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N-propyl-2,2-diphenyl-2-hydroxyacetamide, a novel α-hydroxyamide with anticonvulsant, anxiolytic and antidepressant-like effects that inhibits voltage-gated sodium channels

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ABSTRACT

In patients with epilepsy, anxiety and depression are the most frequent psychiatric comorbidities but they often remain unrecognized and untreated.

We report herein the antidepressant-like activity in two animal models, tail suspension and forced swimming tests, of six anticonvulsants α-hydroxyamides. From these, N-propyl-2,2-diphenyl-2-hydroxyacetamide (compound **5**) emerged not only as the most active as anticonvulsant (ED_{50} = 2.5 mg/kg, MES test), but it showed the most remarkable antidepressant-like effect in the tail suspension and forced swimming tests (0.3–30 mg/kg, i.p.); and, also, anxiolytic-like action in the plus maze test (3–10 mg/kg, i.p.) in mice. Studies of its mechanism of action, by means of its capacity to act via the $GABA_A$ receptor ([^{3}H]-flunitrazepam binding assay); the 5-HT_{1A} receptor ([^{3}H]-8-OH-DPAT binding assay) and the voltage-gated sodium channels (either using the patch clamp technique in hNa_v 1.2 expressed in HEK293 cell line or using veratrine, in vivo) were attempted. The results demonstrated that its effects are not likely related to 5-HT_{1A} or $GABA_A$ergic receptors and that its anticonvulsant and antidepressant-like effect could be due to its voltage-gated sodium channel blocking properties.

1. Introduction

Epilepsy is a common group of neurological disorders whose hallmark is unprovoked seizures that can be distressing, harmful and even fatal. Associated comorbidities contribute greatly to disability and impaired quality of life of people with epilepsy (Gaitatzis et al., 2012). They present a significantly higher rate of anxiety and depression than the general population, and the symptoms of these pathologies might serve as a barrier to appropriate epilepsy care, as they are sometimes treated first or instead of the major pathology that caused them (Kanner, 2011). Several clinical studies have demonstrated the "bi-directional" relationship between depression and epilepsy or the presence of common pathogenic mechanisms that facilitate the incidence of one in the presence of the other (Kanner, 2003). Despite the high prevalence and significant consequences of comorbid depression in people with epilepsy, it often remains under-treated or untreated (Yang et al., 2014). Furthermore, these pathologies, when recognized, are treated with psychotropic drugs which can worsen seizures (Mula and Schmitz, 2009).

Antiepileptic drugs comprise a heterogeneous group of agents with diverse mechanisms of action that can be grouped into four broad categories: modulation of voltage-dependent sodium, calcium or potassium channels; increase in GABAergic inhibition via actions on $GABA_A$ receptors or on GABA synthesis, reuptake, or degradation; decreased synaptic excitation via actions on ionotropic glutamate receptors and/or modulation of neurotransmitter release via presynaptic mechanisms (Landmark, 2007; Rogawski and Loscher, 2004). On the other hand, antidepressant drugs modify the activity of neurons in different ways, by increasing monoamines levels and by modulating ion channels. Voltage-gated sodium channels are involved in many

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psychiatric disorders. Inhibition of voltage-gated sodium channels has emerged as a relevant mechanism since several anticonvulsants are effective to treat depression. Moreover, some antiepileptic drugs are also used as mood stabilizers i.e. lamotrigine, topiramate, phenytoin, carbamazepine and valproic acid (Yatham, 2004). In addition, voltage-gated sodium channels blockers have been proposed to be an effective non-benzodiazepine treatment for anxiety (Bourin et al., 2009; Mirza et al., 2005; Saitoh et al., 2015; Sugiyama et al., 2012).

Continuous efforts are done by researchers to find drugs that can be active as anticonvulsant/antidepressant/anxiolytic to minimize the side effects caused by the co-administration of drugs to treat these pathologies. Our group has built a considerable expertise in the synthesis of new molecular entities with anticonvulsant, antidepressant and anxiolytic-like profiles (Pastore et al., 2013, 2014; Wasowski et al., 2012; Wasowski and Marder, 2012).

We have previously described the synthesis and the anticonvulsant activity of novel heterocycles N-derivative-1,2,3-oxathiazolidine-4-one-2,2-dioxides, bioisosteres of trimethadione (oxazolidine-2,4-dione) and phenytoin, via the cyclization of a series of α-hydroxyamides (Pastore et al., 2014). These α-hydroxyamides also showed excellent anticonvulsant activity and were not neurotoxic (Pastore et al., 2013). The aim of this work was to further expand the anticonvulsant profile and to evaluate the potential antidepressant-like activities of these α-hydroxyamides. Furthermore, a first approximation of their mechanism of action, by means of their capacity to act on the $GABA_A$ receptor, the $5HT_{1A}$ receptor and/or the voltage-gated sodium channels was attempted.

2. Materials and methods

2.1. Drugs and chemistry

N-propyl-2-hydroxyisobutylamide (**1**); N-butyl-2-hydroxyisobutylamide (**2**); N-benzyl-2-hydroxyisobutylamide (**3**); N-phenetyl-2-hydroxyisobutylamide (**4**); N-propyl-2,2-diphenyl-2-hydroxyacetamide (**5**) and N-butyl-2,2-diphenyl-2-hydroxyacetamide (**6**) (Fig. 1) were obtained by microwave assisted free solvent synthesis as previously described (Pastore et al., 2013). Synthesis of N-propyl-2,2-diphenyl-2-hydroxyacetamide (**5**) is shown in Supplementary data. Their chemical purities were confirmed by elemental analysis (99%). Analysis of the ^{1}H NMR and ^{13}C NMR spectra showed that the compounds obtained presented analytical and spectroscopic data in full agreement with its assigned structure. Diazepam was obtained from Roche Diagnostics, [^{3}H]-flunitrazepam and [^{3}H]-8-hydroxy-2-(dipropylamino)tetralin ([^{3}H]-8-OH-DPAT) were obtained from Perkin Elmer Life and Analytical Sciences, Boston, MA, USA, imipramine hydrochloride and veratrine from Sigma-Aldrich. All reagents for electrophysiological recordings were of analytical grade and were purchased from local suppliers, as well as Dulbecco's Modified Eagle's Medium and Fetal bovine serum. Geneticin was obtained from Tecnolab S.A.

2.2. Animals and injection procedures

Both adult male Swiss mice weighing 25–30 g used in the pharmacological tests and adult male Wistar rats weighing 200–300 g used for binding assays were obtained from the Central Animal House of the School of Pharmacy and Biochemistry, University of Buenos Aires.

For behavioral assays mice were housed in groups of five in a controlled environment (20–23 °C), with free access to food and water and maintained on a 12 h/12 h day/night cycle, light on at 07:00 A.M. Housing, handling, and experimental procedures complied with the recommendations set forth by the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Publication No. 8023, revised 1996) and the Institutional Committees for the Care and Use of Laboratory Animals of the University of La Plata, Argentina (code: 014-06-15, 2016) and the Faculty of Pharmacy and Biochemistry, University of Buenos Aires, Argentina (code: 31682/2014). All efforts were taken in order to minimize animal suffering. The number of animals used was the minimum number consistent with obtaining significant data. Mice were randomly assigned to any treatment groups and were used only once. The pharmacological tests were evaluated by experimenters who were kept unaware of the treatment administered and were performed between 10:00 A.M. and 2:00 P.M.

