as tacrine or ESR.

In vivo studies: object recognition test (ORT)

The *in vivo* studies following the ORT for **MJM255** and the reference compound, ESR, were performed in two different groups: mice without pretreatment and mice pretreated with the muscarinic receptor antagonist, SCO (1 mg/kg). In the first case, the behavior of the animals in a new environment was evaluated under normal conditions. In the case of the mice pretreated with SCO, it was evaluated whether **MJM255** acts as an *in vivo* inhibitor of AChE and, if at the administered dose, it is capable of inhibiting the metabolism of ACh. Thereby, the residence time of the ACh in the synaptic cleft would be increased, which would increase its concentration and could reverse the antimuscarinic effect of SCO.

ESR, also called physostigmine, is a tertiary alkaloid amine that occurs naturally in the seed of *Fisostigma venenosum* L., known as Calabar bean or Eseré nut. This drug has a methylcarbamate substituent and basic groups that bind to the anionic site of AChE. The transfer of the carbamoyl group to the serine hydroxyl group of the stearic site of the enzyme is the same as observed for ACh, but the carbamylated enzyme hydrolyzes much more slowly, recovering its enzymatic activity after several minutes, instead of microseconds (Andrade and Gondal 2020; Arens and Kearney 2019). The slow recovery of the carbamylated enzyme implies that it displays a rather prolonged effect, increasing cholinergic transmission.

For the study, the two groups of mice (with and without SCO treatment) were subdivided in three different ones: control (**A**), treated with ESR (**B**), and treated with **MJM255** (**C**). This order was used to present all the results. The study was performed in two different phases: Phase 1 (P1) and Phase (P2). During P1, similar objects were used (OBJ-1 and OBJ-2). During P2, different objects were used (OBJ-1 and OBJ-3). The parameters evaluated for the different groups, in both phases, were three: latency period for the approximation to the object, frequency of approximation and exploration time.

Latency period – Mice without scopolamine pretreatment (scopolamine free)

The results corresponding to the latency period, for the animals without pretreatment with SCO, can be observed in Figure 1. For each individual experimental group (saline, + CMCNa, saline + ESR, saline + **MJM255**), no difference in the phase P1 was observed. There were no statistically significant differences in latency period between the first approach to OBJ-1 *versus* OBJ-2 in any of the three mentioned groups. In a similar way, during phase P2 of the experiment no statistically significant differences in the latency period between OBJ-1 *versus* OBJ-3, were observed. In summary, both objects aroused the same curiosity and equally boosted the animals' interest, resulting in a similar latency period for both, regardless of the received treatment and experimental phase.

Conversely, when comparing the results between the different treatment groups, it was observed clear and visually marked differences. For the group of animals treated with ESR, the latency period was much higher than the control in both experimental phases, the highest values were obtained during the phase P1 of the experiment. The latency period to approach OBJ-1 for the animals treated with ESR increased in a statistically significant manner (97.13 ± 28.86, $P<0.01$, Figure 1B) compared to the control group (19.80 ± 3.90, Figure 1A). Similarly, in the case of OBJ-2, there was also a statistically significant increase in the latency period for the group treated with ESR (116.9 ± 18.21, $P<0.0001$, Figure 1B) *versus* the control group (11.10 ± 4.58, Figure 1A).

The results obtained during the phase P2 of the experiment in the group treated with ESR showed again that the latency period was higher than the control for the two objects (OBJ-1 and OBJ-3). For the group treated with **MJM255** (Figure 1C), the results were identical to the control group, in both phases of the experiment.

Latency period – Mice pretreated with scopolamine

As previously described for the animals without SCO pretreatment, in Figure 2 it can be observed that the animals pretreated with SCO did not show statistically significant differences in both latency periods to approach OBJ-1 *versus* OBJ-2 in phase P1 of the experiment, and to approach OBJ-1 *versus* OBJ-3 in phase P2 of the experiment, in any of the three different groups: control (A), treated with ESR (B) and treated with **MJM255** (C).

Contrary to what happened with animals not previously treated with SCO, in which ESR significantly increased the latency period, in this case that difference was completely eliminated (Figure 2B). This could perfectly correspond to the previously mentioned muscarinic receptor antagonist effect of SCO, which would counteract the indirect cholinergic effect of ESR when AChE is inhibited.

