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Inhibitors of Cyclooxygenase-2, but not Cyclooxygenase-1 Provide Structural and Functional Protection Against Quinolinic-Acid Induced Neurodegeneration

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Running Title: COX-2 inhibitors protect against excitotoxicity

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Abbreviations: AUC, Area under the curve; CNS, Central Nervous System; COX, Cyclooxygenase; DARPP-32, Dopamine and adenosine 3'5'-monophosphate regulated phosphoprotein, 32 kilodaltons; ED_{50}, Effective dose in 50% of animals; FLURBI, Flurbiprofen; HPLC, High-pressure liquid chromatography; NeuN, Neuron specific nuclear protein; NMDA, N-methyl-d-aspartate; NSAIDS, Non-steroidal anti-inflammatories; NS-398, N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide; PG, Prostaglandins; QA, Quinolinic acid; t_{max}, Time to maximum plasma concentration; VS, Valeryl salicylate.

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ABSTRACT

Cyclooxygenases (COX) are implicated in neurodegenerative processes associated with acute and chronic neurological diseases. Given the potential utility of COX inhibitors in treating these disorders, we examined the nonselective COX inhibitor flurbiprofen, the specific COX-1 inhibitor valeryl salicylate (VS), and the COX-2 inhibitor NS-398 for their abilities to protect striatal neurons against a quinolinic acid (QA)-induced excitotoxic lesion. Rats were administered COX inhibitors 10 minutes prior to a unilateral QA lesion of the striatum, then tested 2-3 weeks later in a battery of motor tasks (bracing, placing, akinesia and apomorphine-induced rotations). Lesion volume was assessed using immunohistochemical methods 1 month after lesioning. Orally administered flurbiprofen (50 mg) was highly neuroprotective, preserving 84-99% of motor performance (ED_{50}:8.6-9.7 mg) while reducing lesion volume 75% (ED_{50}:3.2 mg). The identities of the COX isoforms associated with QA-induced neurodegeneration were determined using VS and NS-398. Oral VS was ineffective in virtually all indices of functional neuroprotection. In contrast, oral NS-398 was highly effective, preserving approximately 83% of motor performance at 2 mg (ED_{50}:0.1-0.4 mg), and reducing lesion volume 100% (ED_{50}:0.4 mg). Similar results were obtained using inhaled flurbiprofen (2 mg), which preserved 88-100% of motor performance while reducing striatal lesion size 92%. These results demonstrate that COX-2 inhibition protects neurons from acute, excitotoxic neurodegeneration. Moreover, formulating a non-selective COX inhibitor into an inhaleable preparation dramatically improves its potency in treating acute neuronal damage, a situation where the rapidity of drug delivery and onset of action is critical to clinical efficacy.

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INTRODUCTION

One of the first steps in excitotoxic neuronal damage involves the hyperstimulation of NMDA receptors (Choi, 1992) leading to a massive Ca^{2+} influx that activates, among other processes, the Ca^{2+}-dependent phospholipases A_2. These phospholipases A_2 cleave membrane phospholipids to yield arachidonic acid, which is converted by cyclooxygenases (COX, Hurley et al., 2002) into prostaglandin (PG) G_2. PGG_2 is subsequently reduced to PGH_2 with the production of a free radical intermediate that rapidly converts to a reactive hydroxyl radical (Kukreja et al., 1986). Two isoforms of COX exist, the constitutive isoform, COX-1 and an inducible isoform, COX-2. While the products of COX-1 activity exert a cytoprotective role in the periphery (Hawkey, 2001), their function in the CNS remains unclear (Zhang and Rivest, 2001, Lin et al., 2002).

COX-2 expression is constitutive in some neurons (Seibert et al., 1994), but is induced by glutamate (Manev et al., 2000) and pro-inflammatory stimuli (Bazan et al., 1994) in migratory immune cells, glia and neurons (Nogawa et al., 1997, Luo et al., 1998, Hurley et al., 2002). The resulting increase in COX-2 activity may contribute to neurodegeneration either by oxidative stress, or the neurotoxic actions of prostaglandins such as PGA_1 and PGE_1 (Kukreja et al., 1986, Bezzi et al., 1998). Increased expression of COX-2 is associated with a number of acute and chronic neurodegenerative states, including seizures, ischemia/stroke, Alzheimer's disease (Hurley et al., 2002), Parkinson's disease (Knott et al., 2000) and amyotrophic lateral sclerosis (Yasojima et al., 2001). The involvement of COX-2 in acute and chronic neurodegenerative syndromes has promoted the development of neuroprotective

treatment strategies involving COX inhibitors, such as the non-steroidal anti-inflammatory drugs (NSAIDs). While epidemiological studies suggest that NSAIDS may be protective in chronic neurodegenerative conditions (McGeer et al., 1996), little is known of their clinical efficacy in treating acute neurodegeneration. If COX inhibition blocks aspects of excitotoxicity, then neurodegenerative disorders with an excitotoxic component may benefit from this treatment. Moreover, the rapidity of onset of COX inhibition may play a crucial role in protecting neurons impacted by acute neurological insults, such as those associated with ischemia and/or trauma (Dash 2000, Strauss, 2000, Iadecola et al., 2001). This need may be served by formulations of COX inhibitors that can be delivered by the pulmonary route, allowing rapid entry of a drug into the circulation.

