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Interaction with σ_1 Protein, but not NMDA Receptor, is Involved in the Pharmacological Activity of Donepezil

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Running Title: Donepezil effects on NMDA and σ_1 receptors

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Abbreviations: DHEAS, dehydroepiandrosterone sulfate; $GABA_A$, γ-aminobutyric acid type A receptor; NMDA, N-methyl-D-aspartate; ODN, oligodeoxynucleotide.

Abstract

In the present study, we examined the interaction of donepezil, a potent cholinesterase inhibitor, with two additional therapeutically-relevant targets, N-methyl-D-aspartate (NMDA) and σ_1 receptors. Donepezil blocked the responses of recombinant NMDA receptors expressed in Xenopus oocytes. The blockade was voltage-dependent, suggesting a channel blocker mechanism of action, and was not competitive at either the L-glutamate or glycine binding sites. The low potency of donepezil (IC_{50}s: 0.7 – 3 mM) suggests that NMDA receptor blockade does not contribute to donepezil's therapeutic actions. Of potential therapeutic relevance, donepezil binds to the σ_1 receptor with high affinity (Ki =14.6 nM) in an *in vitro* preparation (Kato et al., 1999). We thus sought to determine whether an interaction with the σ_1 receptor may occur *in vivo* under physiologically relevant conditions by evaluating the σ_1 receptor-dependency of donepezil's effects in behavioral tasks. Donepezil showed antidepressant-like activity in the mouse forced-swimming test as did the σ_1 receptor agonist igmesine. This effect was not displayed by the other cholinesterase inhibitors, rivastigmine and tacrine. The donepezil and igmesine effects were blocked by preadministration of the σ_1 receptor antagonist BD1047 and an *in vivo* antisense probe treatment. The memory enhancing effect of donepezil was also investigated. All cholinesterase inhibitors attenuated dizocilpine-induced learning impairments. However, only the donepezil and igmesine effects were blocked by BD1047 or the antisense treatment. Therefore, donepezil behaved, as an effective σ_1 receptor agonist on these behavioral responses and an interaction of the drug with the σ_1 receptor must be considered in its pharmacological actions.

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Introduction

Cholinesterase inhibitors inhibit the hydrolysis of acetylcholine and elevate its concentration in the synaptic cleft, provoking an increase in the efficacy of cholinergic neurotransmission. (±)-2-[(1-Benzylpiperidin-4-yl)methyl]-5,6-dimethoxy-indan-1-one (donepezil) is a potent cholinesterase inhibitor used for treatment of Alzheimer's disease. Donepezil raises brain acetylcholine concentration as revealed by *in vivo* microdialysis studies in rats (Kosasa et al., 1999). Randomized, double-blind, placebo-controlled clinical studies showed that donepezil produces a significant improvement of cognition and global function in patients with mild-to-moderately severe Alzheimer's disease (Rogers & Friedhoff, 1996; Rogers et al., 1998, 2000; Mohs et al., 2001; Winblad et al., 2001).

The compound, however, may have other pharmacologic properties other than cholinesterase inhibition, and NMDA receptors and σ_1 receptors are two potential sites of interaction for donepezil which may contribute to its therapeutic effects. NMDA receptors are a class of receptors for the neurotransmitter glutamate and their blockade has been shown to be neuroprotective in a variety of neuropathological conditions such as in animal models of stroke (Choi, 1990). Also, as evidenced by memantine, NMDA receptor blockade can have therapeutic benefits in Alzheimer's disease (Fleischhacker et al., 1986; Rossum et al., 2004). Interestingly, the structure of donepezil bears some general similarities to both the NMDA receptor antagonist ifenprodil and to some of the NMDA receptor channel blocker agents. Donepezil inhibits the binding of [^{3}H]-MK801 to NMDA receptor channels with low affinity (IC_{50} = 135 ± 15 μM; Wang, 1999). However, interactions at the channels of individual NMDA receptor subtypes, or at other sites on the NMDA receptor complex,, have not yet been evaluated. Furthermore, the functional significance of donepezil interactions at specific NMDA receptor subtypes has also not yet been defined. Thus, NMDA receptor blockade represents a potential mechanism for the neuroprotective action of donepezil, evidenced in *in vitro* cell toxicity models (Takada et al., 2003).

The σ_1 receptor is a 25 kDa intracellular protein, bearing a transmembrane domain and an endoplasmic reticulum retention signal. Its activation induces both a rapid modulation of

ion channels and neurotransmitter responses, including inositol trisphosphate gated calcium stores, NMDA receptors and K^+ channels (Maurice et al., 1999, 2001a; Hayashi & Su, 2005). After activation, the protein also translocates from the vicinity of the endoplasmic reticulum towards plasma and organelles membranes, where it participates in the reconstitution of lipid microdomains (lipid rafts) and remodeling of plasma membrane composition (Hayashi & Su, 2005). Modulation of the σ_1 receptor leads therefore to a variety of neuromodulatory effects that may differ after acute or chronic activation. While donepezil has previously been shown to have a high affinity for σ_1 receptors in radioligand binding experiments, with a Ki =14.6 nM (Kato et al., 1999), it is not clear whether donepezil can access this intracellular binding site *in vivo* under physiological conditions.

In the present study, we examined the putative activity of donepezil at both NMDA and σ_1 receptors. First, we used two-electrode voltage clamp to examine the effect of donepezil on recombinant NMDA receptors expressed in *Xenopus* oocytes. Such an approach will allow for testing activity at any of a growing number of sites on the NMDA receptor complex, in a physiologically-relevant manner. Second, since it is already known that donepezil binds to σ_1 receptor, we examined whether such an interaction occurs *in vivo*. The effect of donepezil was evaluated in σ_1 receptor-mediated behavioral responses (Maurice et al., 1999, 2001a). The anti-depressant-like effect of donepezil was investigated using the forced swim test and compared with the effects of the reference σ_1 receptor agonist, igmesine, and other cholinesterase inhibitors, tacrine and rivastigmine. The involvement of the σ_1 receptor in the donepezil response was checked in animals pretreated with the σ_1 receptor antagonist BD1047 or with an antisense oligodeoxynucleotide targeting the protein (Maurice et al., 2001b). In addition, the anti-amnesic effect of donepezil was examined in mice treated with the non-competitive NMDA receptor antagonist dizocilpine, a learning impairment model known to be alleviated by selective σ_1 receptor agonists. Two memory tests were used, the spontaneous alternation in the Y-maze, assessing spatial working memory, and the step-through passive avoidance response, assessing contextual long-term memory (Maurice et al., 1994).

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Methods

In Vitro transcription and translation of NMDA receptors in Xenopus oocytes

Plasmids were linearized with *Not*I (NR1a) or *Sal*I(NR2B) and transcribed with T7 (NR1a) or SP6 (NR2B) *in vitro* using the mMessage mMachine kit (Ambion, Austin, TX). Xenopus laevis female frogs were obtained from Xenopus I (Dexter, MI) and oocytes were isolated and prepared as previously described (Monaghan and Larsen, 1997). The handling of frogs was performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. Oocytes were injected with 2-30 ng NR1/NR2 RNA (1:3) in 9 - 50 nL and then incubated in ND-96 (96 mM NaCl, 2 mM KCl, 1.8mM $CaCl_2$, 1mM $MgCl_2$, 5mM HEPES, pH 7.6) at 17°C for 1-4 days.