In summary, in this specific case, the latency period was practically identical for different objects, phases and treatments.

Frequency of approximation – Mice without scopolamine pretreatment (scopolamine free)

As shown in Figure 3, when comparing the results obtained for mice not previously treated with SCO, only for those treated with ESR, a statistically significant decrease between the frequency of approximation to OBJ-1 (16.00 ± 3.60, Figure 3B) and to OBJ-2 (5.38 ± 2.05, $P<0.05$, Figure 3B) in phase P1 of the experiment, was observed. However, this was not reproduced during the phase P2, since there were no statistically significant differences between the animal's approach to OBJ-1 (20.13 ± 5.83, Figure 3B) *versus* OBJ-3 (16.38 ± 6.84, Figure 3B).

For the control group (Figure 3A) and for the group of animals treated with **MJM255** (Figure 3C), there were no differences in any of the two phases of the experiment. During the phase P1 they approached both OBJ-1 and OBJ-2 and, in similar way, in the phase P2 they also approached both OBJ-1 and OBJ-3,

without statistically significant differences in the frequency of approximation for both phases. In conclusion, the mice did not discriminate between objects.

However, the results obtained when comparing the groups based on the treatment received, regardless of the phase of the experiment and the object, were striking. For animals treated with ESR, the frequency of approximation to the objects was clearly lower than for the animals treated with the vehicle (control), both during phase P1 (ESR: 16.00 ± 3.60 and control: 65.38 ± 8.27, P <0.0001 for OBJ-1; ESR: 5.38 ± 2.05 and control: 76.00 ± 7.70, P<0.0001 for OBJ-2, Figure 3B and 3A) and during phase P2 (ESR: 20.13 ± 5.83 and control: 53.75 ± 7.98, P<0.005 for OBJ-1; ESR: 16.38 ± 6.84 and control: 62.25 ± 6.26, P<0.001 for OBJ-3, Figure 3B and 3A).

For the animals treated with **MJM255**, although during the phase P1 of the experiment no significant differences were observed compared to the control group but, conversely, that differences were exhibited during the phase P2. The frequency of approximation of the animals to the different objects was much lower (**MJM255**: 18.00 ± 7.57 and control: 53.75 ± 7.98, P<0.01 for OBJ-1; **MJM255**: 20.50 ± 9.79 and control: 62.25 ± 6.26, P<0.01 for OBJ-3, Figure 3C and 3A) and practically identical to the group of animals treated with ESR (Figure 3B).

As above-mentioned, **MJM255** is an *in vitro* AChE inhibitor. Therefore, a possible reason for the ESR-like effect observed in the frequency of approximation to objects in the phase P2 of the experiment could be due to the fact that **MJM255** takes longer in exerting its inhibitory AChE action and, therefore, the effects appear with a delay when compared to ESR.

Frequency of approximation – Mice pretreated with scopolamine

In the case of the results obtained for mice pretreated with SCO, as can be seen in Figure 4, there were no statistically significant differences in the frequency of approximation between the different objects in any of the groups, during phases P1 or P2 of the experiment.

As already mentioned, for animals without pretreatment, a clear decrease in the frequency of approximation to the different objects for the group treated with ESR was observed, in both phases, compared to the control group. Similarly, this effect was also seen in the group treated with **MJM255** *versus* the control group, but only during the phase P2 of the experiment.

However, this fact is not so remarkable for animals pretreated with SCO. This result would reveal a potential central cholinergic effect, indirect in both ESR and **MJM255**, since the mentioned decrease in frequency of approximation is annulled by a muscarinic receptor antagonist, SCO.

A decrease in the frequency of approximation to the objects of the animals treated with ESR was observed in both phases and for the animals treated with **MJM255**, during the phase P2 of the experiment. During P1, there were statistically significant differences in the frequency of approximation to OBJ-2 for the animals treated with ESR compared to the control group (ESR: 53.14 ± 7.77 and control: 76.86 ± 3.27, P<0.05, Figure 4B and 4A). For OBJ-1, a tendency to decrease the approach frequency of

approximation for animals treated with ESR can be pointed out, but these differences are not statistically significant.