In an attempt to determine whether inhibition of COX activity can suppress acute neurodegeneration, we have employed an animal model of excitotoxicity (Beal et al., 1991) that allows us to investigate the relative contributions of the COX-1 and COX-2 isoforms to excitotoxic neurodegenerative processes. This was done by comparing the neuroprotective efficacy of a currently prescribed, non-selective NSAID (flurbiprofen) with specific, experimental COX-1 and COX-2 inhibitors, (valeryl salicylate and NS-398, respectively). In addition, we examined the neuroprotective efficacy of an NSAID administered using a novel pulmonary delivery system that optimizes the timeliness of delivery and while decreasing the neuroprotective dose of the drug.

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MATERIALS AND METHODS

Male Fischer 344 rats (≈ 280 grams; Taconic Farms, Germantown, NY) were used in all studies. Rats were housed in pairs in polypropylene cages with free access to food and water. The vivarium was maintained on a 12 hour light: 12 hour dark cycle (lights on at 7:00) with a room temperature of 22 ± 1°C and relative humidity level of 50 ± 5%. All studies were approved by Alkermes Institutional Animal Care and Use Committee and were conducted in compliance with the NIH Guide for the Care and Use of Laboratory Animals.

Preparation of Inhaleable Flurbiprofen. Flurbiprofen, excipients (1g/l) and ammonium bicarbonate (8 g/l) were mixed into a spray drying solution with ethanol/water 70/30 (v/v) as the solvent. The solution was then is introduced into a NIRO spray dryer at 40 ml/min and atomized into droplets with a rotary atomizer at 20,000 rpm. The droplets contact the drying gas and the dry particles collected with a 6" cyclone. The final loading density of flurbiprofen in the particles is 20%.

Drug Administration. All animals were fasted for 12 hours prior to drug administration. Rats (n=8) were administered either flurbiprofen (2, 10, or 50 mg), NS-398 (N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide, 2 or 10 mg), valeryl salicylate (2, 10, or 50 mg) by the oral route, or an inhaleable formulation of 20% flurbiprofen (FLURBI 2 mg) 10 to 15 minutes prior to injection of quinolinic acid (QA). Vehicles consisted of either blank powder for inhaleable flurbiprofen, or 1% TWEEN in distilled water for the orally administered agents. All oral drugs were delivered by gavage in a total volume of 1 ml. The inhaleable flurbiprofen formulation was administered using the following insufflation technique. Rats are anesthetized with 2%

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halothane /98% oxygen and a laryngoscope used to visualize the epiglottis. A blunt-tip insufflator (Penn Century, Philadelphia PA) containing the pre-measured dose is then inserted into the airway under visual guidance. A bolus of air (3 cc) from an attached syringe is used to deliver the powder from the chamber of the insufflator into the lungs. A second bolus of air is used to make certain that the entire dose is administered. A total of 10 mg of powder containing 2 mg of flurbiprofen was delivered to each rat.

Similar techniques were used for administering flurbiprofen for pharmacokinetic studies. Rats (N=12) were administered 5 mg of powder containing 1 mg of flurbiprofen, or were orally administered 1 mg of flurbiprofen in 1% carboxymethylcellulose solution. Blood samples (500 µl in heparinized tubes) were obtained at 0, 2, 5, 15, 30, 60, 120, 240, and 360 min after administration. Only 4 samples were taken from each rat. The blood samples were centrifuged, plasma removed then rapidly frozen and stored at -80°C until assayed.