Oocyte electrophysiology

Electrophysiological responses of recombinant receptors expressed in Xenopus oocytes were measured by two-electrode voltage clamp (OC-725B Oocyte Clamp, Warner Instruments, Hamden, CT) at a holding potential of -60mV. Unless indicated otherwise, NMDA receptor responses were evoked by bath application of 10 μM (S)-glutamate/10 μM glycine. Recordings were made in barium Ringer's solution (116 mM NaCl, 2 mM KCl, 2 mM $BaCl_2$ and 5 mM HEPES, pH 7.4) to eliminate calcium-activated chloride currents. Only cells which generated stable plateau responses were used (usually in the 50 to 250 nA range). Antagonist responses were determined after 3 or 4 agonist-alone pulses established a stable baseline and agonist alone was applied after antagonist to confirm that there was not significant response run-down or run-up. Current responses to drug application were recorded on both a strip-chart and by digital capture using an ITC-16 computer interface (Instrutech, Great Neck, NY) and a MacIntosh computer with AxoData software (Axon Instruments, Foster City, CA). Dose-response curves for antagonist blockade of responses were fit (GraphPad Prism, ISI Software, San Diego, CA) to the equation: $I = Imax/[1+(IC_{50}/A)]$, where I is the current response, Imax is the current response in the absence of antagonist, and A is the concentration of antagonist.

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Animals

Male Swiss mice (breeding center of the Faculty of Pharmacy, Montpellier, France), aged 5 weeks and weighing 35 ± 2 g were used in this study. Animals were housed in plastic cages in groups. They had free access to food and water, except during behavioral experiments, and they were kept in a regulated environment (23 ± 1°C, 40-60% humidity) under a 12-hr light/dark cycle (light on at 8:00 a.m.). Experiments were carried out between 9:00 a.m. and 5:00 p.m., in a soundproof and air-regulated experimental room, and mice were habituated 30 min before each experiment. All animal procedures were conducted in strict adherence of European Community Council Directives of 24 November 1986 (86-609).

Drugs

(±)-2,3-dihydro-5,6-dimethoxy-2-[[1-(phenylmethyl)-4-piperidinyl]methyl]-1H-inden-1-one hydrochloride (E2020, donepezil) was provided by Eisai Co Ltd. (Tokyo, Japan). (5S, 10R)-(+)-5-Methyl-10, 11-dihydro-5H-dibenzo[a,d]cyclohepten-5, 10-imine maleate (MK-801, dizocilpine), 1,2,3,4 - tetrahydro-9-aminoacridine chlorhydrate (tacrine) were purchased from Sigma-Aldrich (St-Quentin Fallavier, France). (S)-N-Ethyl-N-methyl-3-[1-(dimethylamino)ethyl]-phenyl carbamate hydrogen-(2R,3R)-tartrate (Rivastigmine, Exelon®) was purchased from Novartis (Basel, Switzerland). (+)-N-cyclopropylmethyl-N-methyl-1,4-diphenyl-1-ethyl-but-3-en-1-ylamine hydrochloride (igmesine, JO-1784) was a gift from Dr François J. Roman (Pfizer GRD, Fresnes, France), and *N*-[2-(3,4-dichlorophenyl)ethyl]-N-methyl-2-(dimethylamino) ethylamine (BD1047) a gift from Dr Wayne D. Bowen (NIDDK/NIH, Besthesda, MD). All drugs were solubilized in physiological saline solution or distilled water and administered intraperitoneally (ip) in a volume of 100 µl/20 g body weight.

Design and administration of oligodeoxynucleotides

Based on the mouse cDNA sequences for the σ_1 receptor, 16-mer phosphorothioate-modified oligodeoxynucleotide (ODN) sequences were designed, as previously described (Maurice et al., 2001b). They were targeted to the area from +15 to +1 around the initiation

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codon, 5'-CGCGGCCCACGGCATT-3' (=antisense oligodeoxynucleotide, aODN). This sequence was selected because no homology was found with any of the other known cDNA sequences in the GeneBank database (Bainbridge Island, WA). As a control, a mismatched analogue, including randomly designed defects, 5'-CACGTCCCTCTCCATT-3', was designed (=mismatch oligodeoxynucleotide, mODN). The ODN were synthesized and purified by high pressure liquid chromatography by Eurobio Laboratoires (Les Ulis, France). They were dissolved in sterile saline solution and stored at -20°C until used.

Under sodium pentobarbital anesthesia, mice were implanted with a polyethylene cannula (0.75 mm inner diameter, 6 mm length), fixed using acrylic cement. The tip of the cannula was placed onto the right ventricle, with stereotaxic coordinates from the Bregma being, in mm, A -0.5, L -1, V 2.5. Injections began 24 h after surgery. Under light ether anesthesia, the needle of a Hamilton microsyringe was inserted through the cannula and ODN (1 µl) were slowly injected over 1 min, followed by an additional 1 min wait before removing the needle. Animals received 2 intracerebroventricular (icv) injections per day, at 12 h time interval, during 3 days. These animals were used for experiments, *in vivo* binding assays or behavioral observations, 10 hr after the last injection, i.e., 4 days after cannulation.

Forced swim test

Each mouse was placed in a glass cylinder (diameter 12 cm, height 24 cm) filled with water at a height of 12 cm. Water temperature was maintained at 22-23°C. The animal was forced to swim for 15 min on the first day. The animal behavior was not recorded during this first session. On the second day, each mouse was placed again into the water and forced to swim for 6 min. This session was videotaped and the duration of immobility during the last 5 min was measured. The mouse was considered as immobile when it stopped struggling and moved only to remain floating in the water. Drugs were administered 30 min before the session on the second day.

Spontaneous alternation performances

The maze consisted in a Y-maze (three arms, 50 cm long, 60° separate), placed on the floor of the experimental room and indirectly lit with a 60W bulb lamp placed 150 cm above. The trial consisted of a single 8-min session. Each mouse was placed at the end of one arm and allowed to move freely through the maze. The series of arm entries, including possible returns into the same arm, was recorded using an Apple IIe computer. An alternation was defined as entries into all three arms on consecutive trials. The number of maximum alternations was therefore the total number of arm entries minus two and the percentage of alternation was calculated as (actual alternations / maximum alternations) x 100. The compounds were administered 30 min before the session or 10 min before dizocilpine, given 20 min before the session.

Step-through passive avoidance test

The apparatus consisted of an illuminated compartment with white PVC walls (15 x 20 x 15 gridfloor and a black compartment with a grid floor, separate each by a guillotine door. A 60 W lamp was positioned 40 cm above the floor of the white compartment during the experimental period. Scrambled footshocks (0.3 mA) could be delivered to the gridfloor using a shock generator scrambler (Lafayette Instruments, Lafayette, MA). The guillotine door was initially closed. Each mouse was placed into the white compartment and after 5 s, the door was raised. When the mouse entered the darkened compartment, the door was gently closed and the scrambled footshock delivered for 3 s. The step-through latency, *i.e.*, the time spent to enter the dark compartment, and the number of vocalizations were recorded. The retention test was carried out 24 h after training. Each mouse was placed again into the white compartment and after 5 s, the door was raised. The step-through latency was recorded up to 300 s. The compounds were administered 30 min before the training session or 10 min before dizocilpine, given 20 min before the training session. Injections were not repeated before the retention session (pre-acquisition protocol).

Statistical analyses

Behavioral data, alternation percentages expressed as means ± S.E.M. or latencies expressed as medians and interquartile range, were analyzed using the Dunn's multiple comparisons test after a Kruskal-Wallis non-parametric analysis of variance (KW values). The level of statistical significance was $p < 0.05$.