Likewise, when analyzing the phase P2 of the experiment, statistically significant differences were observed in the frequency of approximation to OBJ-1, both in the case of ESR (46.57 ± 10.75, $P<0.05$, Figure 4B) and **MJM255** (44.14 ± 6.78, $P<0.01$, Figure 4C) compared to the control group (73.71 ± 6.19, Figure 4A). Furthermore, the results for ESR and **MJM255** were very similar to each other. In the case of OBJ-3, these differences, compared to the control group, were only observed for the animals treated with ESR (44.71 ± 8.87 and control: 73.29 ± 5.89, $P<0.05$, Figure 4B and 4A). In the case of **MJM255**, a trend to decrease the frequency of approximation to OBJ-3 was observed, although this was not statistically significant.

The frequency of approximation values of the group treated with **MJM255** during phase P2 were again very similar to the frequency of approximation values of the group treated with ESR in the two phases of the experiment, reinforcing the idea that both have similar pharmacological mechanisms, but **MJM255** takes longer to manifest its effect.

Exploration time – Mice without scopolamine pretreatment (scopolamine free)

As shown in Figure 5, for mice non-treated with SCO, no statistically significant differences for the exploration time of the different objects were noticed, within any group, during phases P1 or P2 of the experiment. For the group of mice treated with **MJM255**, it seems to be a tendency to increase the exploration time of OBJ-1 during phase P1 of the experiment, when compared to OBJ-2, although these results are not statistically significant (Figure 5C).

If we analyze the results obtained during phase P1 of the experiment between groups (intergroups), we can affirm that the mice treated with **MJM255** showed a tendency to increase the exploration time of OBJ-1 comparing to the control group, although these results are not statistically significant. Also, during phase P1 of the experiment, a statistically significant decrease was observed for the exploration time of OBJ-2, both for the group treated with ESR (26.83 ± 15.72, $P<0.005$, Figure 5B) and **MJM255** (57.40 ± 6.26, $P<0.05$, Figure 5C) comparing to the control group (78.98 ± 5.92, Figure 5A).

When analyzing phase P2 of the experiment, it was observed that ESR (24.11 ± 9.53, $P<0.005$, Figure 5B) statistically significant decreases the exploration time of OBJ-3 compared to the control group (68.73 ± 8.45, Figure 5A). However, this was not reproduced for the group treated with **MJM255** (Figure 5C).

Exploration time – Mice pretreated with scopolamine

In the case of mice pretreated with SCO (Figure 6), no statistically significant differences for the exploration time of the different objects were noticed, within any group, during phases P1 or P2 of the experiment. For the control and **MJM255**-treated groups (Figures 6A and 6C), a slight tendency to decrease the exploration time of OBJ-1 could be observed in both phases, although the differences were not, in any case, statistically significant.

Analyzing the results obtained for the different groups, it can be seen that only the group treated with ESR presented statistically significant differences in both phases of the experiment, compared to the control group. In phase P1 of the experiment, the exploration time of OBJ-2 for the animals treated with ESR (54.43 ± 10.25, $P<0.01$, Figure 6B) decreased comparing to the control group (88.66 ± 3.64, Figure 6A). During phase P2 of the experiment, similar results were observed. The exploration time of OBJ-3 of the animals treated with ESR (56.77 ± 9.45, $P<0.01$, Figure 6B) decreased in a statistically significant manner comparing to the control group (91.91 ± 8.31, Figure 6A).

Despite the variability observed, there is evidence that suggests that **MJM255** could inhibit AChE in mice, increasing the levels of the neurotransmitter ACh in the brain. In summary, the results provided in Figure 3 are especially relevant, since it can be clearly observed that the administration of **MJM255** modifies the frequency of approximation to the objects during the phase P2 of the experiment, reducing it and showing a similar effect to ESR, although this effect takes longer to be manifested. Therefore, **MJM255** is a promising molecule, presenting an interesting *in vivo* activity.