Plasma flurbiprofen levels were assayed using HPLC with UV detection. Briefly, rat plasma samples (200 µl) were spiked with ketoprofen as an internal standard, then extracted using Waters Oasis HLB extraction cartridges. Samples were loaded onto a conditioned column, washed with 1 ml deionized water, then eluted with 1 ml methanol. The eluate was dried and reconstituted with 1 ml 60/40 0.1% trifluoroacetic acid/acetonitrile (v/v). Flurbiprofen and ketoprofen were separated using a Luna C_{18}, 5 µm, (150 mm x 3.0 mm ID, Phenomenex) column with a guard column. The column temperature was 35°C and samples were maintained at 25°C with a refrigerated autosampler. The injection volume was 5 µl and the flow rate was 0.4 ml/minute. Materials were eluted from the column with a gradient consisting of 0.1% trifluoroacetic

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acid (A) and 100% acetonitrile (B), using the following parameters. Initial conditions: 50:50; 4 min, 50:50, isocratic; 6 min, 40:60, linear gradient; 10 min, 40:60, isocratic; 11 min, 20:80, linear gradient; 13 min, 20:80, isocratic. Eluates were detected by monitoring at λ =254 nm

Surgery. Immediately following drug administration, rats were anesthetized with ketamine (25 mg/kg), xylazine (1.3 mg/kg), and acepromazine (0.25 mg/kg intramuscularly) and positioned in a stereotaxic instrument (Kopf, Tujunga CA). A midline incision was made in the scalp and a hole drilled through the skull for injection of QA (225 nmol in phosphate-buffered saline) at the following coordinates: 1.2 mm anterior, 2.6 mm lateral to bregma, and 5.5 mm ventral to the surface of the brain (Emerich, et al., 1996). QA was infused into the striatum using a 28-gauge blunt-tip syringe (Hewlett-Packard, Palo Alto CA) in a volume of 1µl over 5 minutes. The injection cannula was left in place for an additional 2 minutes to allow the QA to diffuse from the needle tip, after which the cannula was removed, the bone window waxed over and the overlying skin sutured closed. A similar procedure was followed on the contralateral side, with the exception that only vehicle was was injected. The rats were then injected with lactated Ringer's solution (10 ml subcutaneous) to prevent dehydration and allowed to recover on a heating pad. Surgery was timed so that QA was injected exactly 10 minutes after administration of COX-inhibitors.

Behavioral Testing. Four tests were used to measure unilateral motor impairment. Placement and akinesia tests (Schallert and Tillerson, 2000) were performed 27 days following QA lesions. The placement test requires holding a rat parallel to the edge of a tabletop in such a way as to allow it to place its forelimb atop

the table in response to stimulation of its whiskers by contact with the table edge. For each trial, the subjects were held with their limbs hanging unsupported, then placed with their bodies parallel to and within the distance of their whiskers (approx. 4 cm) from the edge of the table. Each rat was tested in 10 consecutive trials per forelimb and the total number of times the rat placed its forelimb on top of the table was recorded. In the akinesia test, the rat was supported on one forelimb and allowed to move independently. The number of "steps" taken with each weighted forelimb was recorded over 30 seconds. Rats were tested in the bracing task 34 days after surgery. Subjects were individually placed on a smooth stainless steel surface and gently pushed laterally a distance of 90 cm at a rate of approximately 20 cm/second. The number of braces made with the forelimb opposing the direction of movement was recorded. Each trial involved moving the rat twice on each side.

Apomorphine-induced rotations were recorded 4-weeks after surgery to further assess the extent of damage to the striatum (Carman et al., 1991). Rats were administered apomorphine (1 mg/kg SC) and then placed into a cylindrical acrylic container. Each 360-degree rotation made by the rat was counted over a 30 min trial period. Partial rotations and reversals were not recorded. Rats were tested in this paradigm once a week for 2 weeks, with the data for the third trial presented in the Results. Apomorphine-treated rats with QA-induced lesions of the striatum typically rotate 150 times/30 minutes (Nakao et al., 1998).

Histology. At the conclusion of behavioral testing, all animals were sacrificed for histological analysis. Rats were anesthetized with ketamine, xylazine, and acepromazine solution, then transcardially perfused with heparinized phosphate-

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buffered saline (5000 U/L, 20 ml, pH 7.4, 0-4°C), followed by 4% Zamboni's fixative (500 ml, 0-4°C). The brains were then removed, placed in 30% phosphate-buffered sucrose (pH 7.4) and stored (48 hrs, 0-4°C). Before sectioning, the brains were rapidly frozen in methylbutane (-60°C), mounted on a freezing microtome, and 40 µm thick sections cut and stored in a solution of 30% sucrose/30% ethylene glycol in phosphate-buffered saline at -20°C until processed for assessment of lesion size and DARPP-32 immunohistochemistry.