Compounds **1**–**6** were dissolved by using the sequential addition of dimethylsulfoxide, a solution of 0.25% Tween 80 and saline; up to final concentrations of 5%, 20% and 75%, respectively. The rodents were intraperitoneally (i.p.) injected. Veratrine was dissolved in saline at a dose of 0.125 mg/kg. In each session, a control group receiving only vehicle was tested in parallel with those animals receiving drug treatment. Vehicle control mice showed no significant differences in any of the tests assayed compared to mice treated with saline (data not shown).

2.3. Biochemical assays

2.3.1. Tissue preparation

For [^{3}H]-flunitrazepan binding assays, membranes were prepared according to literature (Wasowski et al., 2012). Briefly the brains were rapidly dissected out on ice and the different structures were homogenized in 10 volumes of 0.32 M sucrose and centrifuged at 900 × *g* for 10 min. The resulting supernatant was centrifuged at 100,000 × *g* for

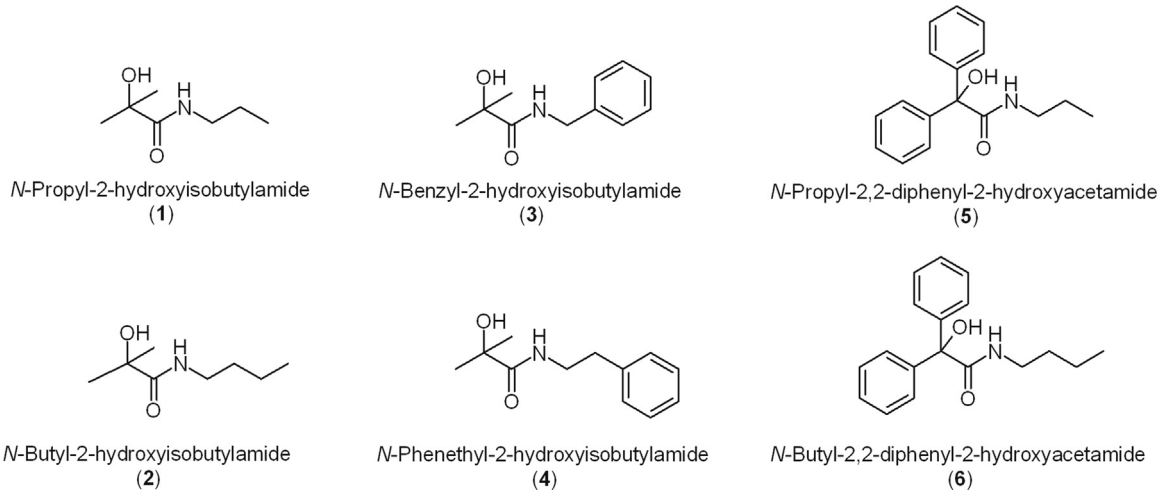

Fig. 1. Molecular structures of the compounds.

30 min and the pellet washed twice in 25 mM Tris–HCl buffer pH 7.4 at 100,000 × *g* for 30 min, and stored at − 20 °C until used.

For $5HT_{1A}$ binding assays the cortex tissues were homogenized in 30 volumes/weight of ice-cold 50 mM Tris–HCl buffer pH 7.4 and the homogenate was centrifuged at 40,000 × *g* for 15 min at 4 °C. The resulting pellets were suspended in 30 volumes/weight of the same buffer and incubated at 37 °C for 20 min. The centrifugation step was repeated twice under the same conditions as described above and the final pellets were suspended in 30 volumes/weight of 50 mM Tris–HCl buffer pH 7.4 and stored at − 80 °C until use. Protein concentration was determined by the method of Bradford using bovine serum albumin as standard (Bradford, 1976).

2.3.2. [^{3}H]-flunitrazepan binding assay

A radioligand binding assay was used to evaluate the putative action of the compounds (**1**–**6**) on the benzodiazepine binding site of the $GABA_A$ receptor complex. The binding of [^{3}H]-flunitrazepam (81.8 Ci/mmol) to the benzodiazepine binding site was performed in washed crude synaptosomal membranes from rat cerebral cortex prepared as described previously (Medina et al., 1990).

The compounds were added to 0.2–0.3 mg membrane protein suspended in 1 ml of 25 mM Tris–HCl buffer in the presence of [^{3}H]-flunitrazepam 0.3 nM. In the screening assays each compound was tested at 300 μM in triplicate. Diazepam was used as positive control. Non-specific binding was measured in the presence of flunitrazepam 10 μM and represented 5–15% of the total binding. The incubations were carried out at 4 °C for 1 h. After incubation, the assays were terminated by filtration under vacuum through Whatman GF/A glass-fiber filters followed by washing three times with 3 ml each of incubation medium. Individual filters were incubated overnight with scintillation cocktail (OptiPhase 'HiSafe' 3) before measuring radioactivity in a Wallac Rackbeta 1214 liquid scintillation counter.

2.3.3. [^{3}H]-8-OH-DPAT binding assays

For the binding assay, membranes were thawed and suspended in 50 mM Tris–HCl pH 7.4, with 1 mM $MnCl_2$ to a final protein concentration of 0.50–0.55 mg/ml. The incubation was carried out at 25 °C for 1 h in a final volume of 1 ml of membrane suspension (in duplicate) in the presence of the sample assayed and with 0.55 nM of [^{3}H]-8-OH-DPAT (170.2 Ci/mmol). Nonspecific binding was determined in parallel incubations in the presence of serotonin (10 μM). The assays were terminated by filtration under vacuum through Whatman GF/A glass-fiber filters and three washes with 3.5 ml each of incubation medium. Radioactivity was determined as described above.

2.4. Electrophysiology

Patch-clamp experiments were performed in HEK293 cell lines stably expressing hNa_v 1.2 α subunit (a kind gift of GlaxoSmithKline, Stevenage, UK) that have been described (Burbidge et al., 2002; Mantegazza et al., 2005). Details of the experiments are described below (Section 2.4.2).

2.4.1. Cell culture

HEK293 cell lines were cultured in minimum essential medium, containing 10% fetal bovine serum, and 0.5% geneticin G418 sulfate. Cells were grown in a 95% O_2/5% CO_2 atmosphere at 37 °C and with 95% humidity. One to two days prior to electrophysiological recordings, the cells were plated on glass coverslips.

2.4.2. Whole-cell voltage-clamp recordings

The cells were grown on glass coverslips and were observed with a mechanically stabilized inverted microscope (Telaval 3, Carl Zeiss, Jena, Germany) equipped with a 40X objective lens. The test solutions were applied through a multi-barreled pipette, by gravity and in continuous flux, positioned close to the target cell. After each experiment on a single cell, the experimental chamber was replaced by another one containing a new sample of cells. All experiments were performed at room temperature (~ 22 °C).

The standard tight-seal whole-cell configurations of the patch-clamp technique (Hamill et al., 1981) was used to record macroscopic currents. Glass pipettes were drawn from WPI PG52165-4 glass on a two-stage vertical micropipette puller (PP-83, Narishige Scientific Instrument Laboratories, Tokyo, Japan) and pipette resistance ranged from 2 to 3 MΩ.

Whole-cell currents were filtered with a 4-pole lowpass Bessel filter (Axopatch 200A amplifier) at 2 kHz and digitized (Digidata 1440, Molecular devices) at a sample frequency of 200 kHz (5 μs). The experimental recordings were stored on a computer hard disk for later analysis. Cells were placed in a recording chamber with 0.5 ml extracellular solution containing (in mM): NaCl 140, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10 and glucose 11; pH was adjusted to 7.4 with NaOH. The patch electrodes were filled with pipette solution containing (in mM): CsF 140, EGTA 10, HEPES 10, NaCl 5, $MgCl_2$ 2; the pH was adjusted to 7.3 with CsOH.

