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Title page

Anti-amnesic and neuroprotective effects of donepezil against learning impairments induced in mice by exposure to carbon monoxide (CO) gas

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Running title page

Running Title: Donepezil in CO-exposed mice

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Abbreviations: CO, carbon monoxide; NMDA, N-methyl-D-aspartate.

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Abstract

Donepezil is a potent acetylcholinesterase inhibitor that also interacts with the sigma$_1$ (σ_1) receptor, an intracellular neuromodulatory protein. In the present study, we analyzed the anti-amnesic and neuroprotective activities of donepezil in a mouse hypoxia model induced by repetitive CO exposure, comparing donepezil's pharmacological profile with other cholinesterase inhibitors tacrine, rivastigmine and galanthamine, and the reference σ_1 agonist igmesine. CO-exposure induced, after 7 days, hippocampal neurodegeneration, analyzed by Cresyl violet staining, and behavioral alterations, measured using spontaneous alternation and passive avoidance responses. When injected 20 min before the behavioral tests, *i.e.* 7-8 days after CO, all drugs showed anti-amnesic properties. Pre-administration of the σ_1 receptor antagonist *N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(dimethylamino)ethylamine (BD1047) blocked only the igmesine and donepezil effects. The neuroprotective activity of the drugs was tested by injection 20 min before the first CO-exposure (pre-insult protection) or by injection 1 h after the last CO-exposure (post-insult protection). All drugs alleviated the hypoxia-induced neurodegeneration and behavioral impairments when injected before CO-exposure. Pre-administration of BD1047 blocked both the igmesine and donepezil effects. However, when injected after CO-exposure, only igmesine and donepezil induced effective neuroprotection and the morphological and behavioral effects were BD1047-sensitive. These results showed that donepezil is a potent anti-amnesic and neuroprotective compound against the neurodegeneration induced by excitotoxic insult and its pharmacological actions as both an acetylcholinesterase inhibitor and σ_1 receptor agonist contribute to its marked efficacy. In particular, the drug is a more potent post-insult protecting agent as compared to more selective cholinesterase inhibitors.

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Introduction

Acetylcholinesterase inhibitors inhibit the hydrolysis of acetylcholine and elevate its concentration in the synaptic cleft, provoking an increase of the efficacy of cholinergic neurotransmission. (±)-2-[(1-benzylpiperidin-4-yl)methyl]-5,6-dimethoxy-indan-1-one (donepezil) is a potent acetylcholinesterase 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 moderate Alzheimer's disease (Rogers & Friedhoff, 1996; Rogers et al., 1998). The compound presents several interesting pharmacological characteristics comparatively with other cholinesterase inhibitors. First, with an IC_{50} value of 5.7 nM for acetylcholinesterase activity *vs*. 7 µM for butyrylcholinesterase activity, donepezil appears to be a very selective acetylcholinesterase inhibitor (Sugimoto et al., 2000). Second, the compound has high affinity for the σ_1 receptor, with an IC_{50} of 14.6 nM, *i.e.*, in the same concentration range as its acetylcholinesterase inhibition potency (Kato et al., 1999). The σ_1 receptor is an intracellular protein involved in modulation of intracellular Ca^{2+} mobilization and neurotransmitter responses (Maurice et al., 1999a). The interaction of donepezil with the σ_1 receptor has been demonstrated at the behavioral level, since the selective σ_1 receptor antagonist *N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(dimethylamino)ethylamine (BD1047) or an antisense probe targeting the σ_1 receptor blocked the anti-amnesic effect of donepezil against dizocilpine-induced learning impairments in mice (Maurice et al., 2006), a behavioral response identifying σ_1 receptor agonist activity (Maurice et al., 1994a). Donepezil and other cholinesterase inhibitors showed neuroprotective effects in both *in vivo* or *in vitro* models of glutamate neurotoxicity, through a mechanism involving mainly an indirect activation of α4β2- and α7-nicotinic receptors (Takada et al., 2003; Fujiki et al., 2005). However, the putative involvement of the σ_1 receptor in the neuroprotective activity of donepezil was never examined.

Repetitive exposure to carbon monoxide (CO) gas constitutes an *in vivo* model of hypoxia. CO exposure induces long-lasting but delayed amnesia in mice that can be measured one week after

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exposure (Nabeshima et al., 1991; Maurice et al., 1994b, 1999b, 2000). The hippocampal cholinergic system appears markedly affected by the hypoxic toxicity involving amino acid excitotoxicity (Nabeshima et al., 1991). In particular, the concentration of acetylcholine and the binding potency of [^{3}H]quinuclidyl benzilate in the frontal cortex and the striatum, but not in the hippocampus, were increased 7 days after CO exposure (Nabeshima et al., 1991). Histological examination of the CA_1 region of the hippocampal formation showed a moderate, but significant neuronal loss, which was augmented by increasing the severity of the CO exposure (Ishimaru et al., 1991). This model involves the neurotoxic effects of excitatory amino acids. Competitive or non-competitive NMDA receptor antagonists acting through glycine or phencyclidine modulatory site prevented the CO-induced amnesia and the concomitant hippocampal neuronal loss (Nabeshima et al., 1991; Ishimaru et al., 1992). On the contrary, the neuroactive steroid pregnenolone sulfate, known to positively modulate the NMDA receptor, worsened the CO-induced neuronal damage and resulting amnesia (Maurice et al., 2000). The CO intoxication model therefore represents a valuable model of excitotoxicity-associated hippocampal damage, which allows evaluating the neuroprotective activity of drugs *in vivo*.

In this study, we used the repetitive CO exposure model to compare the protective effects of donepezil with a reference σ_1 receptor agonist, igmesine, and the acetylcholinesterase inhibitors tacrine, rivastigmine and galanthamine. Compounds were administered either 7 days after CO exposure, 20 min before the first exposure to CO or 1 h after the last exposure, to differently assess the anti-amnesic and neuroprotective activities of the drugs. Cell death in the CA_1 hippocampal area was analyzed in Cresyl violet stained brain sections and the appearance of the learning deficits were examined in CO-exposed mice using both a short-term and a long-term memory tests.

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Methods

Animals

Male Swiss mice, 1-month old and weighing 28-32 g, were purchased from the breeding center of the Faculty of Pharmacy (Montpellier, France). Animals were housed in groups of 20 with access to food and water *ad libitum*, except during experiments. They were kept in a temperature and humidity controlled animal facility on a 12h/12h light:dark cycle (light off at 20:00). Behavioral experiments were carried out between 9:00 and 14:00, in a sound attenuated and air-regulated experimental room, to which mice were habituated at least 30 min. All animal procedures were conducted in strict adherence to the European Communities Council Directive of 24 November 1986 (86-609/EEC).

Drugs

Donepezil hydrochloride was provided by Eisai Co. Ltd (Tokyo, Japan). (+)(S)-N-ethyl-3-[(1-dimethyl-amino)ethyl]-N-methylphenylcarbamate hydrogentartrate (rivastigmine) was from Novartis (Basel, Switzerland). 9-Amino-1,2,3,4-tetrahydroacridine hydrochloride (tacrine) and (4αS,6R,8αS)-4α,5,9,10,11,12-hexahydro-3-methoxy-11-methyl-6H-benzofuro(3α,3,2-ef)(2)benzazepin-6-ol hydrobromide (galanthamine) were from Sigma-Aldrich (St-Quentin-Fallavier, France). (+)-N-cyclopropylmethyl-N-methyl-1,4-diphenyl-1-ethyl-but-3-en-1-ylamine hydrochloride (igmesine) was provided by Dr François J. Roman (Pfizer GRD, Fresnes, France). N-[2-(3,4-dichlorophenyl)ethyl]-N-methyl-2-(dimethylamino)ethylamine (BD1047) was provided by Dr Wayne D. Bowen (Laboratory of Medicinal Chemistry, NIDDK, NIH, Bethesda, MA, USA). All drugs were dissolved in physiological saline solution, 0.9%, were injected intraperitoneally (ip) in a volume of 100 µl per 20 g of body weight. Doses refer to the salt form. For antagonism studies, the σ_1 receptor antagonist was administered 10 min before the experimental drugs. Control animals received only one administration of saline solution, since extensive previous studies have shown no differences in behavioral responses after one or two ip injections of saline (data not shown).

Exposure to CO gas

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Exposure to CO was carried out as previously described (Ishimaru et al., 1991, 1992; Nabeshima et al., 1991; Maurice et al., 1994b, 1999b, 2000). Mice were placed in a transparent plastic vessel (3-cm radius, 10 cm high), with a pipe feeding into it. CO gas was disseminated at the rate of 125 ml/min, and mice were exposed until they began gasping, i.e., between 30 and 40 s. Animals were exposed three times, with 1 h between each exposure. They were kept on a hot plate (Silab, Montpellier, France) immediately after the first exposure and up to 2 h after the third, in order to maintain their body temperature at 38°C and to avoid the hypothermia induced by CO, which lessens the damages induced by hypoxia (Ishimaru et al., 1991).

The anti-amnesic effects of the cholinesterase inhibitors (donepezil, tacrine, rivastigmine, galanthamine) or σ_1 receptor agonist (igmesine) were examined by pre-test injections, 7 days after exposure to CO. The neuroprotective effects of each compound was examined using: (i) injections made 20 min before the first exposure to CO, animals being tested after 7 days; or (ii) injections made 1h after the last exposure to CO, animals being tested on day 7.

Spontaneous alternation performances

The spatial working memory was examined using the spontaneous alternation behavior in the Y-maze (Maurice et al., 1994a,b). The maze was made of black painted wood. Each arm was 40 cm long, 13 cm high, 3 cm wide at the bottom, 10 cm wide at the top, and converged at an equal angle. Each mouse was placed at the end of one arm and allowed to move freely through the maze during an 8 min session. The series of arm entries, including possible returns into the same arm, were checked visually. An alternation was defined as entries into all three arms on consecutive occasions. 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. Neither the exposure to CO, nor the treatments used in the study affected the exploratory activity in the test and the numbers of arm entries were in the 27-34 range for all groups.