Immunohistochemistry: Sections were processed for the histochemical visualization of DARPP-32-like or NeuN-like immunoreactivity using biotin-labeled antibodies (Hsu et al. 1981). Endogenous peroxidases were eliminated with a 20-minute incubation in 0.1M sodium periodate in Tris-buffered saline. Background staining was suppressed with a 1 hour incubation in Tris-buffered saline (TBS: pH 7.4) containing 3% normal goat serum, 2% bovine serum albumin and 0.05% Triton-X100. The sections were then incubated in the primary antibodies, either DARPP-32 (1:25; Cell Signaling Technologies, Inc.), or NeuN (1:100, Chemicon) for 48 hours at room temperature. Following several washes, sections were sequentially incubated in the biotinylated IgG secondary antibody (Vector 1:200) for 60 minutes and the avidin-biotin (ABC "Elite") substrate (1:500) for 75 minutes. DARPP-32 immunostained sections were then reacted in a chromogen solution containing Tris-buffered saline, 0.05% 3,3' diaminobenzidine (DAB) and 0.005% H_2O_2. All sections were mounted on chrome alum-treated slides, air-dried and coverslipped with Permount^{TM}. Control sections were treated in an identical fashion except for the substitution of the primary antibody solvent or an irrelevant IgG matched to the protein concentration of the primary antibody.

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Volumetric measurements*:* Lesion volume was quantified using a point counting procedure, (Cavalieri procedure: see Gundersen and Jensen, 1987) and StereoInvestigator software (MicroBrightField, Inc.). Stereologic measures were made using a microscope (BX-60, Olympus, Tokyo, Japan), integrated with a computer-controlled 3-dimensional motorized stage (Ludl Electronic Products, Hawthorne, NY), and a high-sensitivity CCD video camera system (Hitachi, Tokyo, Japan). Every sixth section throughout the rostrocaudal extent of the NeuN-stained lesion was analyzed. The StereoInvestigator software internally calculates the total volume for each case. Because of the shrinkage of the lesioned striatum over time, we normalized the volume of the lesioned striatum (V_N) to the intact side in all animals by applying the formula $V_N = V_{Lesion} * (V_{Intact\ striatum}/V_{Lesion\ striatum})$.

Neuronal Counts: Counts of DARPP-32-immunopositive neurons were performed using the MicroBrightfield stereological software and stereologic principles (Gundersen et al., 1988). The total number of neurons was estimated by the optical fractionator method using a 100X plan-apo oil immersion objective with a 1.4 numerical aperture. For each tissue section analyzed, section thickness was assessed empirically (approximately 12.5 μm), and upper and lower "guard zones" with a 4-5μm thickness established before each series of measurements**.** The striatum was outlined under a low magnification (4X) and approximately 5% of the outlined region was analyzed using a systematic random sampling design. The total number of neurons (N) for each case was calculated using the following formula: $\Sigma N_{Total} = (N_1 + N_2 + ...N_N) * 12$. The coefficients of error (CE) were calculated according to the procedure of Gunderson and

colleagues as estimates of precision. Values of <0.10 were accepted (West et al., 1996).

Data Analysis The ED_{50} estimates of drug potency in the placement, akinesia and bracing tests were derived using non-linear regression fitting of a sigmoidal dose-response curve to the data (GraphPad Prism, GraphPad Software, San Diego CA). The ED_{50} estimates of drug potency were derived using non-linear regression fitting of a sigmoidal dose-response curve to the data, with the minimum constrained to the value obtained for the mean motor performance of the lesioned side from the vehicle-treated group, or 0 for lesion size. The maximum value of the curve was constrained to the mean level of performance by the unlesioned limb, or the size of the lesion from the vehicle-treated group. The significance of the differences between populations was assessed using multi-way ANOVA followed by Bonferroni's post hoc comparison matrix, except for the placement test, on which Kruskall-Wallis tests were performed as the nature of scoring the placement task skews the distribution.

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RESULTS

Rats receiving vehicle treatment prior to the QA lesion showed significant deficits in the performance of the placement, bracing, and akinesia tests by the limb contralateral to the lesioned side. This was evidenced by an average decrease of 94±1.1%, 31±6.9% and 56±3.1% in the number of placements, braces and steps taken, respectively, relative to the limb ipsilateral to the lesioned hemisphere. In the apomorphine-induced rotation test, all vehicle-treated animals rotated 140 ± 8.1 times in 30 minutes. Lesion volumes in vehicle-treated animals were 6.1±0.6 mm^3, and survival of DARPP-32-immunoreactive neurons was 29±7.5%**.**