Once the whole-cell configuration was obtained, current and series resistance stability were evaluated with a 15 ms-voltage-clamp step from a holding potential of − 80 mV to a test potential of − 20 mV repeated each 10 s. The time needed for the stabilization was variable (approximately 10 min). The series resistance was not electronically compensated, but as it maintains constant during the experiment, the series resistance error contamination is the same before and after drug perfusion. The same voltage-clamp step protocol was applied in the control (vehicle) or in the presence of compound **5**, dissolved in 0.1% dimethylsulfoxide. The voltage dependence of the steady-state inactivation of sodium channels was evaluated using a double voltage-step protocol, where the same depolarization to − 10 mV followed different pre-conditioning steps (from − 130 to − 40 mV). The available fraction of sodium channels at each membrane potential (I_{Vc}/I_{max}) was calculated as the ratio of peak sodium current measured at − 10 mV, at each pre-conditioning voltage test pulse (I_{Vc}) and the maximum peak current observed (I_{max}). The relationship between the available fraction of sodium channels and the pre-conditioning (named *h* curve) was plotted and fitted with a Boltzmann equation (Eq. (1)): $I_{Vc}/I_{max} = 1/(1 + \exp((Vh - V)/k))$ where the available fraction is given as I_{Vc}/I_{max}, Vh is the potential of half-maximal inactivation and k is the slope parameter. Statistical significance of the change in the Vh parameter induced by compound **5** was tested with F method (GraphPad Prism).

2.5. Behavioral assays

2.5.1. Anticonvulsant effect and neurotoxicity

The anticonvulsant activity and neurotoxicity of α-hydroxyamides were performed in acute models, using the procedures proposed by the National Institute of Health (NIH) via the Epilepsy Therapy Screening Program (ETSP) (Rogawski and Loscher, 2004; Stables and Kupferberg, 1997). All procedures were previously published as well they are available for public in NIH website at (https://www.ninds.nih.gov/Current-Research/Focus-Research/Focus-Epilepsy/ETSP). The primary evaluation (phase 1) includes the use of two anticonvulsant tests: Maximal Electroshock Seizure test (MES) and subcutaneous Pentylenetetrazole Seizure test (scPTZ). Toxicity is primary detected using the standardized Rotarod test, which is also included in this phase (Dunham and Miya, 1957). According to this procedure the test substances were administered in 30% polyethylene glycol 400 (PEG) and 10% water. The drugs were administered i.p. in mice in a volume of 0.01 ml/g body weight.

2.5.1.1. scPTZ test. The scPTZ test involves a chemical induction to generate convulsions related to myoclonic seizures and entailed the s.c. administration of 85 mg/kg of PTZ in saline in the posterior midline of

the mice. Afterwards, the animals were placed in 15 cm × 30 cm chambers to record seizure during 30 min of observation. Protection was defined as the failure to observe even a threshold seizure (single episode of clonic spasms of at least 5 s duration). Compounds that are active in scPTZ may act raising seizure threshold (Malawska et al., 2004; Rogawski and Loscher, 2004).

2.5.1.2. MES test. The MES test is associated with the electrical induction of the seizure. The endpoint in this test is tonic hindlimb extension, and the test is thought to be a predictive model for generalized tonic–clonic seizures. The compounds were administrated i.p. to mice at doses of 30 and 100 mg/kg. Quantitative biological studies (phase II) were performed for the most promising compounds from phase I (in MES test). At this stage, the anticonvulsant activity was expressed as median effective dose, ED_{50}, which determines the drug concentration that is effective in the 50% of the tested animals. ED_{50} values were calculated by treating groups of six mice with different doses at the maximal time effect previously determined.

2.5.1.3. Rotarod test. The Rotarod test is used exclusively in mice to assess minimal neurotoxicity. A normal mouse can maintain its equilibrium on a rotating rod (6 rpm) for long periods of time. Motor deficit is indicated by failure to maintain balance on a rotating rod in each of three trials of 1 min each.

2.5.2. Antidepressant-like activities

2.5.2.1. Forced swimming test. The forced swimming test employed was similar to that described previously (Porsolt et al., 1978). This test is currently the most reliable method to screen compounds for antidepressant effects in rodents. Mice were individually dropped in glass cylinders (height: 25 cm; diameter: 10 cm; containing 10 cm of water at 24 ± 1 °C) for 6 min. The immobility time was recorded during the last 4 min of the test. Each mouse was judged to be immobile when it ceased struggling and remained floating in the water, making only the necessary movements to keep its head above water. The test was performed 30 min after the i.p. injection of the α-hydroxyamides, imipramine (control drug) or vehicle.

2.5.2.2. Tail suspension test. The tail suspension test was carried out according to the methodology proposed by Steru et al. (1985). This test is frequently used in laboratory practice to identify compounds with antidepressant-like activity. Mice were individually suspended by the tail to a metal hook (distance from floor: 18 cm) using adhesive tape (distance from tip of tail: 2 cm) for 6 min. Typically, mice demonstrate several escape oriented behaviors interspersed with temporally increasing bouts of immobility. The duration of immobility was recorded during the final 4 min interval of the test. Mice were considered immobile only when they hung passively and completely motionless. The test is based on the fact that animals subject to the short-term, inescapable stress of being suspended by their tails will develop an immobile posture. The test was performed 30 min after the i.p. injection of α-hydroxyamides, imipramine or vehicle.

2.5.3. Locomotor activity test

Because all behaviors measured in the tests performed depend on locomotor activity that is probably the confounding issue; the spontaneous locomotor activity was recorded in separate experiments. The spontaneous locomotor activity was measured in a box made of Plexiglass, with a floor of 30 cm × 15 cm and 15 cm high walls as previously described (Fernandez et al., 2006). The locomotor activity was expressed as total light beam counts per 5 min.

2.5.4. Anxiolytic-like effect

2.5.4.1. Elevated plus-maze test. The elevated plus-maze set-up consisted of a maze of two open arms, 25 cm × 5 cm, crossed by two closed arms of the same dimensions, with free access to all arms from the crossing point. The closed arms had walls 15 cm high all around. The maze was suspended 50 cm from the room floor. Mice were placed on the central part of the cross facing an open arm. The number of entries and time spent in open arms were counted during 5 min under red dim light. An arm entry was defined as all four paws having crossed the dividing line between an arm and the central area. The total exploratory activity (number of entries in both arms) was also determined (Lister, 1987). Diazepam was used as reference compound.

2.6. Effect of pre-treatment with veratrine on the antidepressant effect of N-propyl-2,2-diphenyl-2-hydroxyacetamide (5) in the forced swimming test

The possible role of voltage-gated sodium channels in the mechanism of action of N-propyl-2,2-diphenyl-2-hydroxyacetamide (compound **5**) as antidepressant was investigated using veratrine, a selective activator of voltage-gated sodium channels.

The dose of 0.125 mg/kg of veratrine was chosen for this study as it did not induce increase or decrease in spontaneous locomotor activity nor antidepressant like effects in the forced swimming test, based on a previous study (Prica et al., 2008). Veratrine was administered, in mice, in association with two active doses of compound **5**. The sub active dose of veratrine (or saline) was administered 45 min before testing in the forced swimming test, and compound **5** (or vehicle) were administered 30 min before. The animals were randomly allocated in six experimental groups (number of mice per group = 6–27) as follows (indicated as pretreatment-treatment): (1) saline-vehicle, (2) saline-compound **5** (10 mg/kg), (3) saline-compound **5** (1 mg/kg), (4) veratrine-vehicle, (5) veratrine-compound **5** (10 mg/kg), (6) veratrine-compound **5** (1 mg/kg).