Step-through type passive avoidance response

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The apparatus consisted of an illuminated compartment with white polyvinylchloride walls (15 x 20 x 15 cm high), a darkened compartment with black polyvinylchloride walls (15 x 20 x 15 cm high) and a grid floor. A guillotine door separated each compartment. A 60 W lamp positioned 40 cm above the apparatus lit the white compartment during the experimental period. Scrambled foot shocks (0.3 mA for 3 s) were delivered to the grid floor using a shock generator scrambler (Lafayette Instruments, Lafayette, MA, USA). The guillotine door was initially closed during the training session. Each mouse was placed into the white compartment. After 5 s, the door was raised. When the mouse entered the darkened compartment and placed all its paws on the grid floor, the door was gently closed and the scrambled foot shock was delivered for 3 s. The step-through latency and the number of vocalizations were recorded. The number of vocalizations did not differ among groups, indicating that shock sensitivity was identical. The retention test was carried out 24 h after training. Each mouse was placed again into the white compartment. After 5 s, the door was raised. The step-through latency was recorded up to 300 s. If animals entered the darkened compartment, the escape latency, i.e., the time spent to return into the white compartment, was also measured up to 300 s

Histology

Each mouse was anaesthetised with sodium pentobarbital (100 mg/kg i.p.) and quickly transcardially perfused with 50 ml of phosphate buffered saline solution (PBS), pH = 7.4, followed by 50 ml of PBS containing 4% paraformaldehyde (w/v). Brains were removed and kept overnight in the fixative solution. They were cut in coronal sections (60 µm thickness) using a vibratome (Leica VT1000 S). Serial sections were selected to include the hippocampal formation and placed in gelatin-coated glass-strip. Sections were stained with 0.2% Cresyl violet reagent (Sigma-Aldrich), then dehydrated with graded ethanol, treated with Toluene and mounted with DePeX medium (BDH Laboratory Supplies, England). Examination of the CA1 area was performed using a light microscope (Dialux 22, Leitz), slices being digitalized through a CCD camera (Sony XC-77CE) with the NIH Image, v. 1.63, software, in order to easily process CA1 measurement and pyramidal cells counts. Data are expressed as mean of 6 slices CA1 pyramidal cells per millimeter for each

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group, according to previously reported method (Nabeshima et al., 1991; Ishimaru et al., 1992; Maurice et al., 2000).

Statistical analyses

Y-maze test data were expressed as mean ± S.E.M. Passive avoidance latencies did not show a normal distribution, since a cut-off time was set. They were thus represented as median and interquartile range. Drug doses for antagonism studies were decided *a priori*, based on previous studies (see, for instance, Maurice et al., 2006) and extensive literature in the field. Experiments were thus designed to include dose-response and antagonisms studies in the same set. All behavioral data were therefore analyzed using the Kruskal-Wallis non-parametric ANOVA (*KW* values) for coherence and group comparisons being made with Dunn's non-parametric multiple comparisons test. The level of statistical significance was $p < 0.05$.

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Results

Anti-amnesic effects of igmesine, donepezil and acetylcholinesterase inhibitors against CO-induced learning impairments

The first series of experiments examined the anti-amnesic effects of the drugs. Mice were exposed to CO gas and the learning deficits were measured after 7-9 days using the spontaneous alternation performance, an index of working memory, and the step-through passive avoidance test for long-term memory. CO-exposed mice developed after one week significant memory impairments that could be observed as alternation deficits in the Y maze (Fig. 1A, D) and decrease in step-through latency (Fig. 1B, E) and concomitant increase in escape latency (Fig. 1C, F) in the passive avoidance test. The reference σ_1 receptor agonist igmesine was tested in the 0.3-3 mg/kg dose-range and allowed a complete reversion of the CO-induced deficits in alternation, at the dose of 1 mg/kg (Fig. 1A) and for both passive avoidance parameters (Fig. 1B, C). Pretreatment with the σ_1 receptor antagonist, BD1047 (0.5-3 mg/kg), dose-dependently blocked the beneficial effect of igmesine, but was ineffective by itself on CO-induced deficits (Fig. 1A-C).

Donepezil, administered 20 min before the test also allowed a significant reversion of the CO-induced deficits in alternation, with a maximum effect at the dose of 0.5 mg/kg (Fig. 1D) and for both passive avoidance parameters (Fig. 1E, F). The beneficial effect of donepezil was blocked in a dose-dependent manner by pretreatment with BD1047 (Fig. 1D-F).

The cholinesterase inhibitors tacrine, rivastigmine and galanthamine were tested at the doses of 0.3 and 1 mg/kg ip. All compounds significantly reversed the CO-induced alternation deficits (Fig. 2A), step-through latency deficit (Fig. 2B) and increase in escape latency (Fig. 2C). The drug effects were however not affected by a BD1047 (3 mg/kg) pretreatment, indicating the lack of involvement of the σ_1 receptor in their anti-amnesic potency.

Neuroprotective effects of igmesine, donepezil and acetylcholinesterase inhibitors against CO-induced learning impairments – Pre-insult protection

Drugs were administered 20 min before the first exposure to CO and the learning deficits were measured after 7-9 days using the spontaneous alternation and step-through passive avoidance

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test. Igmesine, 0.3-3 mg/kg dose-range, allowed a complete blockade of the CO-induced deficits in alternation, at the highest doses tested (Fig. 3A) and for both passive avoidance parameters (Fig. 3B, C). Pretreatment with BD1047 (0.5-3 mg/kg) dose-dependently blocked the beneficial effect of igmesine, with the σ_1 receptor antagonist itself ineffective on CO-induced deficits (Fig. 3A-C).

Donepezil, 0.12-1 mg/kg, also allowed a significant prevention of the CO-induced deficits in alternation (Fig. 1D) and for both passive avoidance parameters (Fig. 3E, F), with a maximum effect at the dose of 0.5 mg/kg. The beneficial effect of donepezil against CO-induced alternation deficits was sensitive to the pretreatment with BD1047, but differently depending on the test and parameter. A significant but partial reversion was measured for alternation performances (Fig. 3D). a complete blocked was observed for the step-through latency (Fig. 3E). A non-significant attenuation was observed for escape latency (Fig. 3F).

The cholinesterase inhibitors tacrine, rivastigmine and galanthamine were tested at the doses of 0.3 and 1 mg/kg ip. All compounds significantly reversed the CO-induced alternation deficits (Fig. 4A), step-through latency deficit (Fig. 4B) and increase in escape latency (Fig. 4C). The drug effects were however not affected by a BD1047 (3 mg/kg) pretreatment.

Loss of pyramidal cells in the CA1 hippocampal subfield (Fig. 5A) was measured using Cresyl violet staining, 7 days after CO exposure. Drugs were administered 20 min before the first CO exposure. Stained cell quantification revealed a significant decrease induced by CO exposure (Fig. 5C, I), accompanied with cell swelling and dispersion, as compared to control mice (Fig. 5B). The BD1047 (1mg/kg) was without effect (Fig. 5D, I). Igmesine (1 mg/kg) and donepezil (0.5 mg/kg) treatments blocked the hypoxia-induced cell loss (Fig. 5E, G, I). The igemesine effect was significantly antagonized by the BD1047 pre-treatment (Fig. 5F, I). The donepezil effect was attenuated by BD1047 (-50% cell loss), but the effect remained non-significant (Fig. 5I). Tacrine, 1 mg/kg (Fig. 6C, G), or rivastigmine, 1 mg/kg (Fig. 6D, G), failed to show neuroprotection against neurodegeneration induced by CO exposure (Fig. 6B, G). Galanthamine, 1 mg/kg, showed a slight and significant neuroprotective effect (Fig. 6E, G). This effect of galanthamine was unaffected by the BD1047 pre-treatment (Fig. 6F, G).

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Neuroprotective effects of igmesine, donepezil and acetylcholinesterase inhibitors against CO-induced learning impairments – Post-insult protection

Drugs were administered 1 h after the last exposure to CO and the learning deficits were measured after 7-9 days using the spontaneous alternation and step-through passive avoidance test. Igmesine, 0.3-3 mg/kg dose-range, produced a significant attenuation of the CO-induced deficits in alternation, at the highest doses tested (Fig. 7A) and for both passive avoidance parameters (Fig. 7B, C). Pretreatment with BD1047 (0.5-3 mg/kg) dose-dependently blocked the beneficial effect of igmesine in terms of alternation performance and step-through latency (Fig. 7A, B). However, the σ_1 receptor antagonist was ineffective by itself on CO-induced deficits (Fig. 7A-C).

Donepezil, 0.12-1 mg/kg, also allowed a significant but partial prevention of the CO-induced deficits in alternation (Fig. 7D). The step-though latency deficits in CO-exposed mice was attenuated, but without reaching significance, in contrast to the escape latency parameter (Fig. 7E, F). The beneficial effect of donepezil against CO-induced alternation deficits was blocked by pretreatment with BD1047, since animals treated with the highest dose of BD1047 plus donepezil 0.5 mg/kg performed at a similar level as Veh-treated CO-exposed mice (Fig. 7D-F).

The cholinesterase inhibitors tacrine, rivastigmine and galanthamine were tested at the doses of 0.3 and 1 mg/kg ip. Only rivastigmine and galanthamine showed significant improvement on the CO-induced alternation deficits, effects that were insensitive to BD1047 (Fig. 8A). A tendency toward improvement of the step-through latency and escape latency deficits in CO-exposed mice was observed for the same drugs, but no differences reached statistical significance. The BD1047 treatment was without any effect, in any group (Fig. 8B, C).

Histological examination of the CA1 pyramidal cell layer showed cell loss in CO-exposed mice (Fig. 9B, H) as compared to control mice (Fig. 9A, H). Post-treatment with BD1047, 1 mg/kg, failed to have any protective effect (Fig. 9C, H). Igmesine, 1 mg/kg, and donepezil, 0.5 mg/kg, protected CA1 area against hypoxic insult (Fig. 9D, F, H), and these beneficial effects were blocked by BD1047 administration (Fig. 9E, G, H). The other cholinesterase inhibitors tested, tacrine, 1 mg/kg (Fig. 10C, F), rivastigmine, 1 mg/kg (Fig. 10D, F) or galanthamine, 1 mg/kg (Fig. 10E, F), failed to protect CA1 area against neurodegeneration induced by CO exposure (Fig. 10B, F), as compared to control mice (Fig. 10A, F).