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Results

Donepezil low affinity blockade of NMDA receptor activity

NMDA receptor NR1a subunits were co-expressed in Xenopus oocytes with each of the NR2 subunits. Using two-electrode voltage clamp, cells were held at –60 mV and NMDA receptors were activated by bath application of 10 μM L-glutamate and 10 μM glycine. Agonist-evoked currents were then tested for inhibition by donepezil. As shown in Figure 1, high micromolar concentrations of donepezil inhibit NMDA receptor responses. Dose-response analysis for each of the NR1a-NR2 subunit combinations showed that donepezil is weakly selective among the different NR2-containing NMDA receptors (Fig. 2A). Donepezil IC_{50} values ([95% confidence interval]) were: 0.68 [0.54–0.85] mM for the NR1a/NR2A combination; 1.2 [0.95–1.59] mM for NR1a/NR2B; 3.16 [2.9–3.4] mM for NR1a/NR2C; 1.6 [1.3–2.0] mM for NR1a/NR2D (Fig. 2A).

Ifenprodil and related compounds generally display a significant selectivity for NMDA receptors containing the NR2B subunit. The lack of donepezil selectivity for NR2B subunits, suggests that donepezil is not acting at the ifenprodil binding site. To ensure that our system is ifenprodil-sensitive, we compared ifenprodil and donepezil potency at NR1a/NR2B (Fig. 2B). As expected, ifenprodil displayed a high affinity, 0.36 [0.3–4.3] μM. Ifenprodil was more than 3000-fold more potent than donepezil at NR2B-containing NMDA receptors. Furthermore, in radioligand binding autoradiographic experiments, donepezil was ineffective at displacing [^{3}H]-ifenprodil binding to NMDA receptors (data not shown).

We next sought to determine if donepezil is a competitive antagonist with either L-glutamate or glycine at the respective L-glutamate or glycine binding sites on the NMDA receptor complex. Donepezil potency was determined for inhibition against moderate agonist concentrations (10 μM L-glutamate plus 10 μM glycine) and against high agonist concentrations (300 μM L-glutamate plus 300 μM glycine). If donepezil blocks NMDA receptor responses by binding at either the L-glutamate or glycine binding sites, then we would expect a significant reduction in donepezil potency when tested against high agonist

concentrations. Donepezil potency was not reduced by high L-glutamate and glycine concentrations (Fig. 2C).

NMDA receptor channel blockers commonly display a voltage dependency to their channel blockade such that greater blockade is found at more negative membrane potentials. To evaluate the voltage-dependency of donepezil inhibition of NMDA receptor responses, 1mM donepezil was tested at a range of membrane holding potentials. As shown in Figure 2D, donepezil inhibition is enhanced as the membrane potential becomes more negative.

Involvement of the σ_1 receptor in the antidepressant-like effect of donepezil

Swiss mice submitted to forced swimming rapidly developed a marked immobility response, in the 220-240 s range during the 5 last min of the 6-min duration session (Fig. 3). Donepezil (3-30 mg/kg) significantly decreased the immobility duration(Fig. 3A). This effect was fully blocked in a dose-dependent manner by simultaneous administration of BD1047, which by itself had no effect at the highest dose tested (10 mg/kg) (Fig. 3A). Rivastigmine and tacrine, tested in the 1-30 mg/kg range, failed to affect the immobility duration in the same experimental conditions (Fig. 3B). Igmesine, the reference σ_1 receptor agonist, dose-dependently decreased the immobility duration, and its effect at the highest dose tested was antagonized by BD1047 (Fig. 3C). Moreover, in animals treated repeatedly with the σ_1 antisense ODN, donepezil, 30 mg/kg, failed to decrease the immobility duration, contrarily to mice treated with the mismatch ODN (Fig. 3D).

Anti-amnesic effect of donepezil in dizocilpine-treated mice

The anti-amnesic effects of donepezil in dizocilpine-treated mice were then examined using spontaneous alternation and passive avoidance responses. Donepezil, in the 0.12-1 mg/kg dose range, failed to affect the alternation performances (Fig. 4A) and step-through latency (Fig. 4B). However, when administrered 10 min before dizocilpine, it dose-dependently and significantly reversed the learning deficits in both tests, full effect being observed at the highest doses tested, 0.5-1 mg/kg (Figs. 4A, B). The effect of a co-administration of BD1047 was examined. The σ_1 receptor antagonist blocked the anti-

amnesic effect of donepezil, while having minimal effect in vehicle- and dizocilpine-treated animals (Fig. 4C, D). For comparison, igmesine was also tested (Fig. 5). The σ_1 receptor agonist, in the 0.1-3 mg/kg dose range, failed to affect the behavioral parameters in control animals, but reversed, in a significant but bell-shaped manner, the dizocilpine-induced deficits. Significant effect was observed at 0.3-1 mg/kg in spontaneous alternation (Fig. 5A) and at 1 mg/kg in the passive avoidance response (Fig. 5B). BD1047 dose-dependently blocked the anti-amnesic effects of igmesine, in a highly significant manner in the Y-maze test (Fig. 5C) and non-significantly in the passive avoidance test (Fig. 5D).

The effects of the ODN treatments on the anti-amnesic effect of donepezil , 0.5 mg/kg, were then examined. Dizocilpine treatment led to significant diminution in the spontaneous alternation behaviour in animals treated centrally with mismatch ODN (Fig. 6A). Donepezil did not affect the alternation behaviour by itself, but allowed a significant attenuation of the dizocilpine-induced deficits in mismatch ODN-treated animals (Fig. 6A). In antisense ODN-treated animals, dizocilpine induced deficits of similar extent, but donepezil failed to attenuate the dizocilpine-induced deficit (Fig 6A). Long-term memory capacity was assessed using passive avoidance response. In mismatch ODN-treated animals, donepezil did not affect the latencies by itself, but attenuated the dizocilpine-induced deficits (Fig. 6B). In antisense ODN-treated animals, the dizocilpine-induced decrease in latency was unchanged but remained unaffected by donepezil. It must be noted that neither the cannulation, nor the ODN administration affected the ability of animals to perform the test. This was checked during the training session by measuring the step-through latency or shock sensitivity (data not shown).

Anti-amnesic effects of cholinesterase inhibitors

Rivastigmine and tacrine were tested at the doses of 0.3 and 1 mg/kg in control and dizocilpine-treated animals (Fig. 7). Rivastigmine failed to affect the behavior of control animals and dose-dependently reversed the dizocilpine-induced learning impairments (Fig. 7A for spontaneous alternation and Fig. 7B for passive avoidance). BD1047 failed to affect the anti-amnesic effect of rivastigmine, 1 mg/kg. Similarly, we observed that tacrine failed to

affect the behavior of control mice, but blocked the dizocilpine-learning impairments, at 1 mg/kg (Fig. 7C, D). This effect was also not affected by pre-treatment with BD1047.

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Discussion

The first result of the present study indicates that donepezil is a low-affinity NMDA receptor antagonist. Donepezil blockade of NMDA receptor activity is probably not due to binding at the L-glutamate, glycine, or ifenprodil binding sites on the NMDA receptor complex. Donepezil blockade was not competitively reduced by a 30-fold increase in both glutamate and glycine concentration. Donepezil also did not display ifenprodil-like selectivity for the NR2B-containing NMDA receptors (Jane et al., 2000) and did not displace [^{3}H]-ifenprodil binding to NMDA receptors.

Donepezil displays some general structural similarities to NMDA receptor channel blocker drugs, such as phencycline or CNS-1102. It has a positive charge center surrounded by hydrophobic ring groups, a piperidine and an aromatic ring. Consistent with the actions of a channel blocker, donepezil blockade was voltage-dependent. Thus, the higher negative membrane potentials favor the entering and binding of donepezil in the channel.

Recently, memantine, an NMDA receptor channel blocker drug was approved for use in severe Alzheimer's disease patients. Thus, NMDA receptor channel blockers can have therapeutic benefits in Alzheimer's disease and also present the possibility of being neuroprotective. It is unlikely, however, that a significant component of donepezil's clinical actions are due to NMDA receptor blockade. Therapeutic levels of donepezil are in the low micromolar range while NMDA receptor blocker activity requires millimolar concentrations. Donepezil, however, may have therapeutically-relevant actions on NMDA receptors via indirect mechanisms. Very recently, it has been reported that low concentrations of donepezil (10 – 1000 nM) has a stimulatory action on NMDA receptor function in neurons (Moriguchi et al., 2005) by an indirect, pertussis toxin-sensitive effect. The absence of direct effects of donepezil on NMDA receptor function in the present study is consistent with such an indirect action of donepezil.