Conclusions

The most powerful AChE inhibitor of all the studied 3-amidocoumarins, **MJM255**, was selected for an *in vivo* study, using the ORT. Theoretical physicochemical properties indicate a good bioavailability for this compound and ability to cross the BBB. Mice pretreated with SCO were used to try to elucidate whether **MJM255** is capable of inhibiting AChE *in vivo* and, consequently, increase the residence time of the ACh neurotransmitter in the synaptic cleft and counteract the effects exerted by the muscarinic receptor antagonist, SCO. Although the results are certainly difficult to interpret, they evidence that **MJM255** exerts similar effect as ESR, but in a slower way. This is especially appreciated when evaluating the parameter of frequency of approximation of the animals to objects. This result could indicate that **MJM255** not only acts as an *in vitro* inhibitor of AChE, but it can be compatible with a central indirect cholinergic effect. Further studies are needed to analyze the effect of this compound, and its presence and concentration at the CNS, in order to know the potential of this molecule as a promising hit compound for AD.

Declarations

Ethics approval. Registration Code 15007AE/09/INV MED 02/NER02/JAFG13, authorized by the Consellería do Medio Rural – Autonomous Government of Galicia (Xunta de Galicia).

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Consent to Publish. Not applicable.

Authors Contributions. The authors declare that all data were generated in-house and that no paper mill was used. DV, JAF and MM conceived and designed research. FRE, DV and MM conducted experiments.

DV, JAF, EU and MM contributed new reagents or analytical tools. DV, JAF, EU and MM analyzed data. FRE and MM wrote the manuscript. JAF and EU revised the manuscript. All authors read and approved the manuscript and all data were generated in-house and that no paper mill was used.

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Availability of data and material. All the data is available from the authors under request.

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Tables

Table 1. IC_{50} values for the activity of **MJM255**, tacrine and ESR on recombinant *h*AChE, expressed in HEK 293 cells, and *h*BuChE, isolated from human serum.

Compounds	IC_{50} *h*AChE (µM)	IC_{50} *h*BuChE (µM)
MJM255	12.89 ± 0.86	*
Tacrine	0.45 ± 0.03	0.15 ± 0.01
ESR	0.12 ± 0.01	0.15 ± 0.01

Each IC_{50} value is the mean ± S.E.M. from five experiments (n = 5). * At 100 µM inhibits enzymatic activity below 20%.

Table 2. Molecular properties of **MJM255**, tacrine and ESR calculated using the Molinspiration software and CBLigand-BBB predictor program.

Comp.	LogP	TPSA	n atoms	MW	n ON	n OHNH	n rotb	V	n viol	BBB
MJM255	2.79	105.13	23	310.27	7	1	3	254.72	0	+
Tacrine	3.05	38.92	15	198.27	2	2	0	191.53	0	+
ESR	1.94	44.81	20	275.35	5	1	2	261.48	0	+