Orally administered flurbiprofen dose-dependently spared rats from QA-induced decrements in the performance of a number of motor assessments (Fig. 1, Table 1). Specifically, the number of placements by the impaired limb of rats treated with 50 mg of flurbiprofen were not significantly different from the unlesioned side (Fig. 1A), constituting a 74-fold higher level of performance than observed in vehicle-treated rats. Performance in this test was also improved relative to vehicle ($p<0.05$) following 10 mg of flurbiprofen. Flurbiprofen dose-dependently protected limb function in the bracing and akinesia tests at doses of 10 and 50 mg, but not 2 mg (Fig. 1B, C). Similarly, the 10 ($p < 0.01$) and 50 mg ($p< 0.01$) doses of flurbiprofen resulted in 53% and 69% fewer apomorphine-induced rotations compared to vehicle-treated rats (Fig. 1D). Stereologic assessment of the striatum revealed that pretreatment with 2, 10 and 50 mg flurbiprofen prior to QA administration resulted in 50%, 50% and 70% decreases in lesion volume compared to vehicle treated rats ($p< 0.01$), with no significant differences between the 3

doses (Table 1, Figs 2, 3A). Further, the number of DARPP-32 immunoreactive neurons was 290%, 340% and 355% higher in the striata of rats receiving 2 ($p < 0.05$), 10 ($p < 0.01$) and 50 mg ($p < 0.01$) of flurbiprofen compared to vehicle-treated animals (Fig. 3B).

After establishing the neuroprotective efficacy of flurbiprofen in this model, we attempted to enhance its pharmacokinetics by formulating flurbiprofen into inhaleable microparticles. Comparison of the plasma pharmacokinetics of flurbiprofen (1 mg) administered through oral and pulmonary routes of delivery indicated that inhaled flurbiprofen yielded maximal plasma levels of 79 $\pm$ 9.0 to 84 $\pm$ 7.3 μg/ml (statistically indistinguishable) by 2 to 5 min after inhalation (Fig. 4). Detectable levels of inhaled flurbiprofen (9.5 $\pm$ 1.5 μg/ml) were found in the plasma as long as 6 hrs after administration, with the AUC = 9310 μg ml^{-1}min. In contrast, 1 mg of flurbiprofen administered orally reached a maximum plasma level of 3.6 $\pm$ 0.6 μg/ml by 5 min after dosing. Plasma levels six hours after oral administration were maintained at approximately 3 μg/ml, with the AUC = 1260 μg ml^{-1} min.

Administration of 2 mg of flurbiprofen via the pulmonary route fully protected limb performance as assessed in the placement task (Fig. 5A). Similar results were observed in the akinesia test (Fig. 5C) and apomorphine-induced rotations (Fig. 5D), where performance levels were not significantly different from unlesioned animals. Interestingly, the bracing task did not reveal any lesion effect in the animals administered inhaleable flurbiprofen or blank powder (Fig. 5B). Consistent with the behavioral data, the lesion volumes of rats insufflated with flurbiprofen (Figs 6C, 7A)

were 92% smaller than those in vehicle-treated animals (Figs 6A,B, 7A), and there was a 74% increase in survival of DARPP-32 immunoreactive neurons (Figs 6D-F, 7B).

In order to determine which isoform of COX was responsible for the neuroprotective efficacy of flurbiprofen, the effects of selective COX inhibitors were investigated. The COX-1 selective agent valeryl salicylate exerted no notable neuroprotective effects. Even at the highest dose tested (50 mg), limb performance was not significantly improved in either the placement or akinesia tasks (Figs 8A, C), or in the number of apomorphine-induced rotations (Fig. 8D). Only in the bracing test did the 50 mg dose of VS improve motor function to levels observed on the unlesioned side ($p < 0.05$).

In contrast, rats receiving either 2 or 10 mg of the COX-2 selective inhibitor NS-398 orally before the QA lesion showed significant sparing of limb performance in the placement task compared to vehicle-treated animals (Fig. 9A, Table 1). The 10 mg dose of NS-398 fully protected limb function on the side contralateral to the QA injection. Similar results were observed in the performance of the bracing (Fig. 9B) and akinesia tests (Fig. 9C), where rats pretreated with 10 mg of NS-398 demonstrated contralateral limb performance that was not significantly different from that of the ipsilateral limb. NS-398 also dose-dependently reduced the number of apomorphine-induced rotations relative to vehicle treated rats (Fig. 9D). Stereological analysis indicated that 2 mg of NS-398 resulted in a lesion volume 90% smaller than vehicle ($p < 0.01$), while no lesions were apparent after pretreatment with 10 mg of NS-398 (Figs 10A-C, 11A). Similarly, the 2 and 10 mg doses of NS-398 significantly increased DARPP-32 immunoreactivity compared to vehicle treatment ($p < 0.01$ Figs 10D-F, 11B).

DISCUSSION

High levels of COX-2 expression are routinely observed in association with acute and chronic neurodegenerative diseases. Excitotoxic processes appear to enhance COX-2 activity, both through increased production of substrates and by elevating COX-2 expression, thereby exacerbating neuronal damage through free-radical and prostanoid-mediated mechanisms (Kukreja et al., 1986, Kelley et al., 1999, Manev et al., 2000). Given the close association of COX-2 with neurodegenerative states, we set about comparing the relative neuroprotective efficacy of the currently prescribed, non-selective COX-1/COX-2 inhibitor flurbiprofen, as well as selective inhibitors of COX-1 and COX-2 in an animal model of acute, excitotoxic neurodegeneration.