The second key point of this study is the observation that the antidepressant-like and anti-amnesic effects of donepezil involve an interaction with the σ_1 receptor. The anti-depressant-like activity was measured using the forced swim test. Donepezil is able to shorten

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the immobility duration of animals, dose-dependently but at relatively high doses. This effect was shared by the reference σ_1 agonist igmesine, as previously described (Matsuno et al., 1996; Urani et al., 2001), but not by other cholinesterase inhibitors such as tacrine or rivastigmine. The donepezil effect could be blocked by a selective σ_1 receptor antagonist, BD1047, and by a subchronic pretreatment with an antisense ODN probe, which has been shown to down-regulate σ_1 receptor expression *in vivo* (Maurice et al., 2001b). These observations clearly established that donepezil interacts with the σ_1 receptor to exert its anti-immobility response during forced swimming. Noteworthy, the dose-range for antidepressant-like activity is markedly higher than the doses effective in learning and memory tests. This was previously observed for numerous selective σ_1 receptor agonists (Matsuno et al., 1996, Urani et al., 2001). The requirement of high concentrations of σ_1 receptor agonists in order to elicit antidepressant actions appears to be due to the competing effects of stress-induced release of progesterone, a σ_1 receptor antagonist (Urani et al., 2001).Thus, this behavioral effect of donepezil may not be therapeutically relevant since the dose range is close to donepezil LD_{50} in mice, about 45 mg/kg.

Donepezil was also tested at a second behavioral response known to be mediated by selective σ_1 receptor agonists, the blockade of learning impairment caused by acute administration of dizocilpine, a highly potenty NMDA receptor antagonist (Maurice et al., 1994, 2001b; Ohno and Watanabe, 1995; Zou et al., 1998). Two behavioral tests were used in parallel: a short-term memory procedure involving a spatial working memory component, the spontaneous alternation in the Y-maze, and a long-term memory test involving negatively reinforced contextual memory processes, the step-through type passive avoidance procedure. Results obtained with both tests were similar. Donepezil, administered in the 0.12-1 mg/kg dose range, failed to affect the learning and memory processes when administered alone, but significantly blocked the dizocilpine-induced deficits at the highest doses tested, 0.5 and 1 mg/kg. The donepezil effects were blocked by pre-treament with BD1047 or repeated injections of the antisense ODN probe. These observations demonstrated that the anti-amnesic activity of donepezil involves an interaction with the σ_1 receptor. Igmesine showed a very similar, BD1047-sensitive, profile of efficacy. Moreover, the reversal of the dizocilpine-

induced deficits by rivastimgine and tacrine were not blocked by BD1047. The highest donepezil dose that was tested (1 mg/kg) is a dose that should lead to less than 1 μM in the brain (Geerts et al., 2005), a dose that is well below that necessary to have direct NMDA receptor actions.

The anti-amnesic effects of cholinesterase inhibitors against dizocilpine-induced learning deficits in mice have been previously described (Walker and Gold, 1992; Csernansky et al., 2005). In the latter recent study, physostigmine and donepezil, but not galanthamine, ameliorated the dizocilpine-induced deficits in spatial reversal learning and in contextual and cued memory. Consistent with the widespread distribution of cholinergic neurons and the variety of cholinergic receptor subtypes in multiple brain regions related to learning (e.g. Levin et al., 2003; Tisch et al., 2004), cholinergic drugs exert a complex effect on learning and memory processes. Donepezil, rivastigmine, tacrine, physostigmine and galanthamine act both as cholinesterase inhibitors, thus provoking non-selective activations at all types of nicotinic and muscarinic receptors, and directly as allosteric modulators of nicotinic receptors with varied efficacy. Galanthamine, particularly, is a weak cholinesterase inhibitor (Thomsen et al., 1991). These drugs therefore show marked pharmacological differences in terms of cholinergic activity. Considering that donepezil and physostigmine, but not galathamine, attenuated the dizocilpine–induced deficits in their study, Csernansky et al. (2005) proposed that such anti-amnesic effect may be the result of acetylcholinesterase inhibition and increased synaptic levels of acetylcholine, rather than allosteric nicotinic receptor modulation. We observed that tacrine and rivastigmine were also effective against the dizocilpine-induced learning impairments, in agreement with Csernansky's hypothesis. Moreover, the observation that direct application of nicotine alleviated the dizocilpine-induced learning deficits (Ciamei et al. 2001) is in line with such hypothesis.

The σ_1 receptor is also a target for the pharmacological action of donepezil at therapeutically relevant doses. Interestingly, this σ_1 receptor-mediated effect of donepezil may in turn affect the NMDA receptor, since the modulation exerted by σ_1 receptors on NMDA receptor activation is now well documented. σ_1 Receptor agonists are able to modulate different NMDA-mediated responses, such as enhancing the NMDA-induced

excitatory response of CA_3 pyramidal neurons in the rat hippocampal formation *in vivo* (Monnet et al., 1990; Debonnel and de Montigny, 1996). Thus, donepezil through its interaction with the σ_1 receptor may exert a facilitation of NMDA responses, contrary to our initial hypothesis. Furthermore, the interaction with the σ_1 receptor may also act to indirectly affect cholinergic systems. Indeed, activation of the σ_1 receptor has been shown to exert a direct modulation of cholinergic systems. Selective σ_1 receptor agonists modulate acetylcholine release in striatal and hippocampal slicepreparations *in vitro* (Leventer and Johnson, 1984; Junien et al., 1991) and increase extracellular acetylcholine levels in the rat frontal cortex and hippocampus, *in vivo* (Kobayashi et al., 1996a,b). Interestingly, striatal acetylcholine content is not affected, suggesting that selective σ_1 agonists may present lower cholinomimetic side-effects, than that observed for cholinesterase inhibitors (Matsuno et al.,1997).

Finally, the observation that the anti-amnesic effect of donepezil is fully blocked by antagonism of the σ_1 receptor activation is consistent with the intracellular neuromodulatory role of the protein. The σ_1 receptor is found both pre- and post-synaptically in the hippocampus. Its activation modulates Ca^{2+} mobilizations and, therefore, facilitates acetylcholine release and cholinergic signaling pathways (Junien et al., 1991; Kobayashi et al., 1996a,b). The σ_1 receptor is, however, a pure neuromodulatory receptor and its activation is not a pre-requisite for effective learning ability, as shown in antisense studies (Maurice et al., 2001b). Therefore, selective σ_1 receptor agonists and cholinomimetic drugs share different modes of action. Drugs acting non-selectively through neurotransmitter receptors and σ_1 receptor activation present a complex pharmacological mechanism of action, with their σ_1 component sustaining and constraining the primary effect on neurostransmitter systems. It was previously observed that the anti-amnesic effect induced by the neurosteroid dehydroepiandrosterone sulfate (DHEAS; a negative modulator of $GABA_A$ receptors, a positive modulator of NMDA receptors and a σ_1 receptor agonist) is blocked in mice treated with a σ_1 receptor antisense probe (Maurice et al., 2001b). In a coherent manner, donepezil effect involves both indirect cholinergic activity and σ_1 receptor activation. Its effect is

blocked by σ_1 receptor antagonism, in contrast to the more selective cholinesterase inhibitors that are devoid of σ_1 activity.