Figures

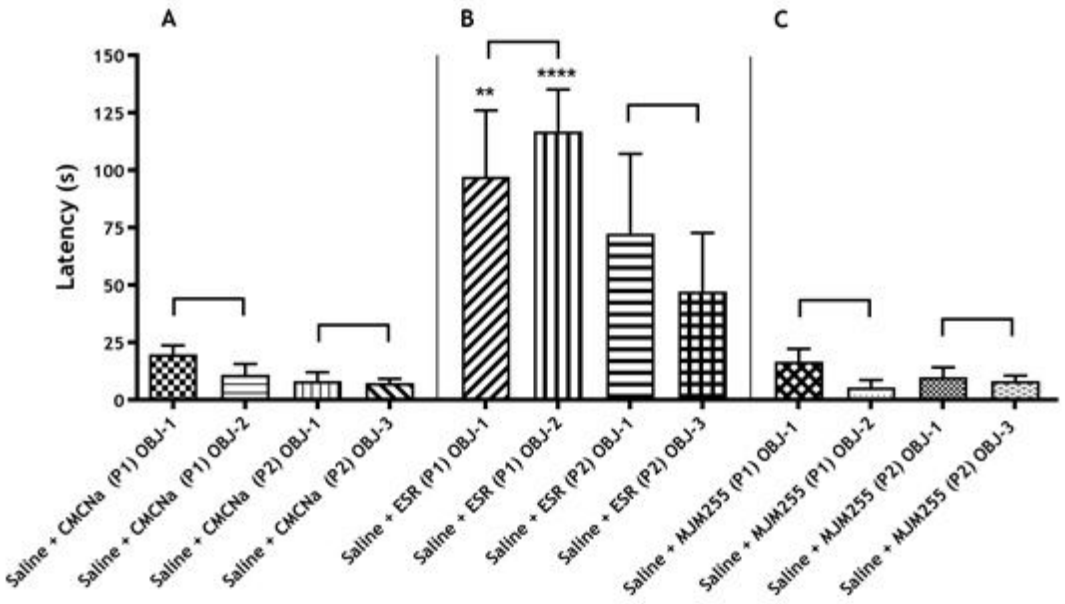

Figure 1

Latency period for approximation to each object in each phase of the experiment for mice without SCO pretreatment, during both P1 (similar objects) and P2 (different objects) phases of the experiment. ** P<0.01, **** P<0.0001. (A) Control, (B) ESR and (C) MJM255.

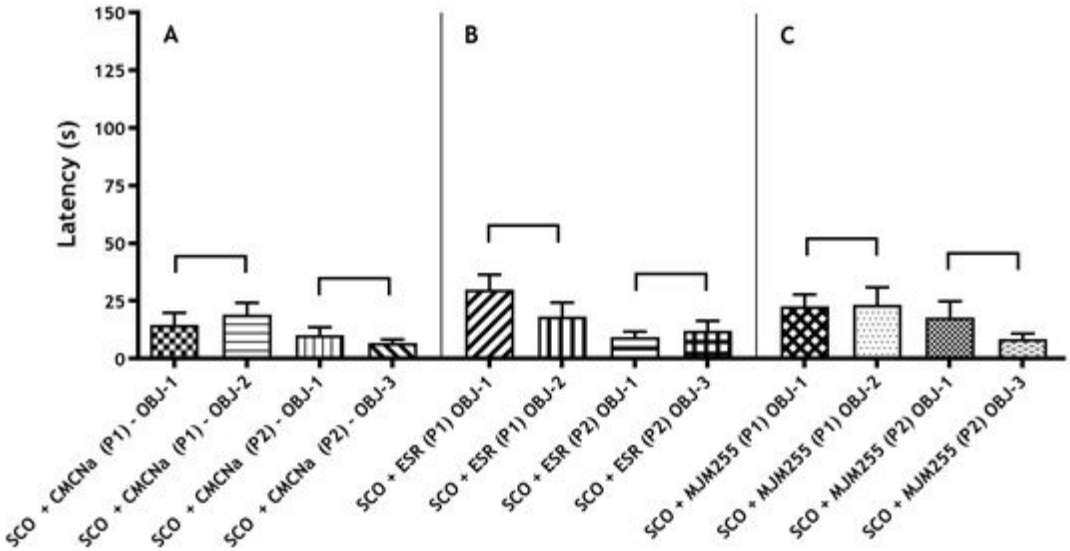

Figure 2

Latency period for approximation to each object in each phase of the experiment for mice pretreated with SCO, during both P1 (similar objects) and P2 (different objects) phases of the experiment. (A) Control, (B) ESR and (C) MJM255.