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Discussion

In the present study, we examined the anti-amnesic and neuroprotective effects of donepezil and other cholinesterase inhibitors against amnesia induced in mice by severe hypoxia. We aimed at determining whether an interaction with the σ_1 receptor is involved in donepezil's anti-amnesic and neuroprotective effects and contributes to its behavioral effects. Indeed, the drug is, with a 14.6 nM affinity for the σ_1 receptor and an IC_{50} of 5.7 nM for inhibition of acetylcholinesterase activity, equipotent for these two targets (Kato et al., 1999). Other cholinesterase inhibitors are more selective cholinomimetics and present only low affinity for the σ_1 receptor. For instance, tacrine shows an affinity of 6 µM for the σ_1 receptor (Kato et al., 1999) as compared with an IC_{50} of 77 nM for the inhibition of acetylcholinesterase activity (Ogura et al., 2000). Repetitive exposure to CO induces in mice a severe hypoxic insult to the hippocampus, leading to an intense excitotoxic neurodegenerative process with synaptic and cellular losses in the CA1-3 pyramidal layers (Nabeshima et al., 1991; Ishimaru et al., 1991, 1992; Maurice et al., 1994b, 1999b). Within one week after exposure, animals gradually develop learning deficits that correlate with these histological changes. Moreover, the model could be used to analyze either anti-amnesic effect of drugs, administered at the time point of the behavioral examination, or neuroprotective effect, with drugs being administered during or immediately after the exposure to CO (Maurice et al., 1994b, 2000). The results of the study were summarized in Table 1.

The first part of the present report examined the anti-amnesic effects of the drugs in CO-exposed mice. Examination of working memory and contextual long-term memory, using the spontaneous alternation and passive avoidance behavior respectively, showed highly significant deficits for all parameters examined. All drugs tested alleviated the deficits, within the 0.1-1 mg/kg dose range, confirming that cholinomimetics as well as σ_1 receptor agonists are effective against amnesia induced by excitotoxic insults. This was previously described for acetylcholinesterase inhibitors in several models, including amino-acid induced lesions (Dokla et al., 1989) and for selective σ_1 receptor agonists (Maurice et al., 1994b, 1999b). The new observation is that donepezil's anti-amnesic effects were blocked by BD1047, a reference σ_1 receptor antagonist. This observation demonstrates that these anti-amnesic effects therefore involve both its cholinomimetic

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and σ_1 agonist properties. Indeed, donepezil, as well as tacrine, galanthamine and rivastigmine, potentiate extacellular levels of acetylcholine in the brain, particularly within the hippocampus, as a result of the inhibition of acetylcholinesterase activity (Kosasa et al., 1999). However, in a recent report, we reported that the anti-amnesic effects of donepezil against dizocilpine-induced learning impairments were blocked not only by BD1047 but also by an antisense probe treatment down-regulating the σ_1 receptor expression within the cortex and hippocampus (Maurice et al., 2006). Therefore, the interaction of donepezil with the σ_1 receptor is effective *in vivo* and contributes to its anti-amnesic effects. Our present results extended this observation in a pathological model of amnesia. Moreover, the inactivation of the σ_1 receptor led to a complete blockade of donepezil's effects, suggesting that cholinergic and σ_1 systems are inter-related and mixed activity does not lead to simple additive effects. This phenomenon has also been observed with drugs acting non-selectively as direct muscarinic agonists and σ_1 receptor agonists (unpublished observation). The σ_1 receptor is present within pre-synaptic neurons where it may facilitate acetylcholine release (Junien et al., 1991) and within the post-synaptic neurons, where it potently modulates intracellular second messenger cascades, particularly facilitating protein kinase C/phospholipase C pathways (Morin-Surun et al., 1999; Monnet et al., 2003). Therefore, mixed compounds may activate post-synaptic neurons by acting synergistically on cholinergic receptors, through increases in acetylcholine concentration in the synaptic cleft and on intracellular pathways via activation of the σ_1 receptor. The physiological consequences of such biphasic action on synaptic transmission have to be examined particularly using electrophysiological approaches.

The second aspect of the present study was to examine the neuroprotective efficacy of donepezil, in comparison with igmesine and other cholinesterase inhibitors (Table 1). Drugs were administered either before the first exposure or 1 h after the last exposure to CO. Histological damage and behavioral deficits were examined after one week. It is hypothesized that the first procedure allows analysis of the drug effects on the acute neurotoxic insult, known to be dependent on excessive glutamate release, whereas the second procedure focuses on the drug effects on the chronic neurodegeneration processes, mainly mediated by long-term oxidative stress in the damage tissue (Nishikawa, 2001). Results show that all drugs are effective neuroprotective agents when they are administered before CO exposure. The CO-induced behavioral deficits and cell loss in the

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CA1 hippocampal layer are blocked by the drug treatments. The most important result of the present study is the observation that donepezil's effects are antagonized by BD1047, in a similar manner as observed for igmesine, suggesting that the drug behaves more likely as a σ_1 receptor agonist than as a cholinomimetic. When drugs were administered 1 h after the last exposure to CO, igmesine remained effective at the morphological and behavioral levels. Donepezil effects were more moderate at the behavioral level but remained significant. Tacrine, galanthamine and rivastigmine showed moderate behavioral efficacy, reaching significance only for the spontaneous alternation parameter with the two last compounds, and no improvement on the morphological measure. Donepezil effects were again sensitive to BD1047, suggesting the importance of the σ_1 receptor in the neuroprotective effect of the compound.

The neuroprotective activity of cholinomimetic drugs has been extensively described using mainly in vitro models. Donepezil, tacrine, galanthamine, neostigmine, pyridostigmine and metrifonate protect rat cortical neurons against glutamate neurotoxicity and apoptosis (Takada et al., 2003). This effect appears mediated in part via up-regulation of α4- and α7-nicotinic receptors (Kume et al., 2005). Moreover, the neuroprotective activity of cholinesterase inhibitors was also examined in vivo, using focal ischemia models in rats. Pretreatment with a single oral dose of donepezil (12 mg/kg) 2 h before permanent middle cerebral artery occlusion attenuated the infarction volume (Fujiki et al., 2005; Geerts et al., 2005). Moreover, a post-ischemic and chronic treatment with methanesulfonyl fluoride (1 mg/kg at 24 and 48 h post occlusion and 0.3 mg/kg once a day during 4 weeks) attenuated the motor (body swing bias) and cognitive (passive avoidance) abilities and biochemical parameters (choline acetyltransferase immunoreactivity) of the animals (Borlongan et al., 2005). On the other hand, σ_1 receptor agonists have also been reported to possess neuroprotective effects (for review, see Maurice et al., 1999a). In particular, σ_1 receptor ligands protected rat hippocampal and cortical neurons against glutamate or NMDA exposure *in vitro* (Pauwels et al., 1992) or against transient focal or permanent global ischemia *in vivo* (O'Neill et al., 1995; Harukuni et al., 2000). Interestingly, σ_1 receptor agonists including igmesine significantly protected rat brain neurons against a hypoxic/hypoglycemic insult more efficiently than toxicity induced by direct application of NMDA (Lockhart et al., 1995; Nakazawa et al., 1998), suggesting that σ_1 receptors exert a potent neuroprotective effect via the regulation of excitatory amino acids release from presynaptic terminals, rather than via an action on the post-synaptic neurons.

We observed here a differential involvement of cholinergic systems, that appeared effective mainly in the pre-treatment procedure in contrast to the σ_1 receptor, that mediated neuroprotection in both the pre- and post-treatment procedures. The neuroprotection induced by cholinomimetic drugs highly effective in the early phase of the glutamate-induced toxicity is likely to involve the α7-nicotinic receptors activation that leads to neuroprotection via the Ca^{2+}-dependent phosphatidylinositol 3-kinase pathway. Indeed, nicotine protected neurons by activating phosphatidylinositol 3-kinase, which activated Akt and up-regulated Bcl-2 (Kihara et al., 2001) and pretreatment with donepezil 24 h before glutamate protected rat cortical neurons in a manner sensitive to the α7-nicotinic receptor antagonist methyllycaconitine (Takada et al., 2003). Moreover, galanthamine has been shown to not only inhibit acetycholinesterase activity but also allosterically modulate both α7- and $\alpha4\beta2$-nicotinic acetylcholine receptors (Pereira et al., 1993, 1994). This last effect is involved in the neuroprotective activity of galanthamine, as shown against glutamate and amyloid β-peptide toxicities (Kihara et al., 2001, 2004), which therefore exerts a more nicotine-like neuroprotective action than an indirect enhancement of cholinergic systems. This difference of mechanism may explain the ability of galanthamine to protect against CO-induced neuronal damage, more significantly than tacrine or rivastigmine.

The neuroprotection induced by activation of the σ_1 receptor by igmesine or donepezil is effective on both early and late phase of the neurotoxicity and putatively through different mechanisms. Acutely, σ_1 receptor ligands, by regulating Ca^{2+} mobilizations from endoplasmic reticulum pools (Hayashi et al., 2000), may exert a presynaptic effect by regulating glutamate release. This effect has been demonstrated in monoaminergic neurons (Gonzalez-Alvear and Werling, 1995). During the delayed phase of the toxicity, administration of σ_1 receptor may allow a long-term trophic effect. Indeed, the σ_1 receptor, when activated, targets the lipid-storing subcompartments of the endoplasmic reticulum (Hayashi and Su, 2004b) and is co-localized with cholesterol and neutral lipids. The σ_1 receptor forms detergent-insoluble lipid microdomains on the endoplasmic reticulum subcompartments and translocates to plasma membrane lipid rafts, where it allows changes in the lipid components and membrane reconstitution therefore affecting the

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functions of proteins residing in plasma membrane lipid rafts including tropic factor receptors and tyrosine kinases. Specifically, it was recently described that σ_1 receptors modulate MAP kinase activation induced by tropic factors, neuritogenesis and oligodendrocyte differentiation (Hayashi and Su, 2004a; Takebayashi et al., 2004). The mechanism and long-term validity of this σ_1 receptor-mediated neuroprotective activity deserves further extensive examination. Moreover, as observed in the anti-amnesic effect, the neuroprotective effect of donepezil is largely sensitive to inactivation of the σ_1 receptor. This observation strongly suggest that mixed cholinomimetic and σ_1 compounds present a very unique pharmacological action, that could not be reduce to the simple addition of to primary pharmacological effects. Activation of the σ_1 receptor intracellularly results in modulation of Ca^{2+} mobilzation and activation of transduction pathways, such as activation of PLC/PKC, and therefore, to shared effects and direct modulation of the signal transduction systems activated by nicotinic or muscarinic receptors. Detailed mechanism studies must be performed to analyze the mode of action of mixed cholinergic/σ_1 receptor drugs.