In conclusion, donepezil is a weak NMDA receptor blocker, with this direct activity unlikely to contribute to its therapeutic actions. However, an *in vivo* interaction of the drug with the σ_1 receptor has now been demonstrated and must be considered in its pharmacological actions. The symptomatic and potential neuroprotective effects of donepezil in Alzheimer's disease (Francis et al., 2005) may therefore result from a both direct and indirect cholinergic mechanisms and an interaction with the σ_1 receptor, known to provide neuroprotection against glutamate and amyloid toxicities.

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Footnotes

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Legends for Figures

Figure 1. A representative recording of donepezil blockade of NMDA receptor-mediated responses in Xenopus oocytes. NR1/NR2A RNA-injected oocytes were voltage-clamped at –60 mV and inward currents were evoked by bath application of 10 μM L-glutamate plus 10 μM glycine (indicated by the bars on top). Increasing concentrations of donepezil caused correspondingly greater levels of current inhibition.

Figure 2. (A) Donepezil inhibits each of the NR2-containing NMDA receptors. NR1/NR2A (2A) NR1/NR2B (2B), NR1/NR2C (2C), and NR1/NR2D (2D) receptors were activated by 10 μM L-glutamate plus 10 μM glycine and tested for inhibition by increasing concentrations of donepezil. 100% Control response represents the current response to agonist alone. (B) Comparison of ifenprodil and donepezil potency at NR1/NR2B NMDA receptors. (C) Donepezil was tested as an antagonist of 10 μM L-glutamate / 10 μM glycine (10 μM Glu/Gly) and of 300 μM L-glutamate / 300 μM glycine (300 μM Glu/Gly). (D) NMDA receptor responses blockade by 1 mM donepezil at various membrane holding potentials. Donepezil blockade was greater at larger negative holding potentials. For all experiments, only cells with stable plateau responses were used.

Figure 3. Antidepressant-like profile of donepezil in comparison with other cholinesterase inhibitors and σ_1 receptor ligands. Mice were submitted to a 15-min duration forced swimming on day 1 and to a 6-min duration swimming on day 2. Donepezil (1-30 mg/kg), rivastigmine (1-30 mg/kg), tacrine (1-30 mg/kg), igmesine (2-40 mg/kg) and/or BD1047 (1-10 mg/kg) were administered ip 30 before the second session. Immobility duration was determined during the 5 last min of the second session. (A) Dose-response effect of donepezil and blockade by BD1047, KW = 47.70, $p < 0.0001$; (B) lack of effect of rivastigmine or tacrine, KW = 3.04, $p > 0.05$; (C) Dose response effect of igmesine and blockade by BD1047, KW = 27.62, $p < 0.0001$; (D) Effect of donepezil (30 mg/kg) in mice centrally administered with the σ_1 receptor antisense ODN probe, KW = 11.03, $p < 0.05$. The number of animals is

indicated within the columns. * $p < 0.05$, ** $p < 0.01$ *vs* the vehicle (V)-treated group; $^{\#\#}$ $p < 0.01$ *vs* the donepezil (30 mg/kg)- or igmesine (40 mg/kg)-treated group; Dunn's test.

Figure 4. Anti-amnesic effect of donepezil against dizocilpine-induced learning impairments, and blockade by BD1047. Donepezil (0.12-1 mg/kg) and/or BD1047 (0.5-1 mg/kg) were administered ip 30 before the Y-maze session or passive avoidance training. Mice were submitted to the spontaneous alternation test in the Y-maze (A, C) or to the step-through passive avoidance training, the retention session being performed after 24 h (B, D). Dose-response effect of donepezil in control and dizocilpine (0.15 mg/kg)-treated animals in the Y-maze alternation test, KW = 50.67, $p < 0.0001$ (A) and in the passive avoidance test, KW = 49.92, $p < 0.0001$ (B). Blockade by BD1047 of the donepezil effect in the Y-maze alternation test, KW = 59.58, $p < 0.0001$ (C) and in the passive avoidance test, KW = 54.67, $p < 0.0001$ (D). The number of animals is indicated within the columns in (A, C). ** $p < 0.01$ *vs* the vehicle (V)-treated group; $^{\#\#}$ $p < 0.01$ *vs* the dizocilpine (0.15 mg/kg)-treated group; ^{o} $p < 0.05$, ^{oo} $p < 0.01$ *vs* the donepezil (0.5 mg/kg)+dizocilpine (0.15 mg//kg)-treated group; Dunn's test.

Figure 5. Anti-amnesic effect of the selective σ_1 receptor agonits igmesine against the dizocilpine-induced learning impairments, and blockade by BD1047. Igmesine (0.1-3 mg/kg) and/or BD1047 (0.5-1 mg/kg) were administered ip 30 before the Y-maze session or passive avoidance training. Mice were submitted to the spontaneous alternation test in the Y-maze (A, C) or to the step-through passive avoidance training, the retention session being performed after 24 h (B, D). Dose-response effect of igmesine in control and dizocilpine (0.15 mg/kg)-treated animals in the Y-maze alternation test, KW = 49.65, $p < 0.0001$ (A) and in the passive avoidance test, KW = 35.28, $p < 0.0001$ (B). Blockade by BD1047 of the igmesine effect in the Y-maze alternation test, KW =51.87, $p < 0.0001$ (C) and in the passive avoidance test, KW = 41.09, $p < 0.0001$ (D). The number of animals is indicated within the columns in (A, C). ** $p < 0.01$ *vs* the vehicle (V)-treated group; $^{\#\#}$ $p < 0.01$ *vs* the dizocilpine (0.15 mg/kg)-

treated group; ^{o} $p < 0.05$, ^{oo} $p < 0.01$ *vs* the igmesine (1 mg/kg)+dizocilpine (0.15 mg//kg)-treated group; Dunn's test.

Figure 6. Effect of the σ_1 antisense ODN treatment on the anti-amnesic effect of donepezil against the dizocilpine-induced learning impairments: spontaneous alternation in the Y-maze (A) and passive avoidance retention (B). Donepezil (0.5 mg/kg was administered 10 min before dizocilpine (0.15 mg/kg) which was given 20 min before the Y-maze session or passive avoidance training. KW = 36.03, $p < 0.0001$ in (A) and KW = 32.15, $p < 0.0001$ in (B). The number of animals is indicated within the columns in (A). ** $p < 0.01$ *vs* the vehicle (V)-treated group; $^{\#\#}$ $p < 0.01$ *vs* the dizocilpine (0.15 mg/kg)-treated group; Dunn's test.

Figure 7. Anti-amnesic effect of rivastigmine (A, B) or tacrine (C, D) against the dizocilpine-induced learning impairments. Rivastigmine (0.3-1 mg/kg), tacrine (0.3-1 mg/kg) and/or BD1047 (0.5-1 mg/kg) were administered ip 30 before the Y-maze session or passive avoidance training. Mice were submitted to the spontaneous alternation test in the Y-maze (A, C) or to the step-through passive avoidance training, the retention session being performed after 24 h (B, D). Dose-response effect of rivastigmine in the Y-maze alternation test, KW =35.92, $p < 0.0001$ (A) and in the passive avoidance test, KW = 31.53, $p < 0.0001$ (B). Dose-response effect of tacrine in control and dizocilpine (0.15 mg/kg)-treated animals in the Y-maze alternation test, KW = 43.32, $p < 0.0001$ (C) and in the passive avoidance test, KW = 32.68, $p < 0.0001$ (D). The number of animals is indicated within the columns in (A, C). ** $p < 0.01$ *vs* the vehicle (V)-treated group; $^{\#\#}$ $p < 0.01$ *vs* the dizocilpine (0.15 mg/kg)-treated group; Dunn's test.