The mixed COX-1/COX-2 inhibitor flurbiprofen was found to be a highly efficacious neuroprotectant from both histological and behavioral standpoints. COX-1 activity apparently has little involvement in excitotoxic neurodegeneration, as the COX-1 selective inhibitor VS showed little or no neuroprotective efficacy over the dose range tested, which should be sufficient to completely inhibit COX-1 activity (Bhattacharyya et al., 1995). Moreover, COX-1 does not appear to produce any neuroprotective agents in the short term (Teisman and Ferger, 2001, Zhang and Rivest, 2001, Lin et al., 2002), as inhibition of COX-1 by VS neither increased lesion size nor impaired motor performance to a greater extent than vehicle treatment. In contrast, the selective COX-2 inhibitor NS-398 was a very potent and efficacious suppressor of excitotoxic neurodegeneration *in vivo*. This was manifested not only by a significant reduction in lesion area and preservation of DARPP-32-ir neurons, but also functionally, as indicated by the preservation of rat performance in four different motor assessments. Histological and

neurobehavioral indices of neuroprotection were not precisely correlated, with evidence of motor dysfunction present despite histological evidence of relatively small lesions. This may reflect the influence of environmental factors upon behavioral performance at any given time, as well as the inability of the histological markers to distinguish between fully healthy versus functionally impaired neurons (Carman et al., 1991). Nonetheless, these data support a significant role for COX-2 in acute neurodegeneration involving excitoxic processes (Hewett et al., 2000).

The potent and significant neuroprotection offered by both NS-398 and flurbiprofen is consistent with the involvement of COX-2 with inflammation and neurodegeneration. Both mixed and COX-2-selective inhibitors suppressed neuronal damage in an MPTP-treated mouse model of Parkinson's disease (Teismann and Ferger 2001), with the COX-2 inhibitor being more potent. Similarly, NS-398 blocks LPS- and NMDA-induced neuron death *in vitro* (Hewett et al., 2000, Araki et al., 2001). While COX-2 inhibitors were acutely neuroprotective in the above studies, selective inhibition of COX-2 may present liabilities. Selective COX-2 inhibition increases chemotactic eicosanoid (leukotriene B_4) formation via 5-lipoxygenase, possibly as a compensatory response to the anti-inflammatory effects of COX-2 inhibition. COX-2 selective inhibitors also carry the burden of unwanted cardiovascular effects resulting from the unbalanced inhibition of cyclooxygenases (Cheng et al., 2002). Further, the efficacy of selective COX-2 inhibitors in treating chronic neurodegeneration has yet to be established in clinical trials, despite the apparent effectiveness of the non-selective COX inhibitors (Rogers et al.,1993, McGeer et al., 1996, Stewart et al., 1997, Veld et

al., 2000, Zandi et al., 2002). Together, these observations support the consideration of non-selective COX inhibitors for the treatment of neurodegenerative syndromes.

The promising therapeutic potential of NSAIDs for treating various neurodegenerative diseases raises the question of how to optimize the delivery method of these drugs to effectively protect neurons, particularly against acute CNS insults. For example, enhancing the rapidity of onset of an NSAID may make a critical difference in preserving neurons following acute trauma or ischemic attacks. Given that drug administration via pulmonary pathways often yields pharmacokinetics comparable to those of intravenous delivery (Vanbever et al., 1999), we created a formulation of flurbiprofen that could be administered by inhalation. Pharmacokinetic studies indicated that the C_{max} for inhaled flurbiprofen (1 mg) was 24 times higher than an equivalent oral dose. Moreover, the increase in plasma levels following pulmonary administration of flurbiprofen was too rapid to accurately resolve. While the t_{max} for oral flurbiprofen was also on the order of 5 min, the oral formulation used in this study (flurbiprofen in aqueous TWEEN) is a departure from the typical tablet or capsule, which would require more time to dissolve and be systemically absorbed (t_{max}: 0.7-2 hrs, Davies, 1995). Therefore, it appears that not only does the pulmonary delivery route allow rapid entry of flurbiprofen into the circulation, it achieves higher plasma concentrations than an equivalent, orally administered dose (Davies 1995).