In conclusion, the present study showed that donepezil is a potent anti-amnesic and neuroprotective compound against the morphological and behavioral impairments induced in mice by severe hypoxia due to repetitive CO-exposure. The pharmacological actions of donepezil as both an acetylcholinesterase inhibitor and σ_1 receptor agonist contribute to its marked efficacy. In particular, the drug is a more potent post-insult protecting agent as compared to more selective cholinesterase inhibitors.

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Footnotes

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Legends for Figures

Figure 1. Anti-amnesic effects of igmesine (A-C) and donepezil (D-F) in CO-exposed mice. Mice were exposed three consecutive times to CO (125 ml/min, 30-40 s) at 38°C. After 7 days, animals were examined for spontaneous alternation performances (A, D). On day 8 after exposure, animals were trained for passive avoidance task and retention was examined on day 9, in terms of step-through latency (B, E) and escape latency (C, F). Igmesine (0.3-3 mg/kg ip), donepezil (0.12-1 mg/kg ip) and/or BD1047 (0.5-1 mg/kg ip) were administered 20 min before the test. The number of animals per group was n = 8-11. Kruskal-Wallis ANOVA: KW = 63.46, $p < 0.0001$ in (A); KW = 46.20, $p < 0.0001$ in (B); KW = 52.07, $p < 0.0001$ in (C); KW = 52.83, $p < 0.0001$ in (D); KW = 31.80, $p < 0.0001$ in (E); KW = 39.86, $p < 0.0001$ in (F). * $p < 0.05$, ** $p < 0.01$ *vs* the vehicle-treated non-CO exposed group; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ *vs* the vehicle-treated CO-exposed group; ^{o} $p < 0.05$, ^{oo} $p < 0.01$ *vs* the igmesine (1 mg/kg)- or donepezil (0.5 mg/kg)-treated CO-exposed group; Dunn's test.

Figure 2. Anti-amnesic effects of tacrine, rivastigmine and galanthamine in CO-exposed mice. Mice were exposed three consecutive times to CO (125 ml/min, 30-40 s) at 38°C. After 7 days, animals were examined for spontaneous alternation performances (A). On day 8 after exposure, animals were trained for passive avoidance task and retention was examined on day 9, in terms of step-through latency (B) and escape latency (C). Tacrine (0.3-1 mg/kg ip), rivastigmine (0.3-1 mg/kg ip), galanthamine (0.3-1 mg/kg ip) and/or BD1047 (3 mg/kg ip) were administered 20 min before the test. The number of animals per group was n = 8-11. KW = 53.68, $p < 0.0001$ in (A); KW = 30.29, $p < 0.001$ in (B); KW = 47.96, $p < 0.0001$ in (C). * $p < 0.05$, ** $p < 0.01$ *vs* the vehicle-treated non-CO exposed group; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ *vs* the vehicle-treated CO-exposed group; Dunn's test.

Figure 3. Pre-insult neuroprotective effects of igmesine (A-C) and donepezil (D-F) in CO-exposed mice. Mice were administered with igmesine (0.3-3 mg/kg ip), donepezil (0.12-1 mg/kg ip) and/or BD1047 (0.5-3 mg/kg ip) 20 min before being exposed three consecutive times to CO (125 ml/min,

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30-40 s) at 38°C. After 7 days, animals were examined for spontaneous alternation performances (A, D). On day 8 after exposure, animals were trained for passive avoidance task and retention was examined on day 9, in terms of step-through latency (B, E) and escape latency (C, F). The number of animals per group was n = 8-11. KW = 54.97, $p < 0.0001$ in (A); KW = 40.23, $p < 0.0001$ in (B); KW = 42.56, $p < 0.0001$ in (C); KW = 42.01, $p < 0.0001$ in (D); KW = 32.32, $p < 0.0001$ in (E); KW = 25.58, $p < 0.001$ in (F). * $p < 0.05$, ** $p < 0.01$ *vs* the vehicle-treated non-CO exposed group; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ *vs* the vehicle-treated CO-exposed group; ^{o} $p < 0.05$, ^{oo} $p < 0.01$ *vs* the igmesine (1 mg/kg)- or donepezil (0.5 mg/kg)-treated CO-exposed group; Dunn's test.

Figure 4. Pre-insult neuroprotective effects of tacrine, rivastigmine and galanthamine in CO-exposed mice. Mice were administered with tacrine (0.3-1 mg/kg ip), rivastigmine (0.3-1 mg/kg ip), galanthamine (0.3-1 mg/kg ip) and/or BD1047 (3 mg/kg ip) 20 min before being exposed three consecutive times to CO (125 ml/min, 30-40 s) at 38°C. After 7 days, animals were examined for spontaneous alternation performances (A). On day 8 after exposure, animals were trained for passive avoidance task and retention was examined on day 9, in terms of step-through latency (B) and escape latency (C). The number of animals per group was n = 8. KW = 43.31, $p < 0.0001$ in (A); KW = 33.15, $p < 0.001$ in (B); KW = 41.76, $p < 0.0001$ in (C). * $p < 0.05$, ** $p < 0.01$ *vs* the vehicle-treated non-CO exposed group; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ *vs* the vehicle-treated CO-exposed group; Dunn's test.

Figure 5. Pyramidal cell loss in hippocampal CA1 area, 7 days after CO exposure. Pre-insult treatment with igmesine or donepezil. (A) Low magnification of Cresyl violet-stained section of the hippocampal formation. (B-H) High magnification of the CA1 hippocampal subfield. (I) Averaged levels of viable cells. Experimental groups included vehicle-treated non CO-exposed mice (no CO+Veh) and CO-exposed animals (CO+Veh), treated with igmesine (CO+IGM), 1 mg/kg ip, donepezil (CO+DPZ), 0.5 mg/kg ip, BD1047 (CO+BD), 1 mg/kg, igmesine+BD1047 (CO+IGM+BD), and donepezil+BD1047 (CO+DPZ+BD). (A) Scale bars = 450 µm; (B-H) scale bar shown in (B) = 85 µm. The number of mice used is indicated within the columns in (I), $F_{(6,28)}$ =

12.52, $p < 0.0001$. ** $p < 0.01$, * $p < 0.05$ *vs* (no CO+Veh) group; ## $p < 0.01$ *vs* (CO+Veh) group; oo $p < 0.01$ *vs* (CO+IGM) or (CO+DPZ) group; Dunnett's test.

Figure 6. Pyramidal cells death in hippocampic CA1 area 7 days after CO exposure. Pre-insult treatments with acetylcholinesterase inhibitors. (A-F) Representative microphotographs of coronal sections of Cresyl violet-stained hippocampal CA1 subfield. (G) Averaged levels of viable cells. Experimental groups included vehicle-treated non CO-exposed mice (no CO+Veh) and CO-exposed animals (CO+Veh), treated with tacrine (CO+THA), 1 mg/kg ip, rivastigmine (CO+RIVA), 1 mg/kg ip, galantamine (CO+GAL), 1 mg/kg ip, and galanthamine+BD1047 (CO+GAL+BD). (A-F) Scale bar shown in (A) = 85 µm. The number of mice used is indicated within the columns in (G), $F_{(6,25)} = 6.23$, $p < 0.001$. ** $p < 0.01$, * $p < 0.05$ *vs* (no CO+Veh) group, Dunnett's test.

Figure 7. Post-insult neuroprotective effects of igmesine (A-C) and donepezil (D-F) in CO-exposed mice. Mice were exposed three consecutive times to CO (125 ml/min, 30-40 s) at 38°C. Igmesine (0.3-3 mg/kg ip), donepezil (0.12-1 mg/kg ip) and/or BD1047 (0.5-1 mg/kg ip) were administered 1 h after the last exposure to CO. After 7 days, animals were examined for spontaneous alternation performances (A, D). On day 8 after exposure, animals were trained for passive avoidance task and retention was examined on day 9, in terms of step-through latency (B, E) and escape latency (C, F). The number of animals per group was n = 8. KW = 51.50, $p < 0.0001$ in (A); KW = 31.78, $p < 0.001$ in (B); KW = 38.07, $p < 0.0001$ in (C); KW = 34.18, $p < 0.0001$ in (D); KW = 16.18, $p < 0.05$ in (E); KW = 41.12, $p < 0.0001$ in (F). * $p < 0.05$, ** $p < 0.01$ *vs* the vehicle-treated non-CO exposed group; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ *vs* the vehicle-treated CO-exposed group; ^{o} $p < 0.05$, ^{oo} $p < 0.01$ *vs* the igmesine (1 mg/kg)- or donepezil (0.5 mg/kg)-treated CO-exposed group; Dunn’s test.

Figure 8. Post-insult neuroprotective effects of tacrine, rivastigmine and galanthamine in CO-exposed mice. Mice were exposed three consecutive times to CO (125 ml/min, 30-40 s) at 38°C. Tacrine (0.3-1 mg/kg ip), rivastigmine (0.3-1 mg/kg ip), galanthamine (0.3-1 mg/kg ip) and/or BD1047 (0.5-1 mg/kg ip) were administered 1 h after the last exposure to CO. After 7 days, animals

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were examined for spontaneous alternation performances (A). On day 8 after exposure, animals were trained for passive avoidance task and retention was examined on day 9, in terms of step-through latency (B) and escape latency (C). The number of animals per group was n = 8. KW = 38.93, $p < 0.0001$ in (A); KW = 31.58, $p < 0.001$ in (B); KW = 38.65, $p < 0.0001$ in (C). * $p < 0.05$, ** $p < 0.01$ *vs* the vehicle-treated non-CO exposed group; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ *vs* the vehicle-treated CO-exposed group; Dunn's test.

Figure 9. Pyramidal cell loss in hippocampal CA1 area, 7 days after CO exposure. Post-insult treatments with igmesine or donepezil. (A-G) Representative microphotographs of coronal Cresyl violet-stained sections of CA1 hippocampal subfield. (H) Averaged levels of viable cells. Experimental groups included vehicle-treated non CO-exposed mice (no CO+Veh) and CO-exposed animals (CO+Veh), treated with igmesine (CO+IGM), 1 mg/kg ip, donepezil (CO+DPZ), 0.5 mg/kg ip, BD1047 (CO+BD), 1 mg/kg, igmesine plus BD1047 (CO+IGM+BD), and donepezil plus BD1047 (CO+DPZ+BD). (A-G) Scale bar shown in (A) = 85 µm. The number of mice used is indicated within the columns in (H), $F_{(6,28)} = 13.17$, $p < 0.0001$. ** $p < 0.01$, * $p < 0.05$ *vs* (no CO+Veh) group; ## $p < 0.01$ *vs* (CO+Veh) group; oo $p < 0.01$ *vs* (CO+IGM) or (CO+DPZ) group; Dunnett's test.