2.7. Statistical analyses

The effects of the compounds in mice were analyzed by one-way analysis of variance (ANOVA) and post-hoc comparisons between treatments and control groups were made using Dunnett's multiple comparison test. The association study with veratrine was analyzed by two-way ANOVA (pre-treatment vs. treatment) and post-hoc comparison was made using Bonferroni post test. As a significant interaction between pre-treatment and treatment was observed, subsequent one-way ANOVAs and Newman-Keuls multiple comparison post-hoc test were applied. Electrophysiological experiments were analyzed using paired *t*-test. A P value < 0.05 was considered statistically significant. All data were expressed as mean ± S.E.M. and analyzed with GraphPad Prism 5.00 software.

3. Results

3.1. Anticonvulsant effect of α-hydroxyamides

Table 1 shows the anticonvulsant profile of compound **5**. Profiles of compounds **1**, **2**, **3**, **4** and **6** were previously reported (Pastore et al., 2013). The results demonstrated that the administration of these compounds presented absence of tonic extension of the hind legs after an electroshock in the MES test, even at doses lower than 100 mg/kg, being compound **5** the most potent with an ED_{50} of 2.5 mg/kg. Compound **5** did not present anticonvulsant activity in the scPTZ test. However, compounds **2**, **3**, and **4** were able to reduce convulsions induced in this assay. Importantly, none of the mice treated with α-hydroxyamides died during the assays or showed any failure to maintain balance on the Rotarod assay, evidencing that they did not produce neurotoxicity or motor impairment, at the doses tested.

3.2. Antidepressant effects of α-hydroxyamides

An initial antidepressant-like effect screening of all α-hydroxyamides was performed, at a fixed dose of 10 mg/kg, using the forced

Table 1
Anticonvulsant profile of N-propyl-2,2-diphenyl-2-hydroxyacetamide (**5**) in mice.

Dose	MES test[a]				scPTZ test[b]			Rotarod test[c]	
(mg/kg)	0.5 h	4 h	Class[d]	MTE (h)[e]	0.5 h	4 h	Class[d]	0.5 h	4 h
30	1/3	2/3	**1**	4	0/3	0/3	3	0/3	0/3
100	3/3	2/3	ED_{50} = 2.5 mg/kg		0/3	0/3		0/3	0/3

Values represent number of mice protected from seizures or with neurotoxic effects divided by the number of mice tested.
ED_{50}: median effective dose.
MTE and ED_{50} (MES test) values for phenytoin and trimethadione are 1 h and 5.54 mg/kg (White et al., 2002) and 0.5 h and 627 mg/kg (Krall et al., 1978) respectively. Values for compounds **1**, **2**, **3**, **4** and **6** were previously reported (Pastore et al., 2013).

[a] Maximal electroshock seizure.
[b] Pentylenetetrazol test.
[c] Toxicity evaluated in Rotarod test.
[d] The tested compounds can be classified into three classes according to their activity (Malawska et al., 2004): (1) anticonvulsant activity at 100 mg/kg or less; (2) anticonvulsant activity at doses higher than 100 mg/kg; (3) compound inactive at any doses up to 300 mg/kg.
[e] MTE: maximal time effect on MES test determined at intervals of 0.5, 1, 2 and 4 h at 30 mg/kg.

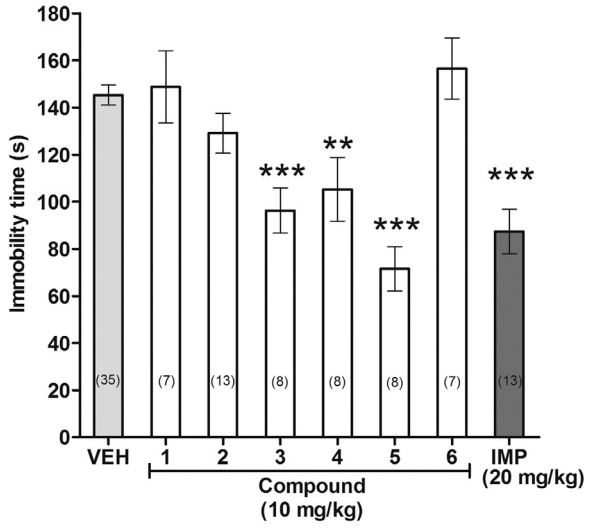

Fig. 2. Effects of acute administration of N-propyl-2-hydroxyisobutylamide (**1**), N-butyl-2-hydroxyisobutylamide (**2**), N-benzyl-2-hydroxyisobutylamide (**3**), N-phenethyl-2-hydroxyisobutylamide (**4**), N-propyl-2,2-diphenyl-2-hydroxyacetamide (**5**) and N-butyl-2,2-diphenyl-2-hydroxyacetamide (**6**) (10 mg/kg) and imipramine (IMP, 20 mg/kg) in the forced swimming test. Results are expressed as mean ± S.E.M. of the immobility time (in s) in comparison to control animals (injected with vehicle, VEH). The number of mice used in each group is shown in the column. Statistical analysis was performed by one-way ANOVA followed by Dunnett's test. **P < 0.01, ***P < 0.001 compared with the control group.

swimming test, and compared to vehicle control group and imipramine (20 mg/kg) treated group (Fig. 2).

The results revealed that compounds **1**, **2** and **6** did not showed an antidepressant-like effect at this dose, as they could not significantly decrease the immobility time of mice compared to control group. Meanwhile, compounds **3**, **4** and **5** and imipramine, significantly reduced the immobility time of mice.

Subsequently, these three α-hydroxyamides and compound **2**, that although not showing a significantly effect at 10 mg/kg revealed a tendency in decreasing the immobility time of mice (Fig. 2); were evaluated by the effect of acute administration at different doses; compound **2** at 10, 30 and 60 mg/kg, compounds **3** and **4** at 1, 10 and 30 mg/kg and compound **5** at 0.3, 1, 10 and 30 mg/kg (Fig. 3A–D, respectively). Fig. 3A shows that compound **2** significantly reduced the immobility time of mice at 30 and 60 mg/kg compared to control mice, evidencing an antidepressant-like effect at higher doses. Compounds **3** and **4** significantly reduced the immobility time of mice at 10 and 30 mg/kg compared to control mice. From these derivatives, compound **5** resulted as the most active α-hydroxyamide, showing a decrease in the immobility time of mice even at a dose of 0.3 mg/kg (Fig. 3D). Also, all tested compounds demonstrated a clear dose dependent effect.

Moreover, the antidepressant–like effect of compounds **2**, **3**, **4** and **5**, at 30 mg/kg, was evaluated using another antidepressant test of immobility, the tail suspension test (Fig. 4). The results revealed that α-hydroxyamides **2**, **3** and **5** and imipramine, at 20 mg/kg, significantly reduced the immobility time of mice in this test. Compound **5** was also tested at 10 mg/kg in the tail suspension test, but no antidepressant effect was found at this dose (data not shown).

3.3. Locomotor activity test

In order to discard any effect of compounds **2–5** on locomotion, the locomotor activities of mice were also evaluated. The performance of α-hydroxyamides treated mice on the locomotor activity test was unaffected at doses up to 30 mg/kg. The results for compound **5** (at 1 mg/kg, 10 mg/kg and 30 mg/kg) are shown in Fig. 5B.