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Figure 1

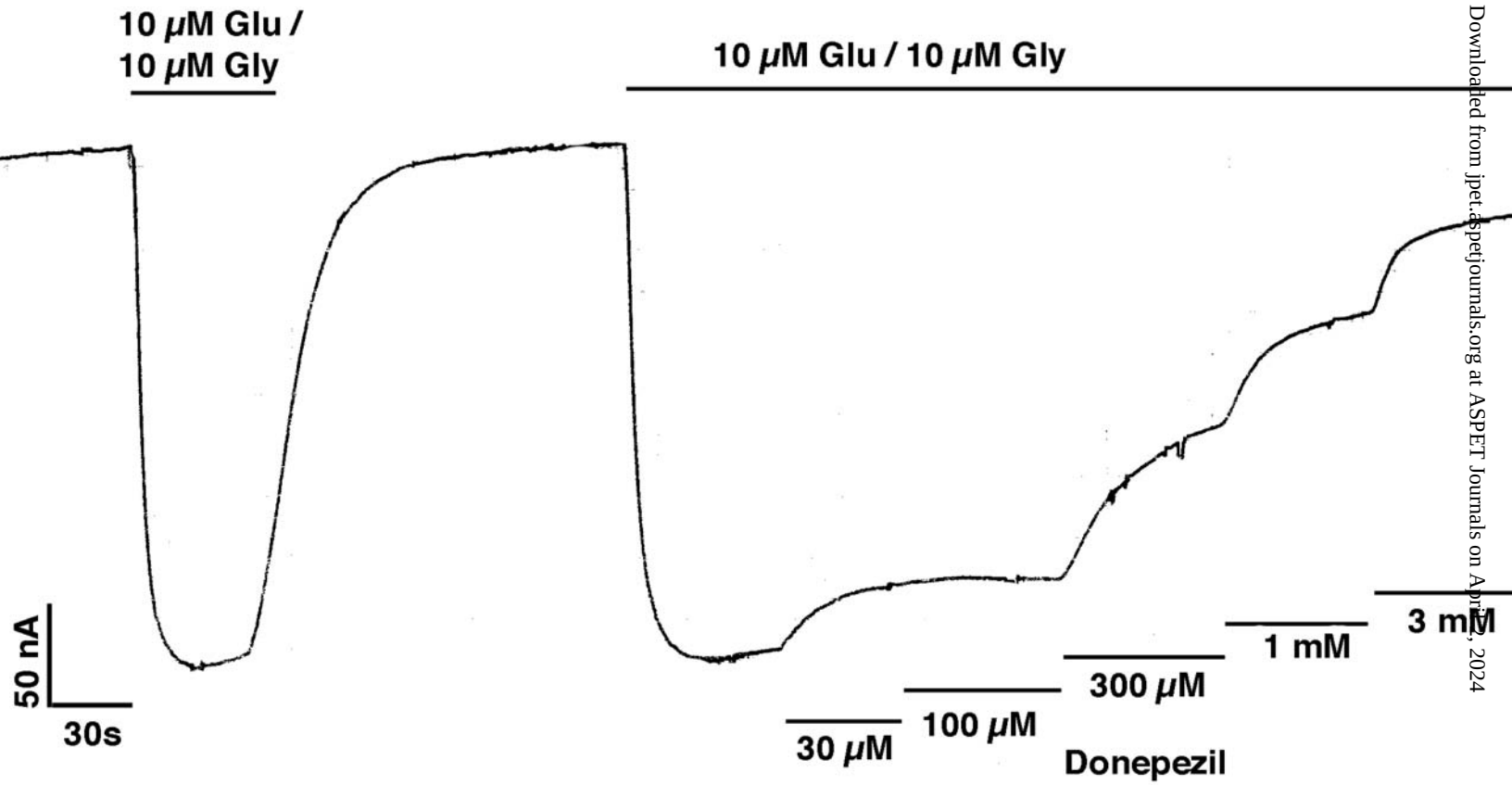

Figure 2

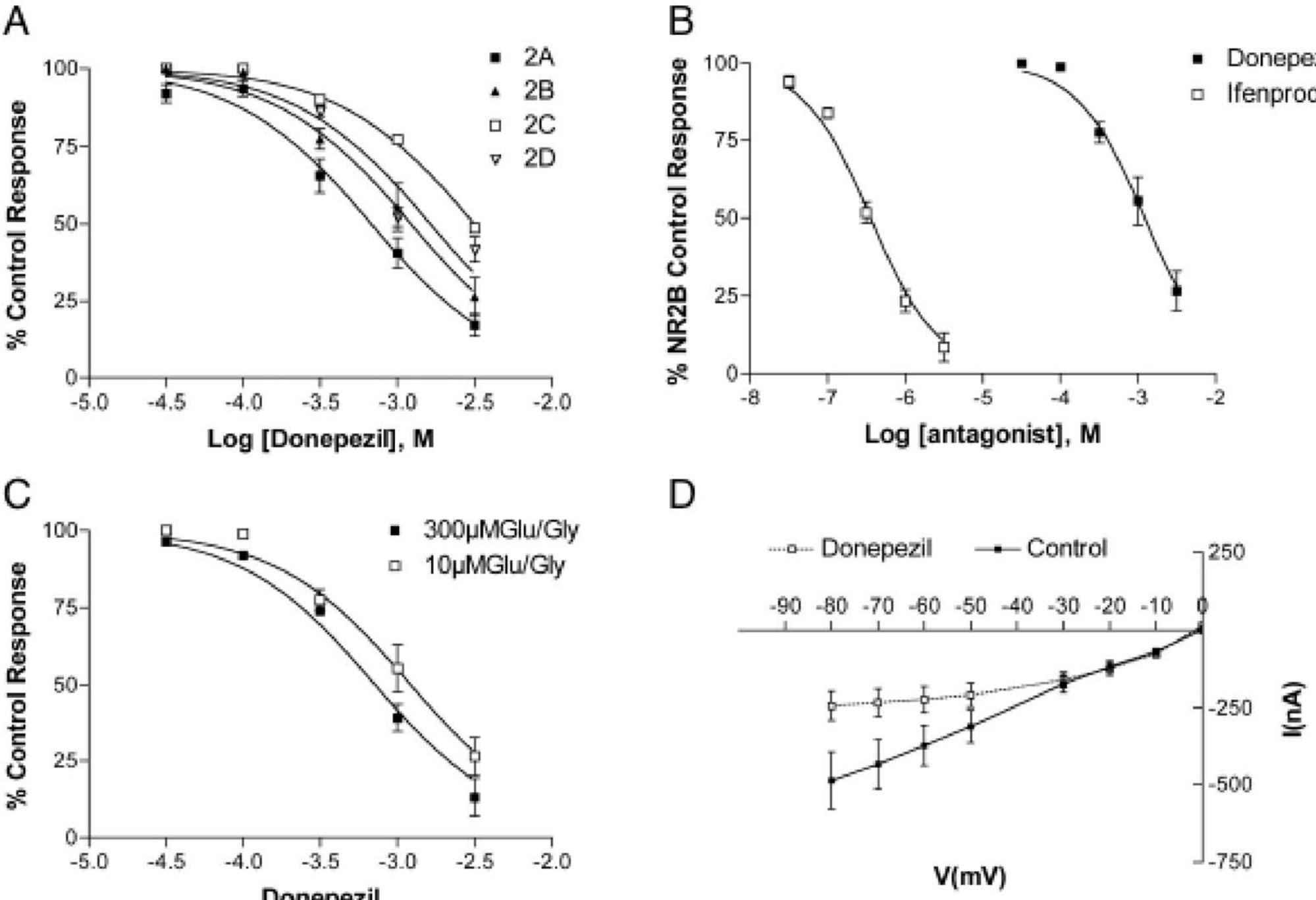