Figure 10. Pyramidal cells death in hippocampic CA1 area 7 days after CO exposure. Post-insult treatments with acetylcholinesterase inhibitors. (A-E) Representative microphotographs of coronal sections of Cresyl violet-stained hippocampal CA1 subfield. (F) Averaged levels of viable cells. Experimental groups included vehicle-treated non CO-exposed mice (no CO+Veh) and CO-exposed animals (CO+Veh), treated with tacrine (CO+THA), 1 mg/kg ip, rivastigmine (CO+RIVA), 1 mg/kg ip, and galantamine (CO+GAL) , 1 mg/kg ip. (A-E) Scale bar shown in (A) = 85 µm. The number of mice used is indicated within the columns, $F_{(4,15)} = 3.40$, $p < 0.05$. ** $p < 0.01$, * $p < 0.05$ *vs* (no CO+Veh) group, Dunnett's test.

Table 1. Effect of acetylcholine inhibitors and σ_1 receptor ligands against CO-induced learning impairments in mice

Drug treatment	Learning and memory			Morphological examination	
	Test	Active dose (or dose range tested)	BD1047 sensitivity	cell loss protection	BD1047 sensitivity
Anti-amnesic effect					
Igmesine	SA, PA	0.3-3 mg/kg	++		
Donepezil	SA, PA	0.25-1 mg/kg	++ (SA), + (PA)		
Tacrine	SA, PA	1 mg/kg	-		
Rivastigmine	SA, PA	0.3-1 mg/kg	-		
Galanthamine	SA, PA	1 mg/kg	-		
Pre-insult neuroprotective effect					
Igmesine	SA, PA	1-3 mg/kg	++	++	++
Donepezil	SA, PA	0.25-1 mg/kg	++	++	+
Tacrine	SA, PA	0.3-1 mg/kg	-	-	
Rivastigmine	SA, PA	0.3-1 mg/kg	-	-	
Galanthamine	SA, PA	1 mg/kg	-	+	-
Post-insult neuroprotective effect					
Igmesine	SA, PA	0.3-1 mg/kg	++ (SA), + (PA)	++	++
Donepezil	SA	0.25-1 mg/kg	+	++	++
	PA	0.5-1 mg/kg	+		
Tacrine	SA, PA	(0.5-1 mg/kg)		-	
Rivastigmine	SA	1 mg/kg	-	-	
	PA	(0.3-1 mg/kg)			
Galanthamine	SA	1 mg/kg	-	-	
	PA	(0.3-1 mg/kg)			

Abbreviations used: SA, spontaneous alternation; PA, passive avoidance; -, ineffective; +, partially effective; ++, fully effective.

Figure 1

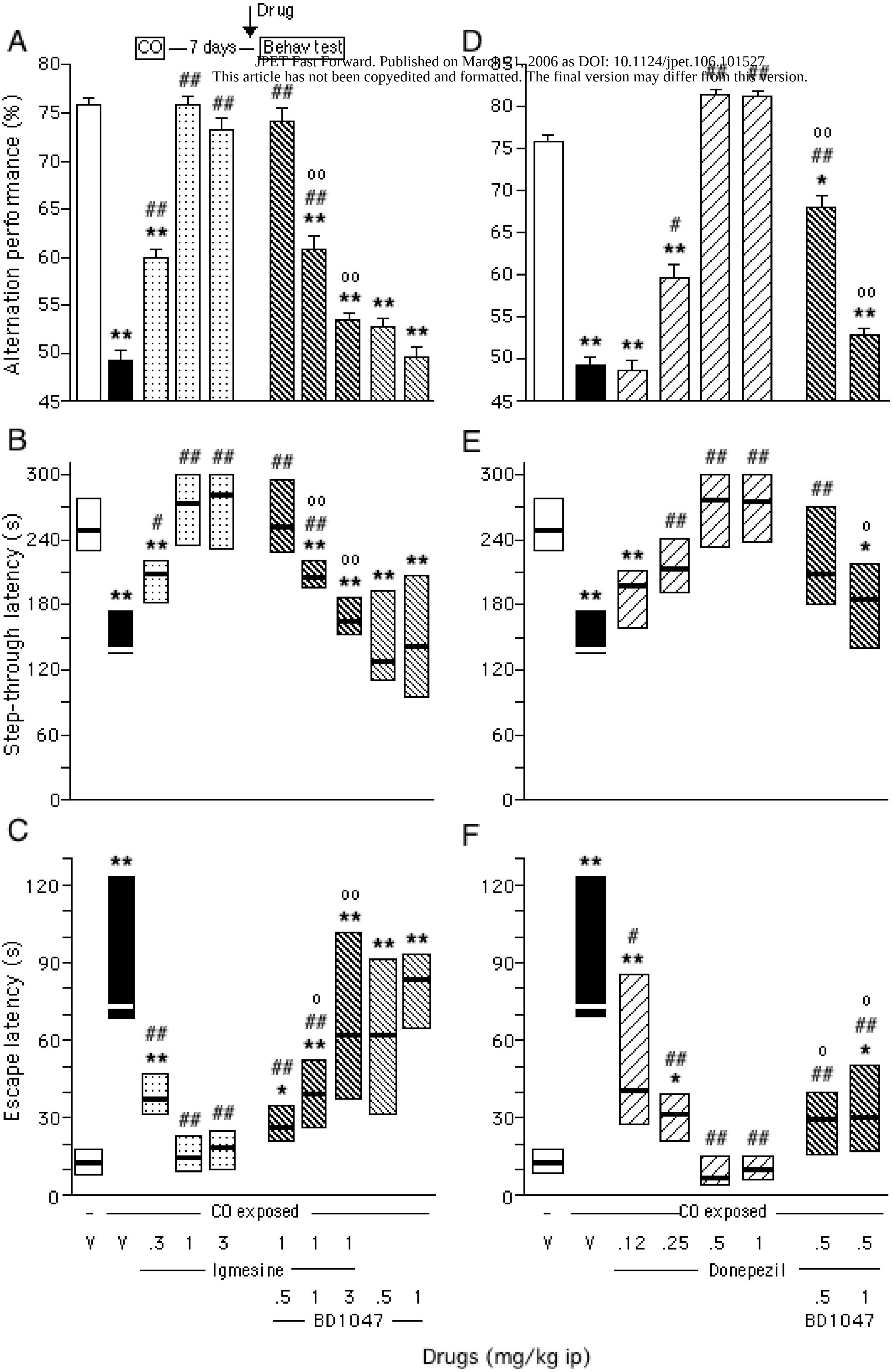
A
B
C
D
E
F
Drug
CO
7 days
Behav test
Alternation performance (%)
Step-through latency (s)
Escape latency (s)
CO exposed
Igmesine
Donepezil
BD1047
Drugs (mg/kg ip)

Figure 2

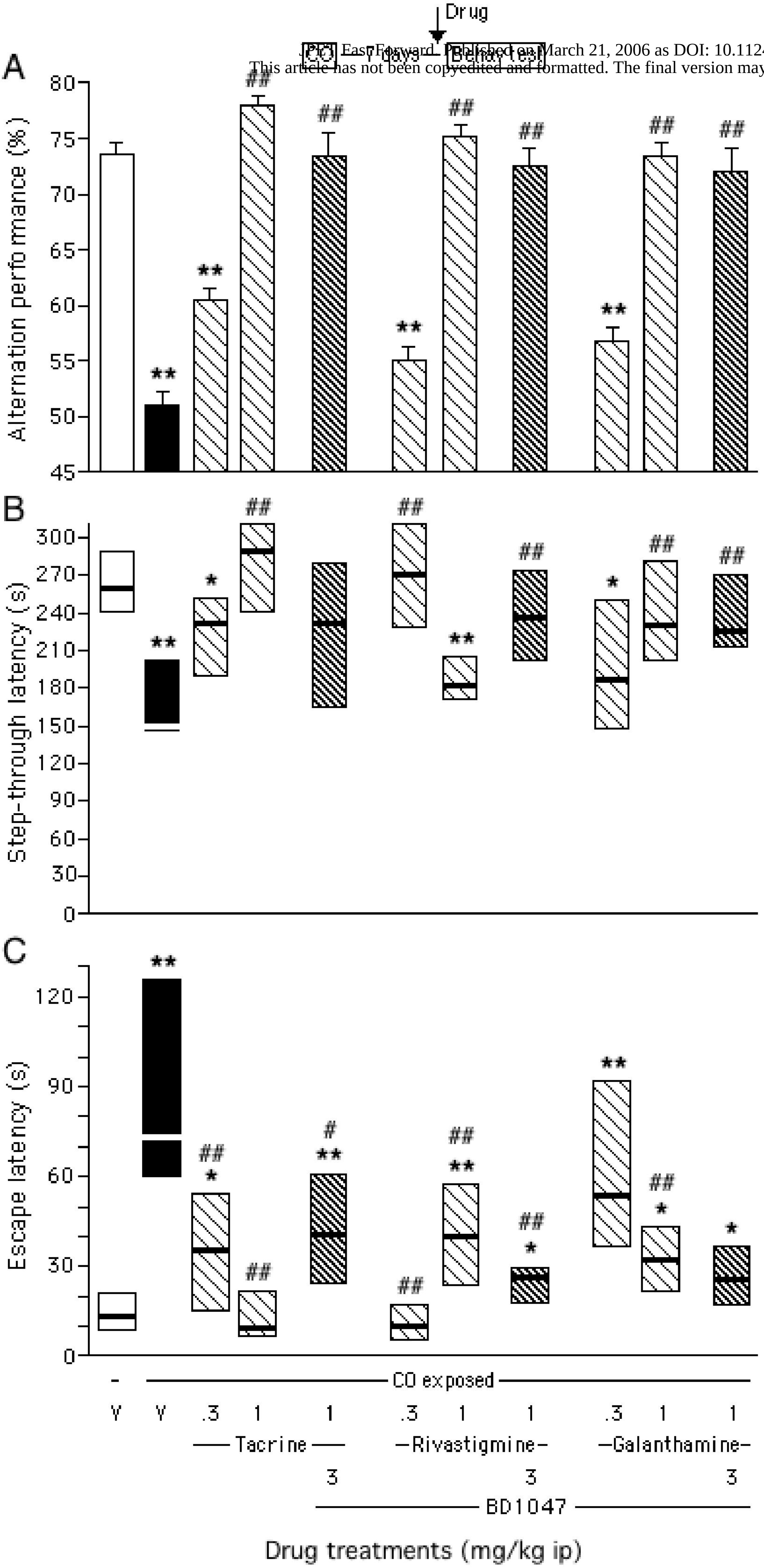
Drug
CO
7 days
Behav test
A
Alternation performance (%)
80
75
70
65
60
55
50
45
**
**
##
##
**
##
##
**
##
##
B
Step-through latency (s)
300
270
240
210
180
150
120
90
60
30
0
**
*
##
##
**
##
*
##
##
C
Escape latency (s)
120
90
60
30
0
**
##
*
##
#
**
##
##
**
##
*
**
##
*
*
-
CO exposed
V
V
.3
1
1
.3
1
1
.3
1
1
Tacrine
Rivastigmine
Galanthamine
3
3
3
BD1047
Drug treatments (mg/kg ip)