The pharmacodynamic characteristics of the inhaleable flurbiprofen formulation are as dramatic as its pharmacokinetics. Inhalation of 2 mg of flurbiprofen afforded almost complete neuroprotection, as indicated by >90% retention of motor function relative to the unlesioned side, and >90% reduction of lesion volume compared to

vehicle-treated control. Moreover, 2 mg of inhaled flurbiprofen was 2-6 times more effective than the same dose administered orally, which was only slightly more efficacious from a neurobehavioral standpoint than vehicle. Indeed, oral administration of 50 mg of flurbiprofen was necessary to provide almost complete neuroprotection (66-86% preservation of behavioral and histological indices). Together, the pharmacokinetic and pharmacodynamic observations indicate that the pulmonary route of drug administration is capable of rapidly delivering an agent into the circulation and hence, the brain, while achieving higher plasma levels than oral administration. Moreover, acute, as opposed to chronic, administration of a COX-2 inhibitor in close temporal association with the onset of a neurological insult may prove to be the most effective way to minimize neuronal damage (Gilroy et al., 1998, Dash et al., 2000).

The underlying mechanisms responsible for the profound neuropreservation observed following the pulmonary administration of such small amounts of flurbiprofen remain unclear. After an acute insult to the brain, COX-2 expression increases in two phases. Initially, glutamate receptor activation rapidly increases neuronal COX-2 expression (Manev et al., 2000, Hewett et al, 2000) and the production of reactive oxygen species. In addition to suppressing necrosis, reducing free-radical damage to the mitochondria by COX-2 inhibition would reduce the probability of neuronal apoptotsis manifested many days after the initial insult (Luetjens et al., 2000). Subsequent to this initial insult, cellular inflammatory processes would increase the amount of active COX-2 available at the lesion site (Luo et al., 1998), expanding the neurodegeneration beyond the initial area impacted (Barone and Feuerstein, 1999). Based on our observations, rapid suppression of the initial, glutamate receptor

stimulated activation of COX-2 expression by inhaled flurbiprofen appears sufficient to reduce the immediate neuronal damage which would trigger subsequent neurodegeneration by inflammatory mechanisms. Therefore, an inhaleable, rapidly acting preparation of flurbiprofen holds promise as a neuroprotectant in cases where the time to achieve effective concentrations in the target organ is a critical factor, as in acute CNS insults.

In summary, the current investigation compares the efficacy of 3 different classes of COX inhibitors dispensed using two different administration modalities to an animal model of excitotoxic neurodegeneration. We demonstrate significant histological preservation and functional protection with both non-selective and COX-2 selective inhibitors, with even more robust effects achieved by using pulmonary over oral routes of administration. Thus, an inhaleable formulation of NSAID may have a significant impact on the severity of acute neurological insults, such as stroke and trauma (Hurley et al., 2002), where time is critical in establishing a therapeutically effective dose and where COX-2 activity in the CNS is the target.

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Table 1. Relative Potency of COX Inhibitors as Neuroprotectants.

Assessment	Drug	$ED_{50,}$ 95% CI (mg)
Placement Test	Flurbiprofen	9.7, 6.5-14
	Valeryl salicylate	> 50 (ND)
	NS-398	0.37, 0.26-0.51
Bracing Test	Flurbiprofen	8.6, 7.4-9.9
	Valeryl salicylate	25, 3.7-172
	NS-398	0.1, 0.01-1.1
Akinesia Test	Flurbiprofen	9.5, 6.5-13.9
	Valeryl salicylate	> 50 (ND)
	NS-398	0.13, 0.12-0.13
Apomorphine-Induced Rotations	Flurbiprofen	11, 4.6-27
	Valeryl salicylate	41, 10-165
	NS-398	1.6, 0.01-30
Lesion Volume	Flurbiprofen	3.2, 0.23-46
	NS-398	0.42, 0.31-0.56
DARPP-32 Immunoreactivity	Flurbiprofen	1.2, 0.14-8.9
	NS-398	0.8, 0.3-2.3

The ED_{50} estimates of drug potency were derived using non-linear regression fitting of a sigmoidal dose-response curve to the data, with the minimum constrained to the value obtained for the mean performance of the lesioned side from the vehicle-treated group, or 0 for lesion size. The maximum value of the curve was constrained to the mean performance of the unlesioned limb, or of the vehicle-treated lesion size. ND: Could not be determined.

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Figure Legends

Fig. 1. Pretreatment with oral flurbiprofen (FLURBI) suppresses the deterioration of motor performance scores in rats receiving QA lesions. Rats were administered vehicle (Veh) or FLURBI (2, 10, or 50 mg, PO) 10 min prior to receiving unilateral QA lesions of the striatum. Data represent the mean ± SEM of motor performance scores of rats (n=8) in the placement test (A), bracing test (B), akinesia test (C), and apomorphine-induced rotations (D). Performance of the impaired limb (■, contralateral to brain side receiving lesion) is compared to that of the unimpaired limb (□, ipsilateral to lesion) in A, B, and C. Rotation scores are compared to vehicle group in D. *,**: Performance is significantly different from corresponding unimpaired limb, $p<0.05$, 0.01, respectively; or ^{a,aa}: vehicle control group, $p<0.05$, 0.01, respectively, 2-way ANOVA + Tukey's test (A-C), 1 way ANOVA + Dunnet's test (D).