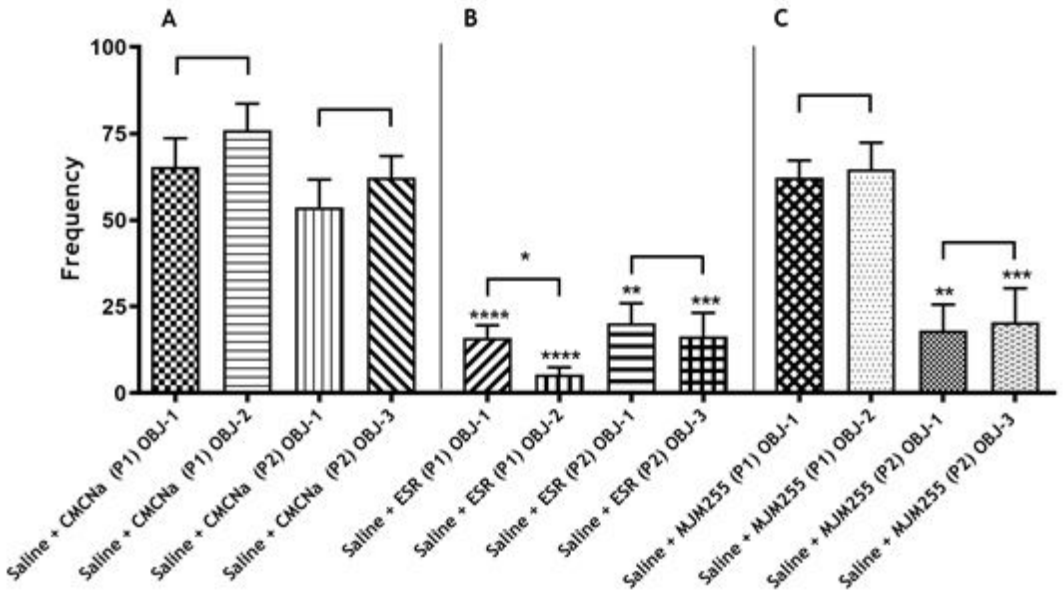

Figure 3

Frequency of approximation for each object in each phase of the experiment for mice without SCO pretreatment, during both P1 (similar objects) and P2 (different objects) phases of the experiment. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. (A) Control, (B) ESR and (C) MJM255.

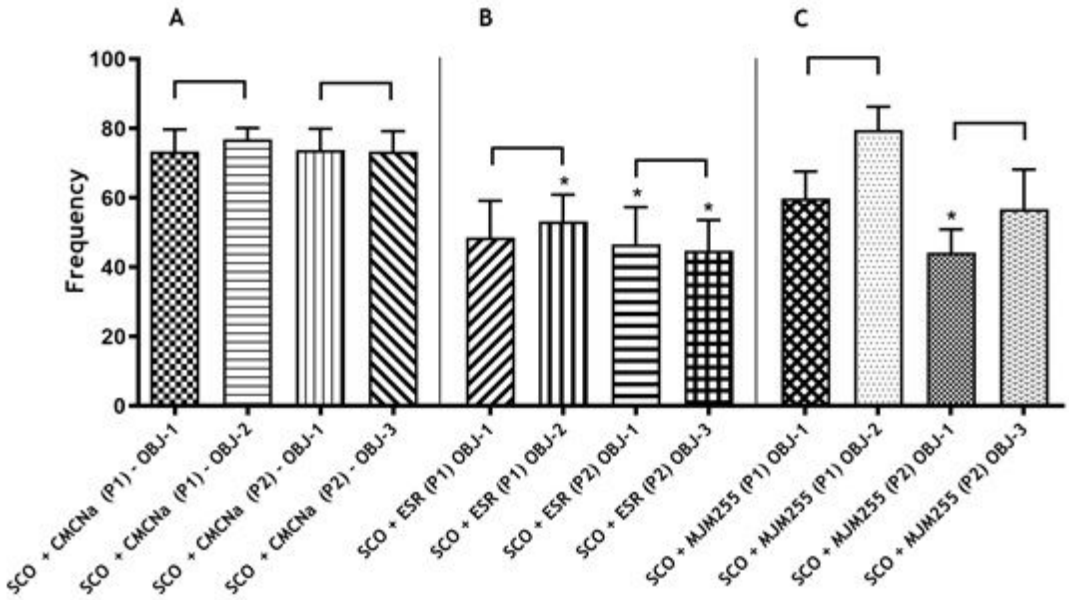

Figure 4

Frequency of approximation for each object in each phase of the experiment for mice pretreated with SCO, during both P1 (similar objects) and P2 (different objects) phases of the experiment. * P<0.05. (A) Control, (B) ESR and (C) MJM255.