Figure 3

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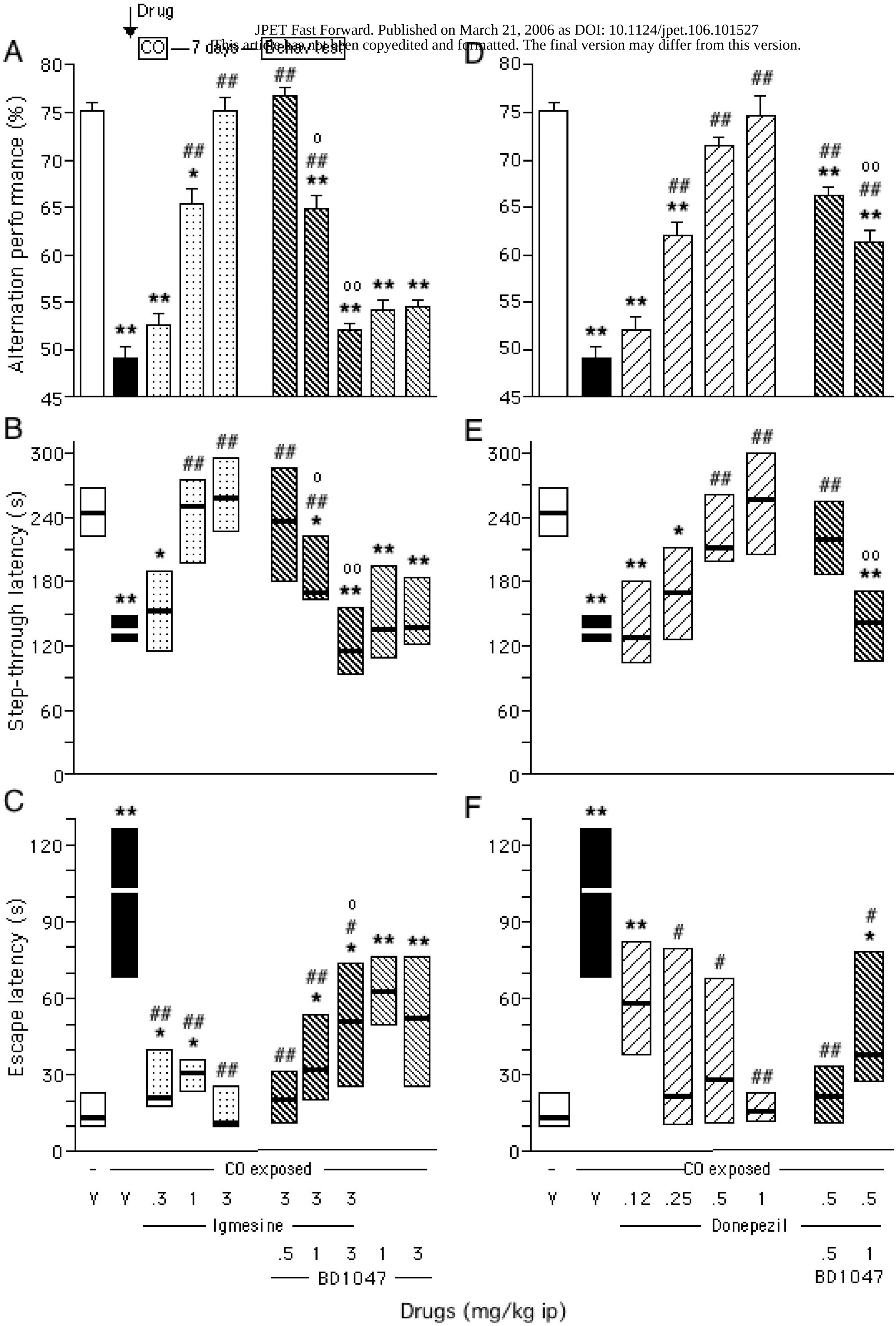
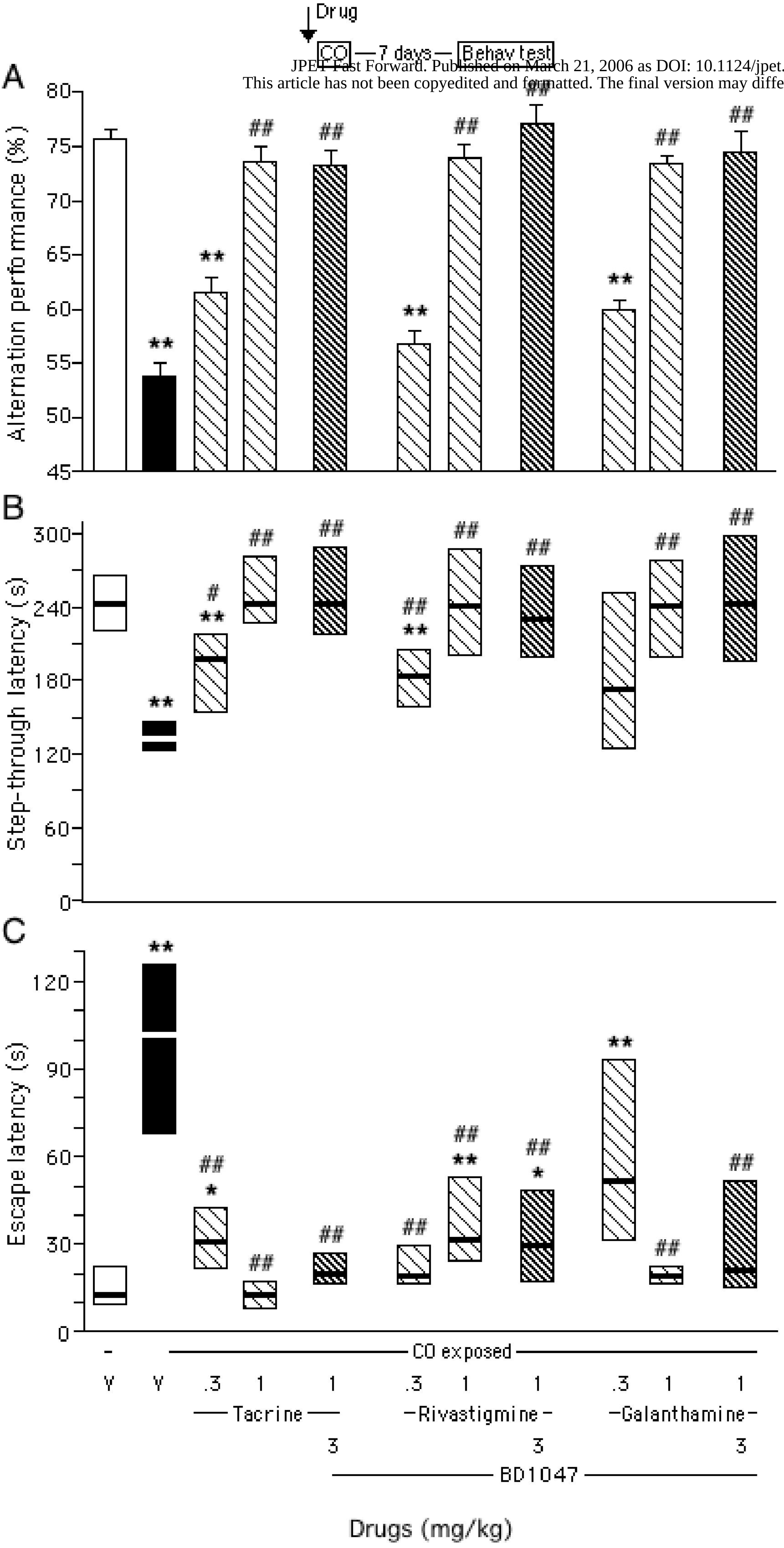
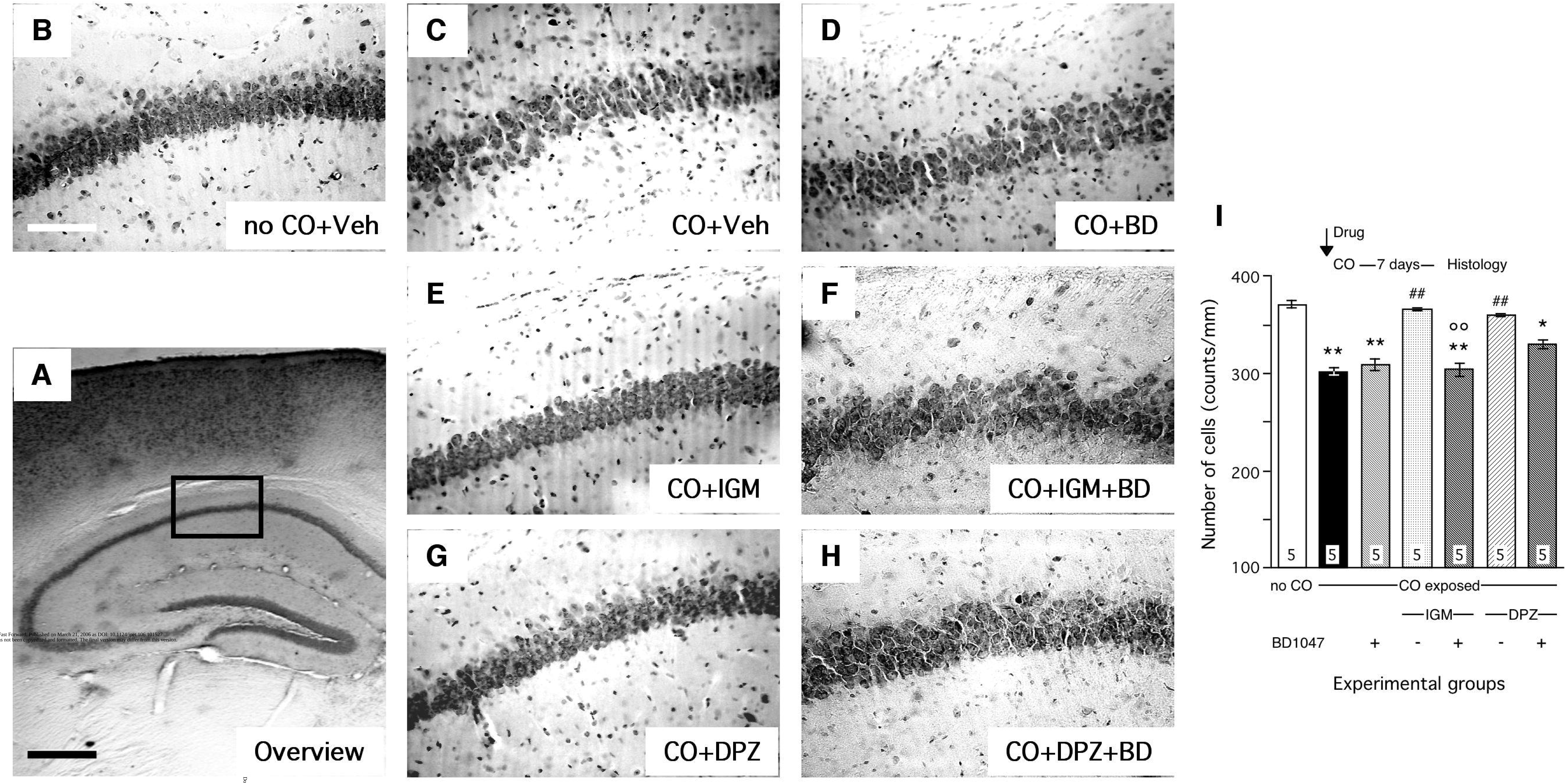