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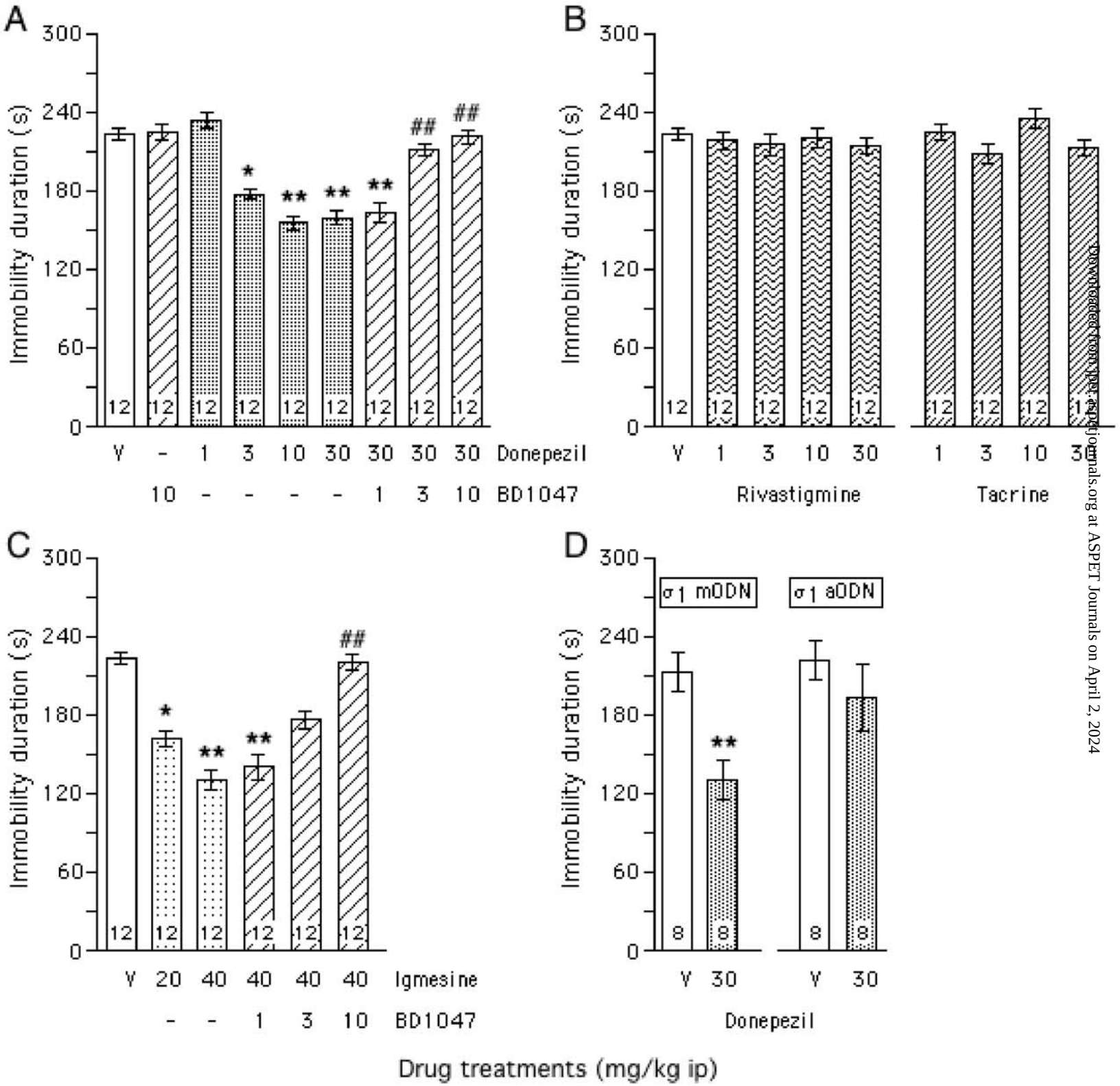
Figure 3



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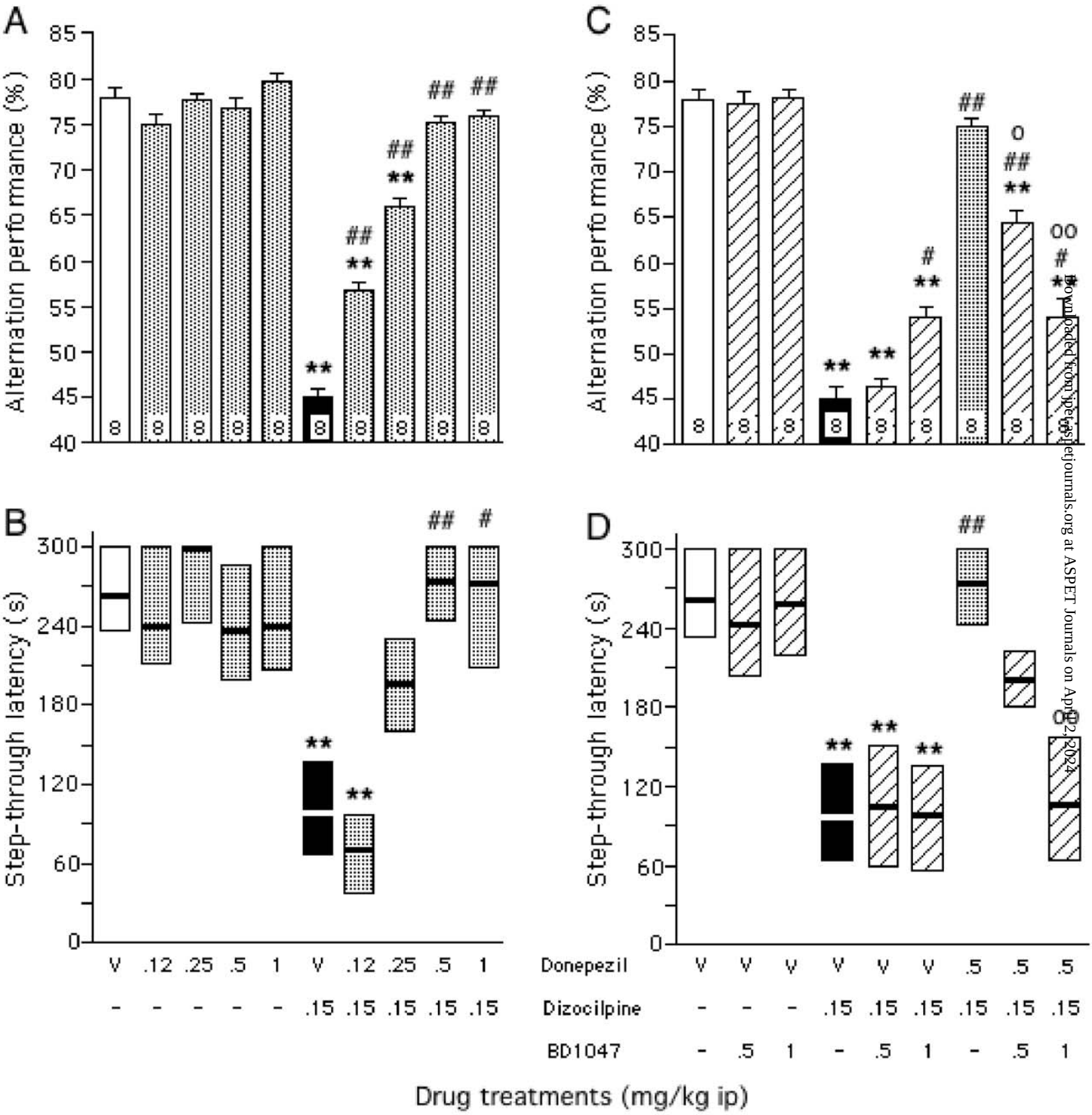