3.4. Anxiolytic-like effect of N-propyl-2,2-diphenyl-2-hydroxyacetamide (compound 5)

As anxiety is a comorbid pathology in people with epilepsy, the potential anxiolytic-like activity of compound **5** was also evaluated in the plus maze test. The anxiolytic-like effect of compound **5** was compared to vehicle control group and the effect of diazepam, a classical benzodiazepine used as a reference anxiolytic drug (Fig. 5A). Compound **5** significantly increases the percentage of open arms entries, at 3 and 10 mg/kg, and the percentage of time spent in open arms, at 10 mg/kg, when compared to vehicle treated mice. This result revealed an anxiolytic-like effect of compound **5** at these doses. Nevertheless, the administration of compound **5** at doses of 1 mg/kg and 30 mg/kg produce no effect in mice in this assay. Meanwhile, diazepam, at 1 mg/kg, increased the percentage of open arm entries, the percentage of time spent in open arms, the total arm entries (Fig. 5A) and the locomotor activity of mice (Fig. 5B).

3.5. Study of the mechanism of action of compound 5

Among all α-hydroxyamides tested, compound **5** emerged as the most active candidate, showing significant activity as anticonvulsant in the MES test (ED_{50} of 2.5 mg/kg), but not in the PTZ test, and even antidepressant (0.3–30 mg/kg) and anxiolytic-like (3–10 mg/kg) activities.

In order to explore possible molecular targets involve in this compound activities, binding assays for the benzodiazepine binding site in the $GABA_A$ and in the $5HT_{1A}$ receptors were carried out using [^{3}H]-flunitrazepam and [^{3}H]-8-OH-DPAT as radioligands, respectively. These assays showed that compound **5**, up to a concentration of 300 μM, could not displace [^{3}H]-flunitrazepam and [^{3}H]-8-OH-DPAT from synaptosomal rat brain membranes.

Considering that anticonvulsant drugs, which also have antidepressant-like effects, such as lamotrigine, prevent seizures induced by MES test, and that their mechanism of action is related to voltage-gated sodium channels inhibition, it is possible to postulate that sodium channels could be a target for compound **5**. The ability of compound **5** to inhibit sodium current in hNa_v 1.2 channels stably expressed in the HEK293 cell line was examined by patch-clamp technique in whole-cell

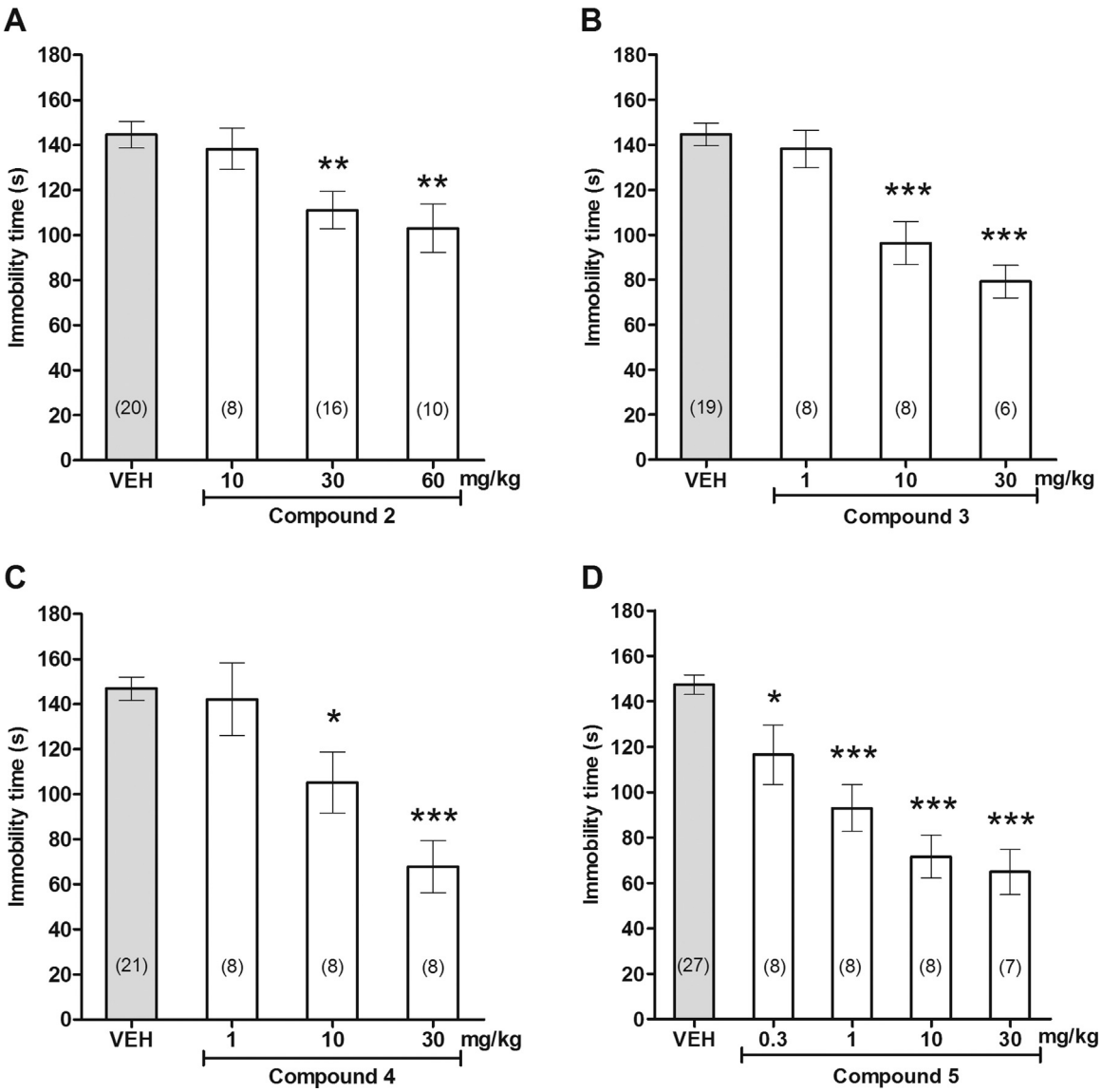

Fig. 3. Effects of acute administration of A) N-butyl-2-hydroxyisobutylamide (**2**) (10, 30, and 60 mg/kg), B) N-benzyl-2-hydroxyisobutylamide (**3**) (1, 10 and 30 mg/kg), C) N-phenethyl-2-hydroxyisobutylamide (**4**) (1, 10 and 30 mg/kg) and D) N-propyl-2,2-diphenyl-2-hydroxyacetamide (**5**) (0.3, 1, 10 and 30 mg/kg) in the forced swimming test. Results are expressed as mean ± S.E.M. of the immobility time (in s) in comparison to control animals (injected with vehicle, VEH). The number of mice used in each group is shown in the column. Statistical analysis was performed by one-way ANOVA followed by Dunnett's test. *P < 0.05, **P < 0.01, ***P < 0.001 compared with the control group.

configuration (Fig. 6). The current evoked by a 15 ms voltage-step from a holding potential of − 80 mV to − 20 mV was significantly inhibited by compound **5** at a concentration of 100 μM. The effect of compound **5** was washed-out as long as the seal remained stable (Fig. 6A). Moreover, as it could be observed in Fig. 6B and C, the compound **5** is able to produce a significant shift of h curve to more hyperpolarized membrane potentials, suggesting that it stabilizes the inactivated state decreasing the available fraction of sodium channels to open when membrane is more depolarized (voltage-dependent inhibition).