Figure 4



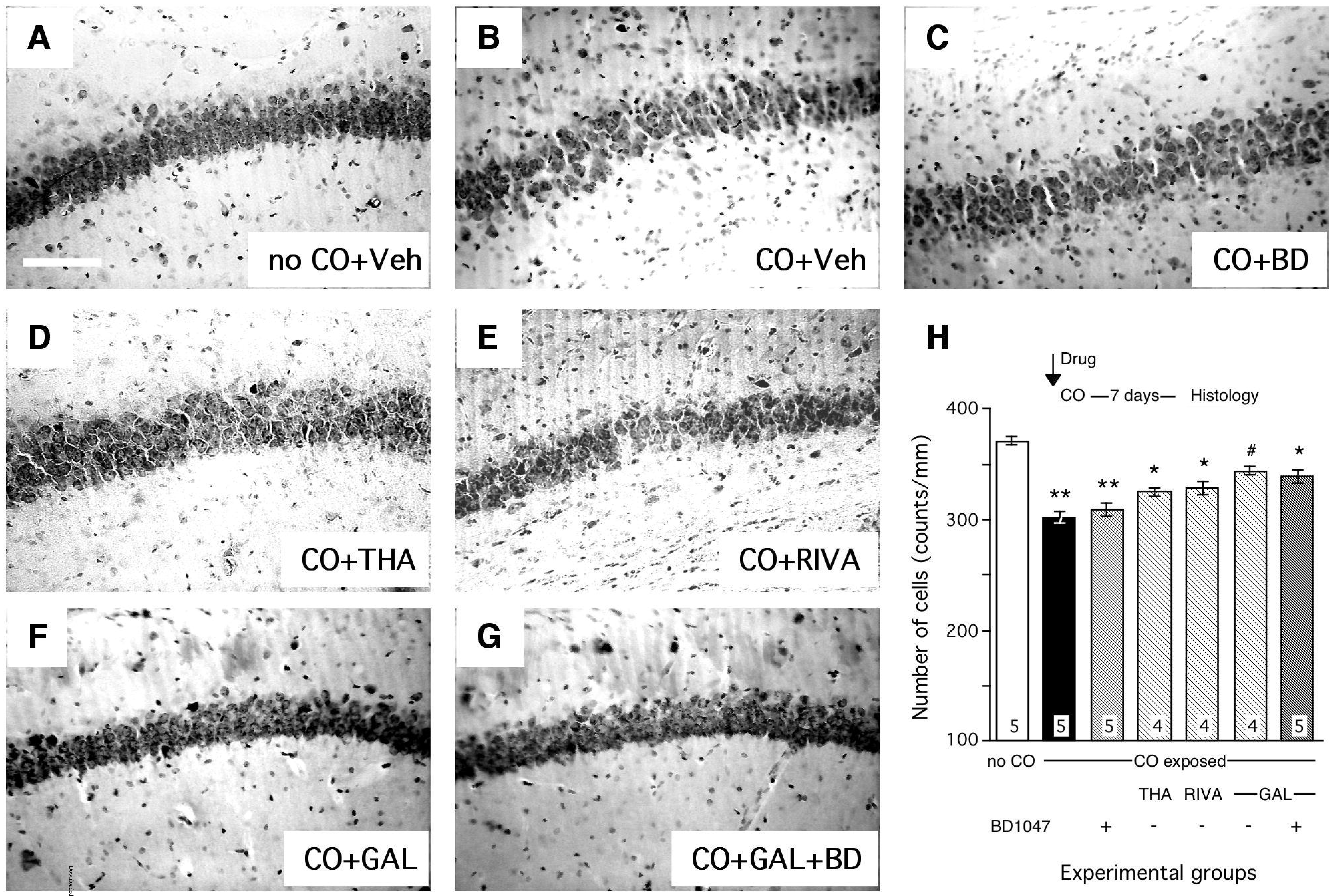
Drug
CO — 7 days — Behav test
A
Alternation performance (%)
80
75
70
65
60
55
50
45
B
Step-through latency (s)
300
240
180
120
60
0
C
Escape latency (s)
120
90
60
30
0
- CO exposed
V V .3 1 1 .3 1 1 .3 1 1
Tacrine
Rivastigmine
Galanthamine
3 3 3
BD1047
Drugs (mg/kg)

Figure 5



B
no CO+Veh
C
CO+Veh
D
CO+BD
A
Overview
E
CO+IGM
F
CO+IGM+BD
G
CO+DPZ
H
CO+DPZ+BD
I
Drug
CO —7 days— Histology
Number of cells (counts/mm)
400
300
200
100
**
**
##
°°
**
##
*
5
no CO
CO exposed
IGM
DPZ
BD1047
+
-
+
-
+
Experimental groups

Figure 6



A
no CO+Veh
B
CO+Veh
C
CO+BD
D
CO+THA
E
CO+RIVA
F
CO+GAL
G
CO+GAL+BD
H
Drug
CO —7 days— Histology
Number of cells (counts/mm)
400
300
200
100
**
**
*
*
#
*
5
5
5
4
4
4
5
no CO
CO exposed
THA
RIVA
GAL
BD1047
+
-
-
-
+
Experimental groups