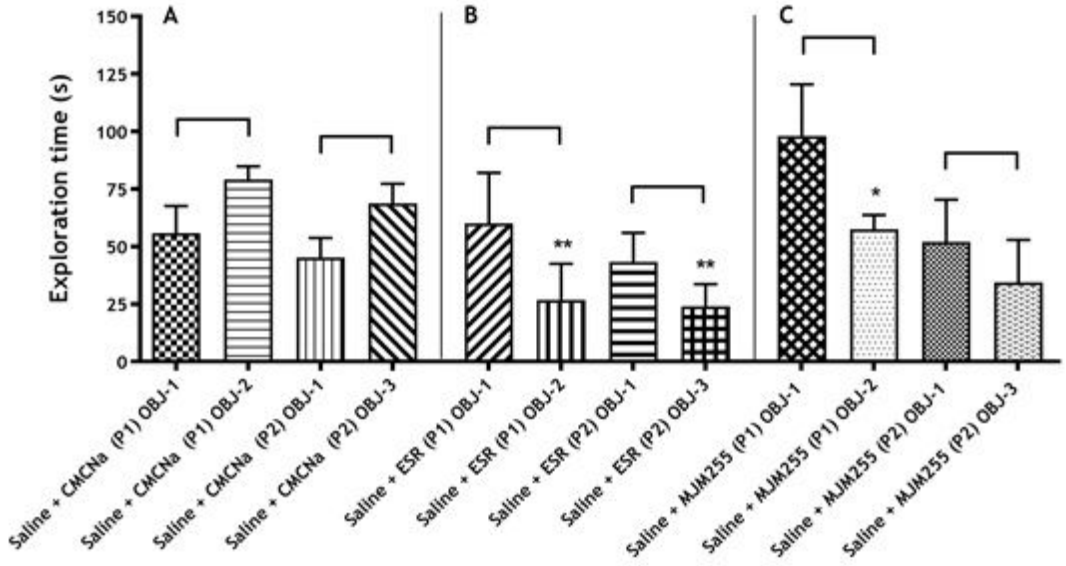

Figure 5

Exploration time for each object in each phase of the experiment for mice without SCO pretreatment, during both P1 (similar objects) and P2 (different objects) phases of the experiment. * P<0.05, ** P<0.01. (A) Control, (B) ESR and (C) MJM255.

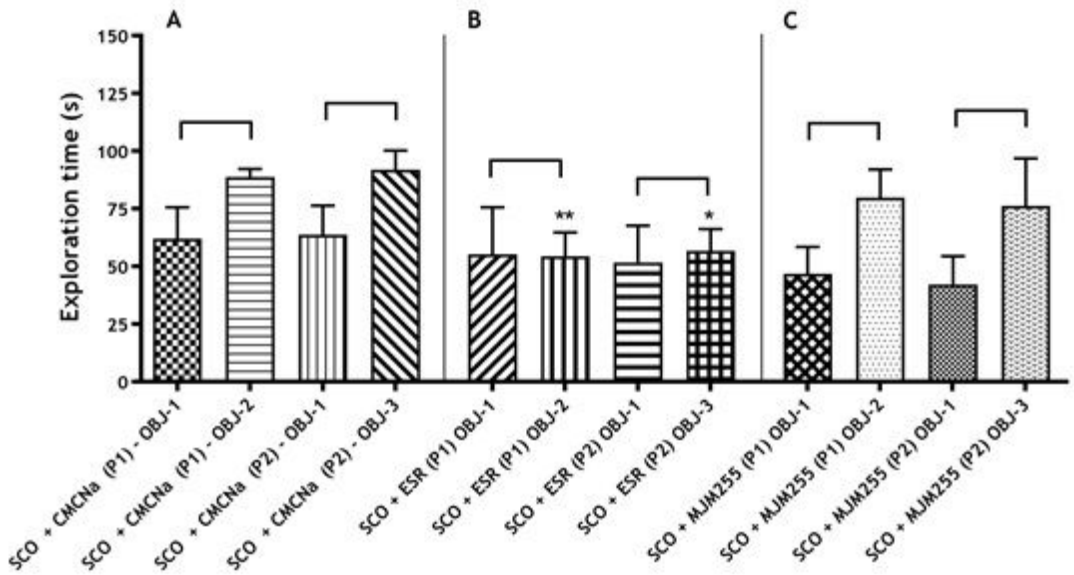

Figure 6

Exploration time in the selected area for each object in each phase of the experiment for mice pretreated with SCO, during both P1 (similar objects) and P2 (different objects) phases of the experiment. * P<0.05, ** P<0.01. (A) Control, (B) ESR and (C) MJM255.

Supplementary Files

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