The in vivo antidepressant-like effect of lamotrigine is able to be blocked by the pre-treatment with veratrine (a voltage-gated sodium channel agonist), suggesting that voltage activated sodium channels are involved in its in vivo action (Bourin et al., 2009). The study conducted in order to assess the effects of veratrine on the antidepressant-like effect of compound **5** in the forced swimming test is shown in Fig. 7. It was already reported that veratrine tested alone in this assay, and up to a dose of 2 mg/kg, did not modify the immobility time of mice, and that veratrine 0.125 mg/kg had no effect on the locomotor activity of mice (Prica et al., 2008). As shown in Fig. 7, veratrine (0.125 mg/kg, i.p.) did not evoke any response by itself. Compound **5** given alone induced a significant antidepressant-like effect at 1 mg/kg and 10 mg/kg. The pre-treatment with veratrine (0.125 mg/kg) significantly reversed the antidepressant-like effect of compound **5** at both doses. Therefore, the antidepressant-like effect of compound **5** in the forced swimming test was able to be blocked by veratrine, suggesting that voltage activated sodium channels are involved in the in vivo action of compound **5**.

4. Discussion

In this work the novel synthetic compound N-propyl-2,2-diphenyl-2-hydroxyacetamide (compound **5**), which induced anticonvulsant, antidepressant and anxiolytic-like effects in mice, was presented. Moreover, we showed that this compound could inhibit voltage-gated sodium channels.

Our previous and present studies demonstrated that of a series of 6 anticonvulsant α-hydroxyamides (**1**–**6**), not all showed the same

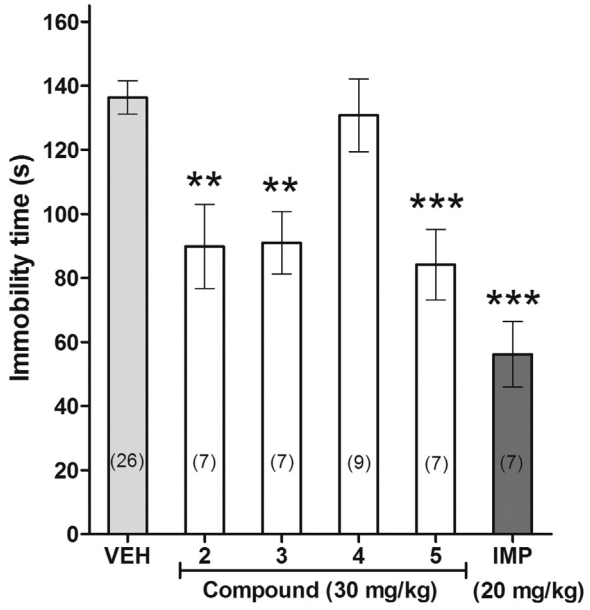

Fig. 4. Effects of acute administration of N-butyl-2-hydroxyisobutylamide (**2**), N-benzyl-2-hydroxyisobutylamide (**3**), N-phenethyl-2-hydroxyisobutylamide (**4**), N-propyl-2,2-diphenyl-2-hydroxyacetamide (**5**) and imipramine (IMP) (20 mg/kg) in the tail suspension test. Results are expressed as mean ± S.E.M. of the immobility time (in s) in comparison to control animal (injected with vehicle, VEH). The number of mice used in each group is shown in the column. Statistical analysis was performed by one-way ANOVA followed by Dunnett's test. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the control group.

anticonvulsant profile. While compounds **2**, **3** and **4** are effective in both MES and PTZ test, an anticonvulsant profile similar to valproic acid; α-hydroxyamides **1, 5** and **6** are only effective in the MES test as phenytoin and trimethadione, probably due to their distinctive mechanisms of action (Krall et al., 1978; Pastore et al., 2013; White et al., 2002).

Among the different tests used in animal models to evaluate antidepressant activity the forced swimming test remains one of the most used tool for assessing pharmacological antidepressant effect due to its relative reliability across laboratories and its ability to detect activity in a broad spectrum of clinically effective antidepressants. The tail suspension test is theoretically similar to the forced swimming test; it is inexpensive and methodologically unsophisticated (Cryan and Mombereau, 2004). Although both tests show common behavioral measure of feeling despair, the underlying pathophysiology seems to be different (Bai et al., 2001). The synthetic compounds **1–6** were evaluated in the forced swimming test in mice, being compounds **2–5** active as antidepressants in this test. α-Hydroxyamides **2**, **3**, **4** and **5**, at a dose of 30 mg/kg, were also evaluated in the tail suspension test. Except for compound **4,** all of them showed a decrease in the immobility time in this assay. It is not unusual for antidepressant compounds to have different responses in both tests due to their differential sensitivity to the immobility-reducing effects. Some agents such as rolipram and levoprotiline reduced immobility in the forced swimming test but have been reported inactive in the tail suspension test (Porsolt et al., 1992). Further, the pattern of the dose response curves may be substantially different between both procedures (Bai et al., 2001; Porsolt et al., 1978).

As psychomotor stimulants, and drugs enhancing motor activity, may give false effects in the forced swimming and tail suspension tests, by means of a decrease in the immobility time via stimulating locomotor activity, an additional measurement of the possible effects of these compounds on the locomotion of mice was carried out. This assay showed that these compounds did not produce disturbances in the locomotor activity up to a dose of 30 mg/kg, evidencing a lack of sedative effect. Even more, Rotarod test results indicated that they did not induce neurotoxicity at doses up to 100 mg/kg.

The elevated plus-maze stands as one of the most used in vivo animal test to screen anxiolytic effects of drugs. It is claimed to be an ethologically valid animal model of anxiety because it uses natural stimuli that can induce anxiety in human (Calatayud et al., 2004). Compound **5**, the α-hydroxyamide most active as antidepressant, was chosen for further studies to evaluate its capacity to act as anxiolytic in the plus maze test at a dose range from 1 mg/kg to 30 mg/kg. This assay showed that compound **5** increased the percentage of entries (at doses of 3 mg/kg and 10 mg/kg) and the time spent (at a dose of 10 mg/kg) in open arms of mice suggesting that this compound has an anxiolytic-like action at these doses, quite similar to those related to the reference benzodiazepine, diazepam; even though it showed no effect at doses of 1 mg/kg and 30 mg/kg in this assay. Anxiolytic drugs usually display hormetic-like biphasic dose responses, independent of the test and animal model employed (Calabrese, 2008).

The GABA system is the main inhibitory neurotransmitter system in the brain and is the target for many clinically used drugs to treat epilepsy and anxiety disorders. However, compound **5** (at 300 μM) was not able to displace the binding of [^{3}H]-flunitrazepam, a $GABA_A$ receptor-specific ligand, from synaptosomal rat brain membranes. This data suggest that the behavioral effects induced by this compound could not involve the benzodiazepine binding site of the $GABA_A$, at least not directly.

Moreover, many studies support evidence for a role of the serotonergic system in various behavioral responses related to depression and anxiety, as well as other psychiatric disorders (Krishnan and Nestler, 2008). The serotonergic hypothesis of depression suggests that depressive symptoms are related to a reduced 5-HT concentration in the brain synapse and an enhancement in the concentration of this neurotransmitter is able to induce antidepressant action. It has been reported, also, that 5-HT receptors were involved in the action mechanism of antidepressants in different behavioral studies (Chenu et al., 2008; Sugimoto et al., 2010). Nevertheless, α-hydroxyamide **5** (at 300 μM) did not show any direct effect on serotonergic $5\text{-}HT_{1A}$ receptor excluding a possible role of the serotonergic receptor in the behavioral activity of this α-hydroxyamide. However, the possibility that compound **5** could act on 5-HT uptake cannot be discarded.