Figure 7

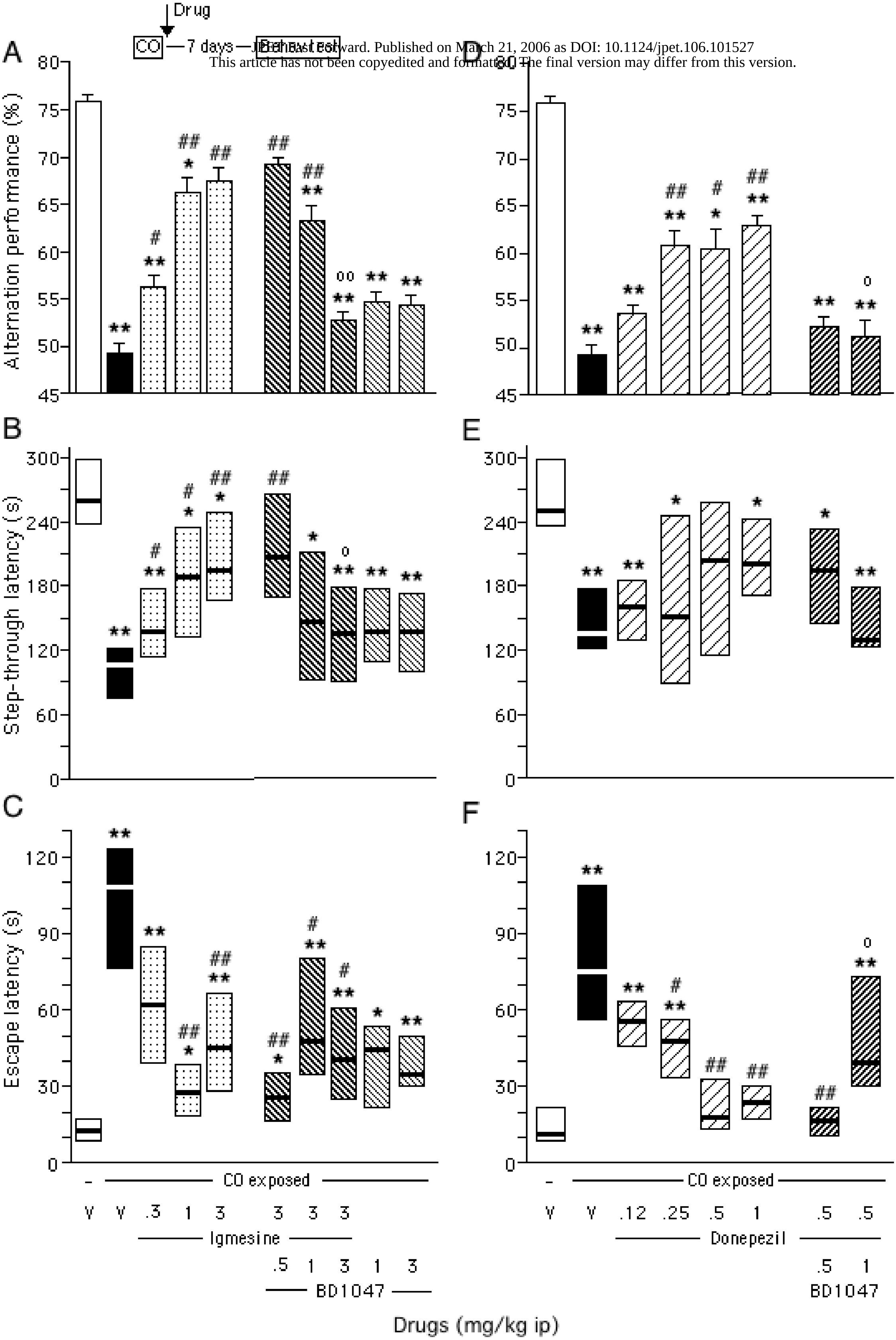

Drug
CO — 7 days — Behav test
A
D
B
E
C
F
Alternation performance (%)
Step-through latency (s)
Escape latency (s)
80
75
70
65
60
55
50
45
300
240
180
120
60
0
120
90
60
30
0
- CO exposed
V V .3 1 3 3 3 3
Igmesine
.5 1 3 1 3
BD1047
V V .12 .25 .5 1 .5 .5
Donepezil
.5 1
BD1047
Drugs (mg/kg ip)

Figure 8

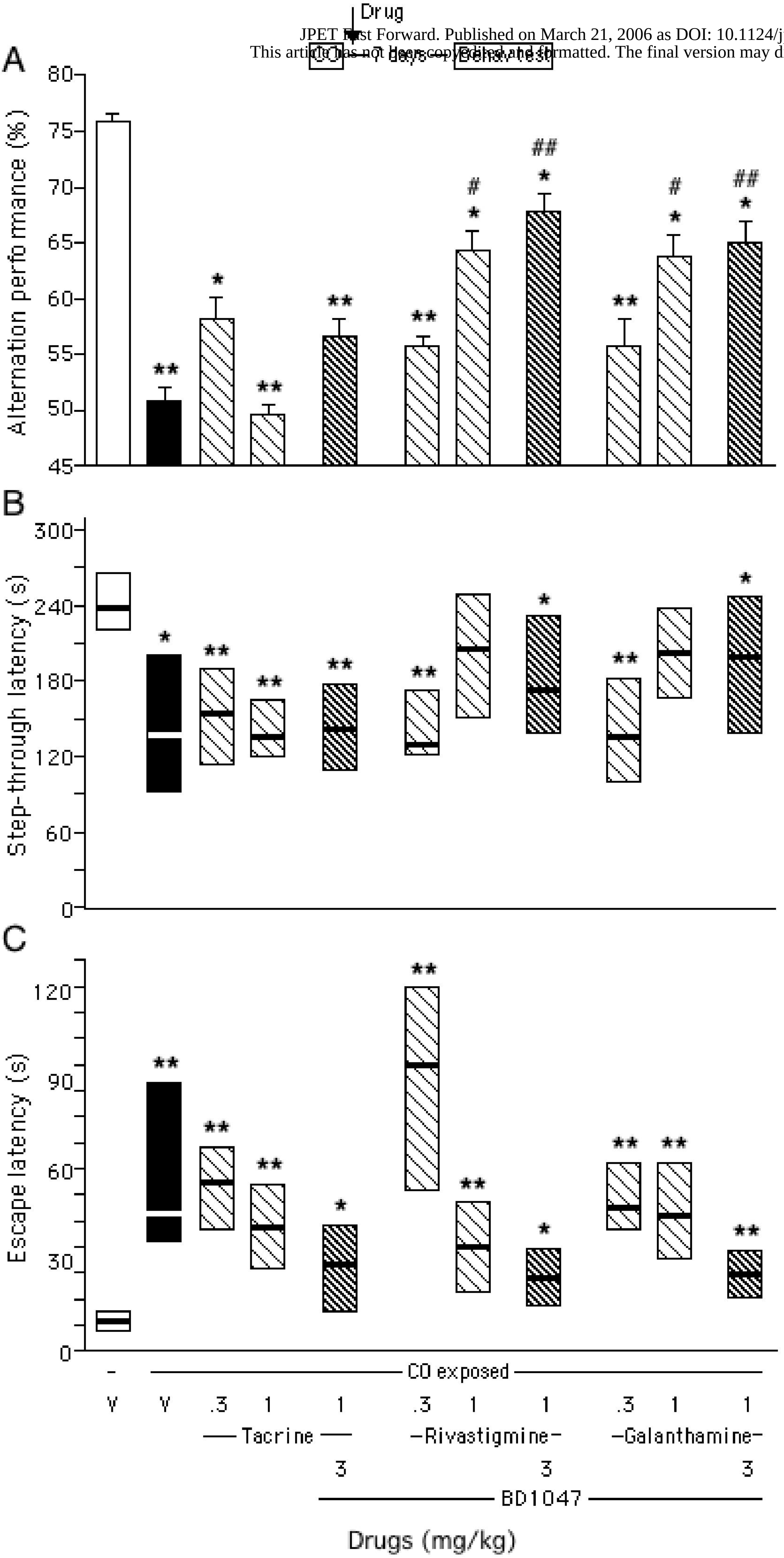
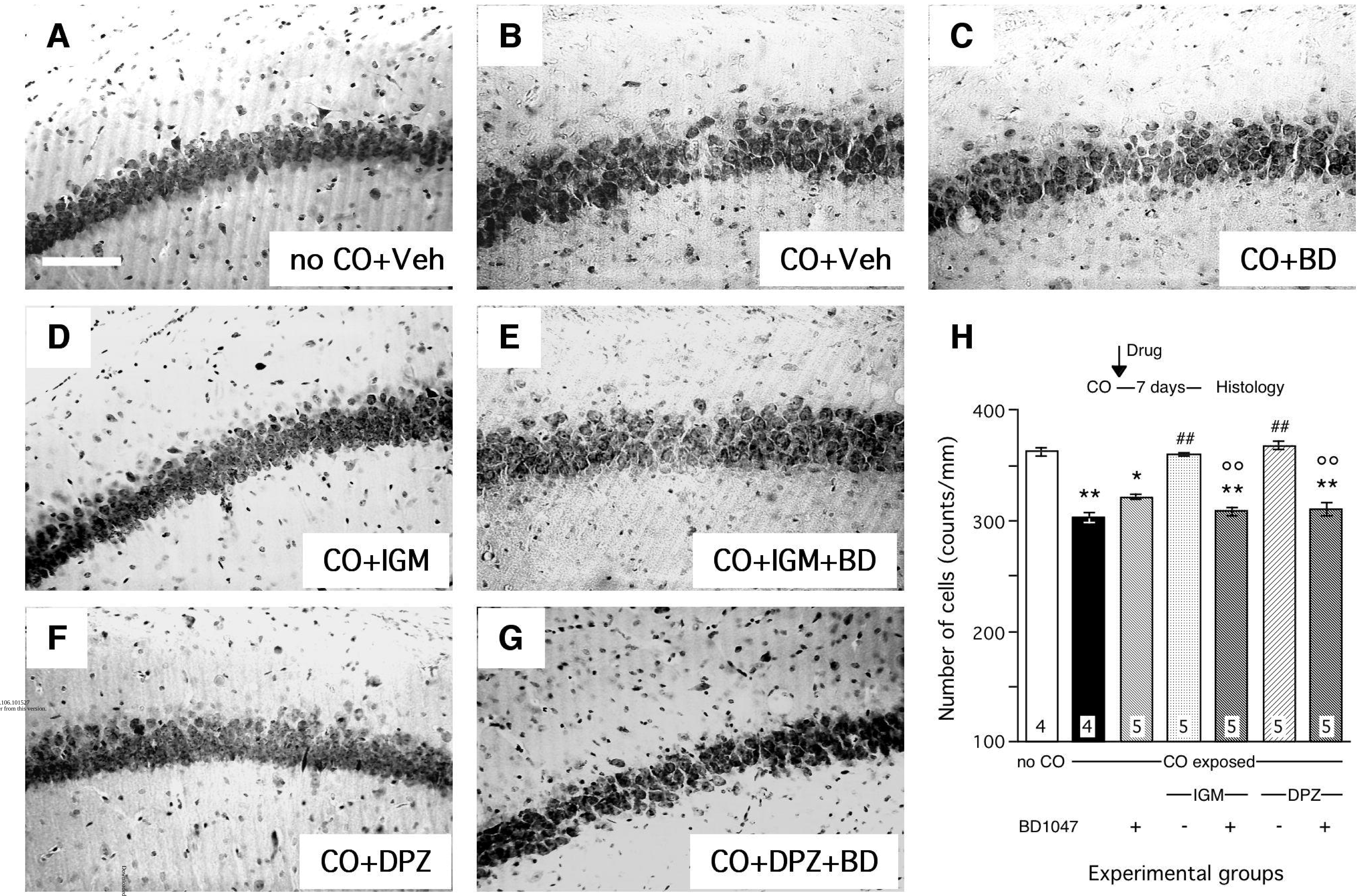

Drug
CO
7 days
Behav. test
A
Alternation performance (%)
80
75
70
65
60
55
50
45
B
Step-through latency (s)
300
240
180
120
60
0
C
Escape latency (s)
120
90
60
30
0
CO exposed
-
V
V
.3
1
1
.3
1
1
.3
1
1
Tacrine
Rivastigmine
Galanthamine
3
3
3
BD1047
Drugs (mg/kg)

Figure 9


A
no CO+Veh
B
CO+Veh
C
CO+BD
D
CO+IGM
E
CO+IGM+BD
F
CO+DPZ
G
CO+DPZ+BD
H
Drug
CO —7 days— Histology
Number of cells (counts/mm)
400
300
200
100
**
*
##
oo
**
##
oo
**
4
4
5
5
5
5
5
no CO
CO exposed
IGM
DPZ
BD1047
+
-
+
-
+
Experimental groups

JPET Fast Forward. Published on March 21, 2006 as DOI: 10.1124/jpet.106.101527

Figure 10