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Figure 5

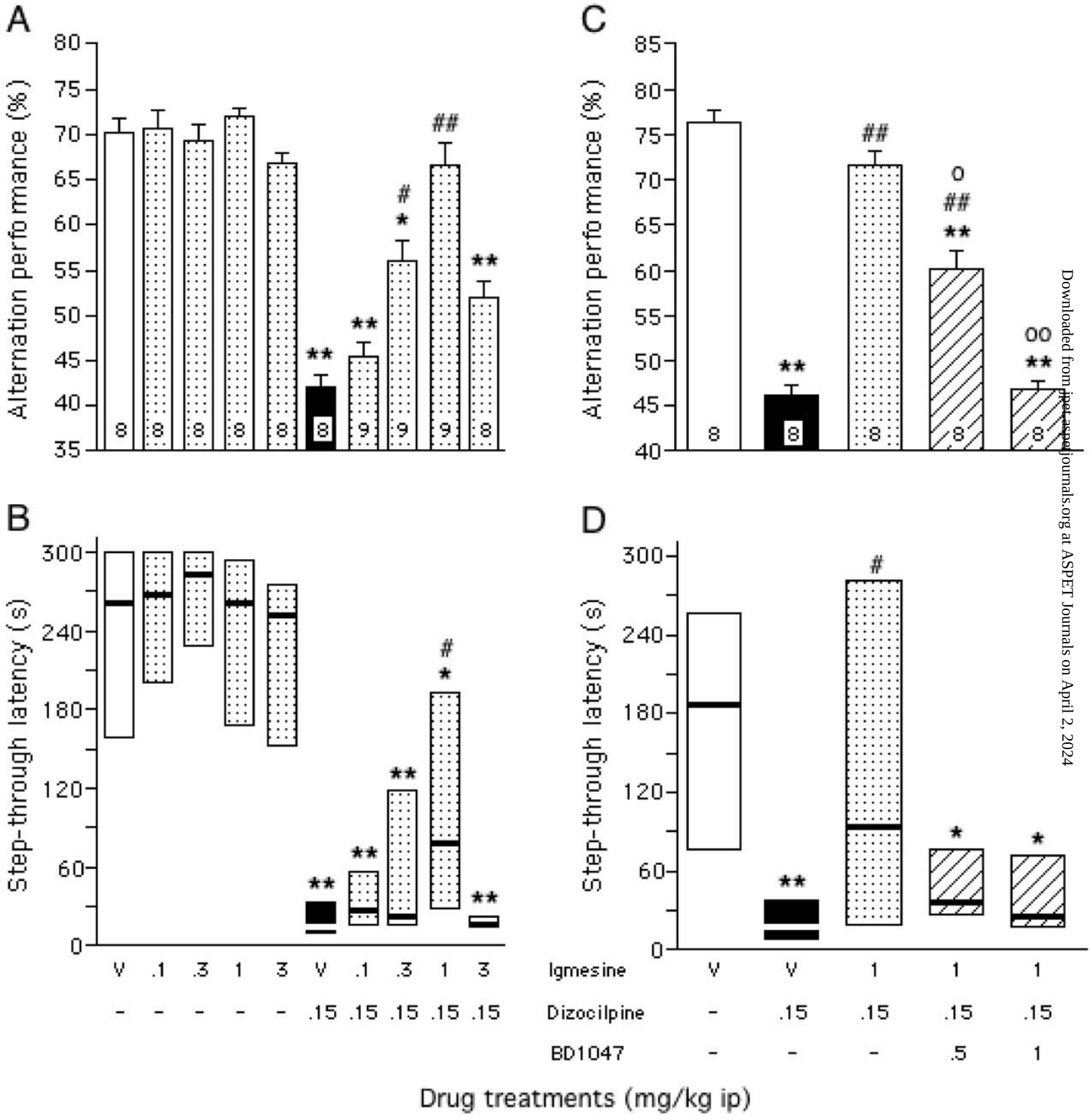

Figure 6

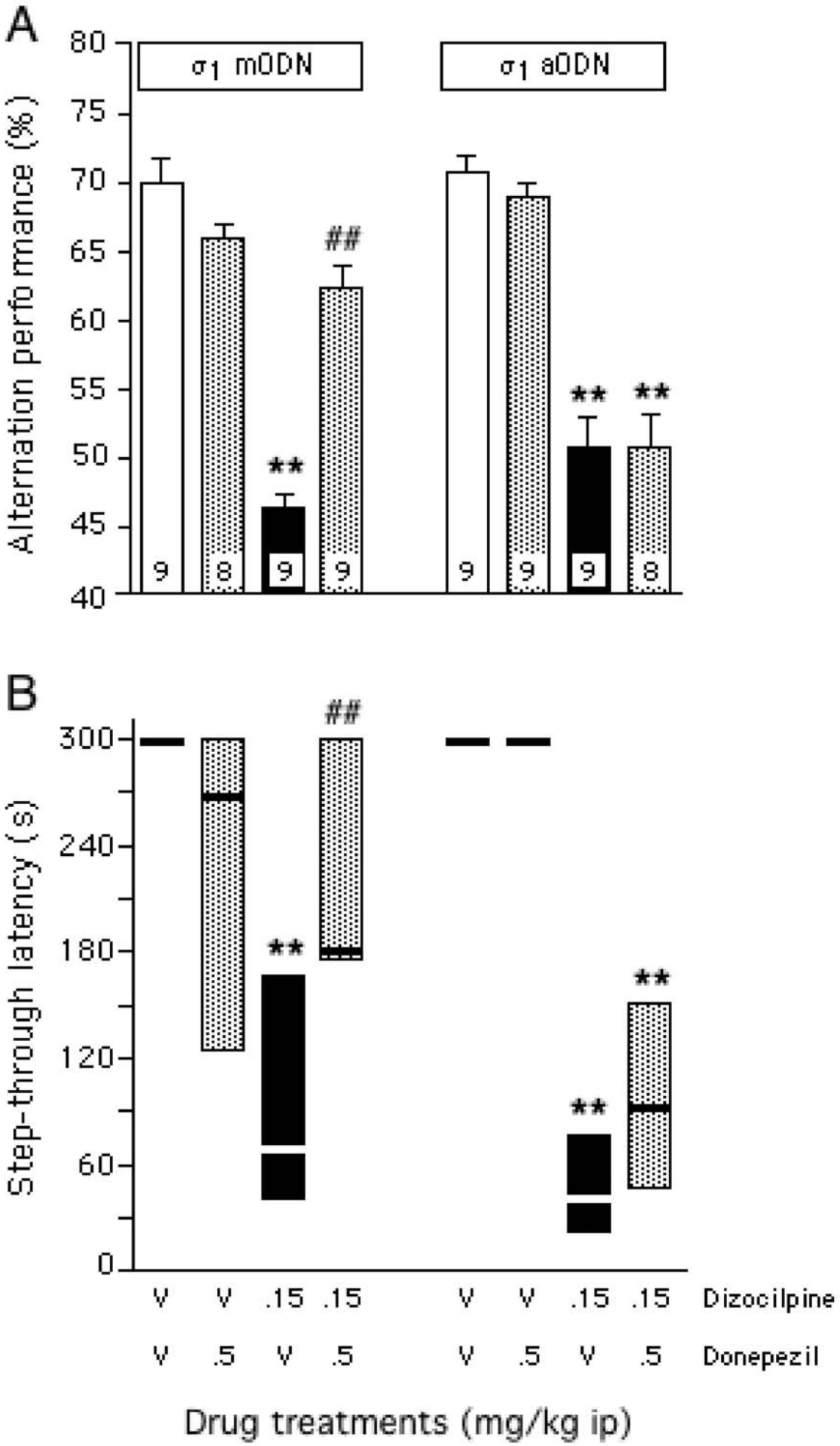

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Figure 7