The voltage-gated sodium channels initiate action potentials in neurons and other excitable cells are responsible for propagation of action potentials along nerves, muscle fibers and neuronal somatodendritic compartment. Voltage-gated sodium channels are the molecular targets for drugs used in the treatment of epilepsy, cardiac arrhythmias, bipolar disorder, migraine, and in the prevention of acute and chronic pain. Many antiepileptic drugs in use nowadays are specifically voltage-gated sodium channels blockers i.e phenytoin, topiramate, carbamazepine, lamotrigine and their mechanisms of action have been recently described in detail (Mantegazza et al., 2010; Qiao et al., 2014). Our results showed that the compound **5** (at 100 μM) induces a reversible inhibition of hNav 1.2 sodium channels expressed in HEK293 cell line, suggesting that this compound has a direct action on this ion channel. Moreover, the observed shift in h curve towards more hyperpolarizes voltages indicated that the compound **5** has a channel state dependent inhibition effect, stabilizing the inactivated state. This mechanism is the same that was previously described for AEDs, like lamotrigine, carbamazepine and phenytoin, which effectively block the sodium conductance and delay recovery from inactivation, which prevents synchronized high frequency firing. (Ragsdale and Avoli, 1998; Rogawski and Loscher, 2004).

Moreover, in vivo results demonstrated that this mechanism could be involved in the antidepressant effect of compound **5**, since veratrine, a sodium channel agonist, was able to revert the antidepressant effect of this α-hydroxyamide.

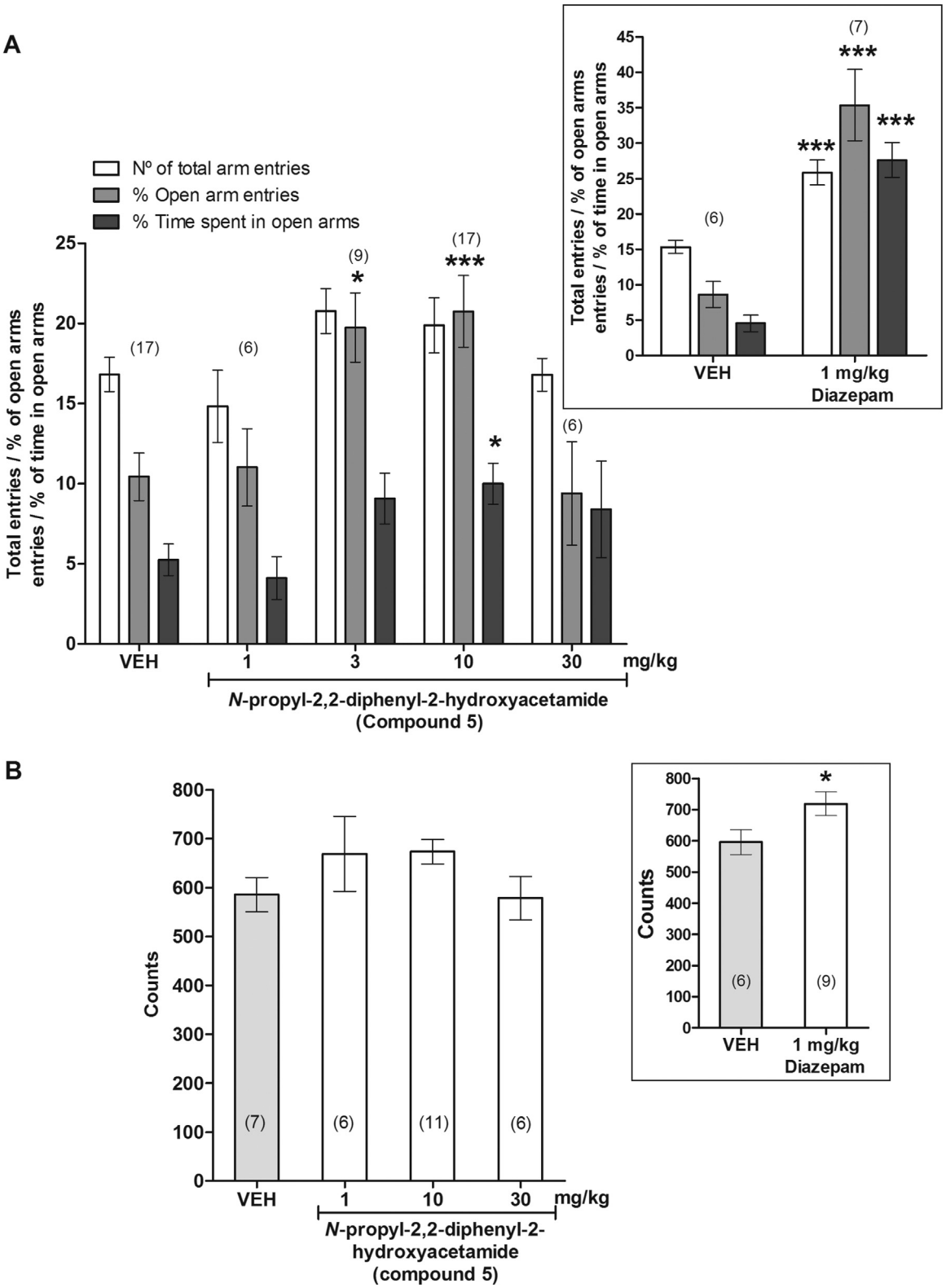

Fig. 5. Effect of the i.p. injection of N-propyl-2,2-diphenyl-2-hydroxyacetamide (compound **5**) and diazepam (inset) in the A) plus-maze and B) locomotor activity tests in mice. Results are expressed as mean ± S.E.M. of A) total arm entries, percentage of open arm entries and percentage of time spent in open arms and B) spontaneous locomotor activity counts; registered in 5 min sessions. The number of mice used in each group is shown in the column. The symbols denote significance levels: ***P < 0.001, **P < 0.01, *P < 0.05 significantly different from vehicle (VEH); Dunnett's multiple comparison test after one-way ANOVA.

Veratrine is an alkaloid able to increase sodium currents through voltage-sensitive sodium channels. It was demonstrated that veratrine induced an increase in the release of glutamate, the major CNS excitatory neurotransmitter, in cortical and cerebellum brain slices. Inhibition of glutamate release is considered as a potentially important mechanism for anticonvulsants (Bourin et al., 2009). Since it is known that veratrine antagonizes the antidepressant-like effects of lamotrigine, topiramate and phenytoin (Prica et al., 2008; Bourin et al., 2009) it was

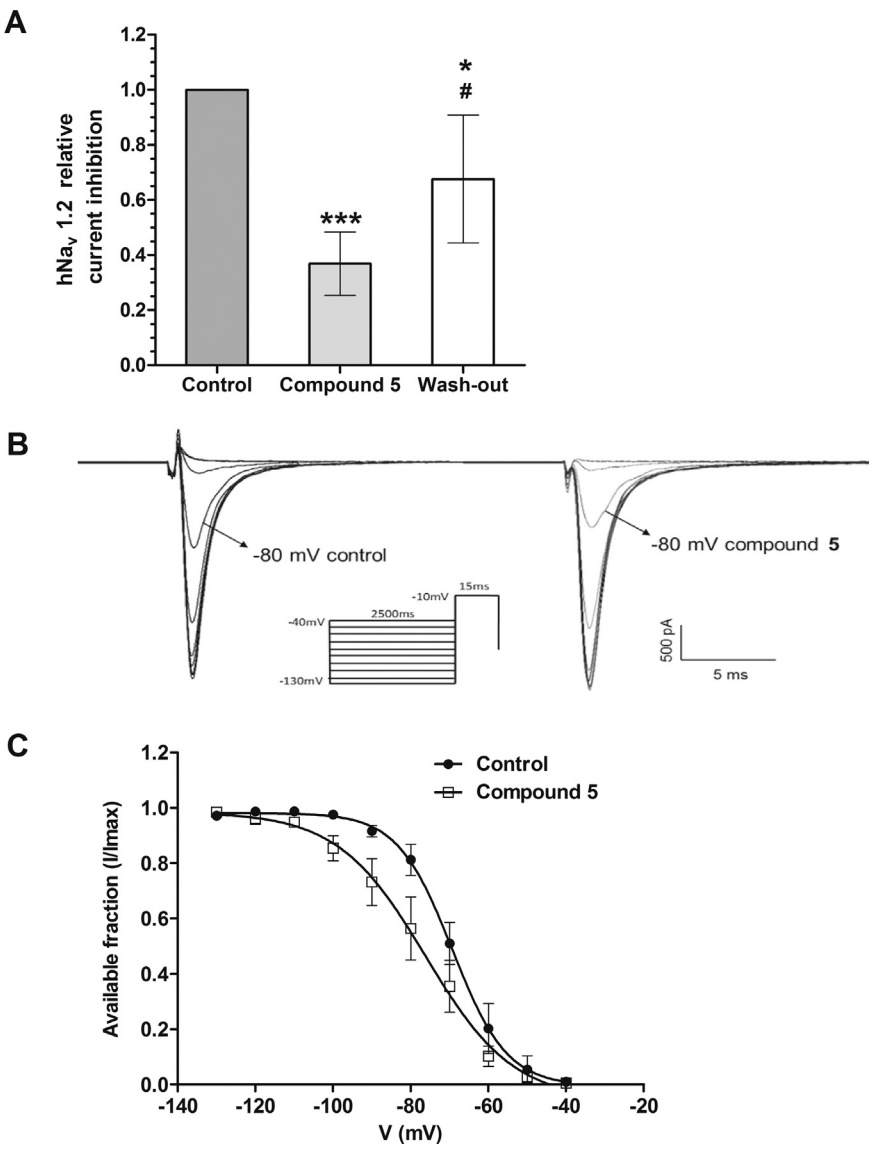

Fig. 6. (A) Inhibition of sodium current in cells treated with compound **5** (100 μM) relative to control. * Indicates a significant difference vs. control group, #indicates a significant difference between wash-out and compound **5**, ***P < 0.0001, inhibition = 36.9% ± 11.5%, *P < 0.05; #P < 0.05; Newman-Keuls after one way ANOVA, n = 8. (B) Sodium currents were activated by a depolarizing voltage step to − 10 mV for 15 ms following 2500 ms hyperpolarizing pre-pulses ranging from − 130 to − 40 mV (protocol given as inset). (C) The mean available fraction (I/Imax) values for compound 5 and control are plotted as function of membrane voltage and fitted to the Boltzmann function (Eq. (1)), $Vh_{control}$ = −(69.3 ± 1.3) mV, $Vh_{compound\ 5}$ = −(76.2 ± 2.6) mV.

supposed that their antidepressant-like effects were dependent on the glutamate release. On the other hand, it was also reported that the antidepressant-like effect of paroxetine (a selective serotonin reuptake inhibitor), desipramine and imipramine (tricyclic antidepressants) was not reversed by veratrine, and not dependent on glutamate release, suggesting that the mechanism of action involved in the antidepressant-like effect of anticonvulsants is different from that of classical antidepressants. Furthermore, the antidepressant-like effect of compound **5** seems to be dependent of the glutamate release (since the antidepressant like effect of this drug was reversed by veratrine) and not related to a serotonin reuptake inhibition.

Also, it was already demonstrated that veratrine induced an anxiogenic-like behavior of mice in the plus maze test, and that its effect could be reversed by riluzole (a sodium channel blocker) (Saitoh et al., 2015). So, voltage-gated sodium channels could be involved in the mechanism of action of drugs active in the plus maze test.

5. Conclusion

The results of the present study provided convincing evidence that i.p. administration of the anticonvulsant α-hydroxyamides **2** to **5**, at doses that do not interfere with the motor performance, exerted clear dose dependent antidepressant action when assessed in two models of depression in mice. From this series of α-hydroxyamides, N-propyl-2,2-diphenyl-2-hydroxyacetamide (compound **5**) emerged as the most promising anticonvulsant, antidepressant and anxiolytic-like derivative. Both in vitro (on sodium current hNa$_v$ 1.2) and in vivo (using veratrine) effects could be associated with a blockade of voltage-gated sodium channels. The capacity of compound **5** to stabilize the inactivated state of the channel was also demonstrated. The combined anticonvulsant, antidepressant and anxiolytic- like profile of compound **5** may represent a valuable tool for the treatment of these disorders. Further studies might be necessary to determine the effect of this compound in chronic models of epilepsy, as a kindling-test. Animals from these experiments could also be tested looking for antidepressant and anxiolytic-like effects. Studies of survivals, after chronic treatments with compound **5**, compared to reference drugs usually use in clinical nowadays, may be also a useful data. Moreover, it would be necessary to determine if these compounds could exhibit enhanced pharmacokinetic or pharmacodynamic properties with advantages as pharmacological therapeutic agents for the treatment of various neuropsychiatric disorders.

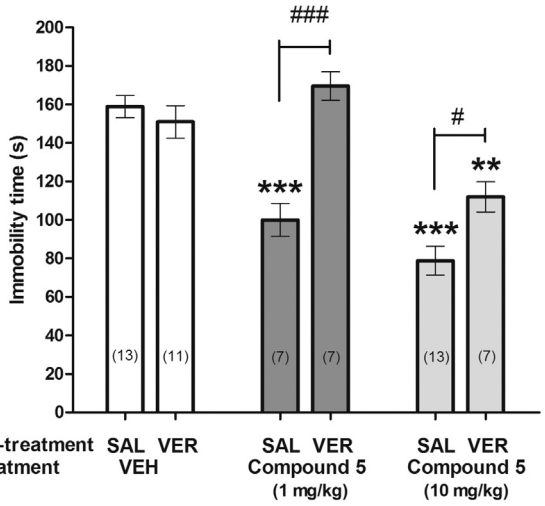

Fig. 7. Effects of N-propyl-2,2-diphenyl-2-hydroxyacetamide (compound **5**) (1 and 10 mg/kg, i.p. 30 min before the test) in the forced swimming test in mice pretreated with veratrine (VER, 0.125 mg/kg, i.p. 45 min before the test). Results are expressed as mean ± S.E.M. of the immobility time (in s) in comparison to control group. The number of mice used in each group is shown in the column. Statistical analysis was performed by two way ANOVA followed by Bonferroni test. Asterisks indicate a significant difference with the control group saline-vehicle (SAL–VEH). **P < 0.01, ***P < 0.001 compared with the control group; # indicates a significant difference between animals receiving saline-compound **5** (SAL-compound **5**) and veratrine-compound **5** (VER-compound **5**) #P < 0.05, ###P < 0.001.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ejphar.2017.11.048.

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