Fig. 2. Photomicrographs of NeuN immunoreactive neurons in the rat striatum. Note the almost complete loss of NeuN-positive neurons in the section from a rat receiving a unilateral QA injection and vehicle treatment (A, E). In contrast, substantial neuroprotection is seen in QA lesioned rats receiving 2 (B, F), 10 (C, G), or 50 (D, H) mg of oral flurbiprofen. The high magnification photomicrographs in panels E-H were taken from the corresponding areas enclosed by the squares illustrated in panels A-D. Scale bar in E: 2500 μm for A, B, C, and D; 125 μm for E, F, G, and H.

Fig. 3. Histological indices of QA-induced neurodegeneration are reduced in rats treated with oral flurbiprofen. Data represent the mean ± SEM of NeuN immunoreactive lesion

volume (A) and DARPP-32 immunoreactivity (B) of rats (n=10) treated with vehicle (Veh) or flurbiprofen (2, 10, or 50 mg, PO) 10 min prior to receiving unilateral QA lesions of the striatum. *,**: Indices are significantly different from vehicle group, $p<0.05$, 0.01, respectively, 1-way ANOVA + Dunnet's test. Dashed line equals control level.

Fig. 4. Plasma pharmacokinetics of flurbiprofen (1 mg) administered by oral (□) and pulmonary (■) routes. Plasma samples were taken at 0, 2, 5, 15, 30, 60, 120, 240, 360 min after administration, then analyzed by HPLC. The t_{max} for inhaled flurbiprofen was between 2 and 5 min after administration, with a C_{max} of 84 ± 7.3 μg/ml. The t_{max} for orally administered flurbiprofen was 5 min and the C_{max} was 3.6 ± 0.6 μg/ml.

Fig. 5. An inhaleable formulation of flurbiprofen potently protects the motor performance scores of rats receiving QA lesions. Groups of rats received no treatment (Lesion only), or were insufflated with either the inhalant vehicle (Veh) or an inhalant formulation containing 2 mg of flurbiprofen 10 min prior to receiving unilateral lesions of the striatum with QA. Data represent the mean ± SEM of motor performance scores of rats (n=8) in the placement test (A), bracing test (B), akinesia test (C), and apomorphine-induced rotations (D). Performance of the impaired limb (■, contralateral to brain side receiving lesion) is compared to that of the unimpaired limb (□, ipsilateral to lesion) in A, B, and C. Rotation scores are compared to vehicle group in D. *,**: Performance is significantly different from corresponding unimpaired limb, $p<0.05$, 0.01, respectively, or ^{a}, ^{aa}: vehicle/lesion groups, $p<0.05$, 0.01, respectively, 2-way ANOVA + Tukey's test (A-C), 1 way ANOVA + Dunnet's test (D).

Fig. 6. Photomicrographs of NeuN- (left panels) and DARPP-32-(right panels) immunoreactive neurons in the striata of rats receiving unilateral QA injections. The high power photomicrographs (insert) are from the area enclosed in the square in the corresponding panels. Sections from animals who received either no additional treatment (A, D) or vehicle (B, E) prior to QA administration showed a significant depletion of immunoreactive neurons (a-e). In contrast, pre-treatment with 2 mg flurbiprofen (FLURBI) administered via pulmonary insufflation provided significant neuroprotection against QA-induced excitotoxic damage (c, f). Scale bar (panel F): 1.75 mm (Panels A-F), 85 μm (a-f).

Fig. 7. Histological indices of QA-induced neurodegeneration are reduced in rats treated with an inhaleable formulation of flurbiprofen. Data represent the mean ± SEM of the lesion volume of rats (n=8) that received only QA, or were insufflated with either the inhalant vehicle (Veh) or an inhalant formulation containing 2 mg of flurbiprofen (FLURBI) 10 min prior to receiving unilateral lesions of the striatum with QA. *,**: Indices are significantly different from vehicle group, $p<0.05$, 0.01, respectively, 1-way ANOVA + Tukey's test. Dashed line equals control level.

Fig. 8. Orally administered valeryl salicylate is less effective than flurbiprofen or NS-398 in mitigating the motor performance deficits of rats receiving QA lesions. Rats received either vehicle (Veh) or valeryl salicylate (2, 10 or 50 mg, PO) 10 min prior to receiving unilateral lesions of the striatum with QA. Data represent the mean ± SEM of motor performance scores of rats (n=8) in the placement test (A), bracing test (B), akinesia

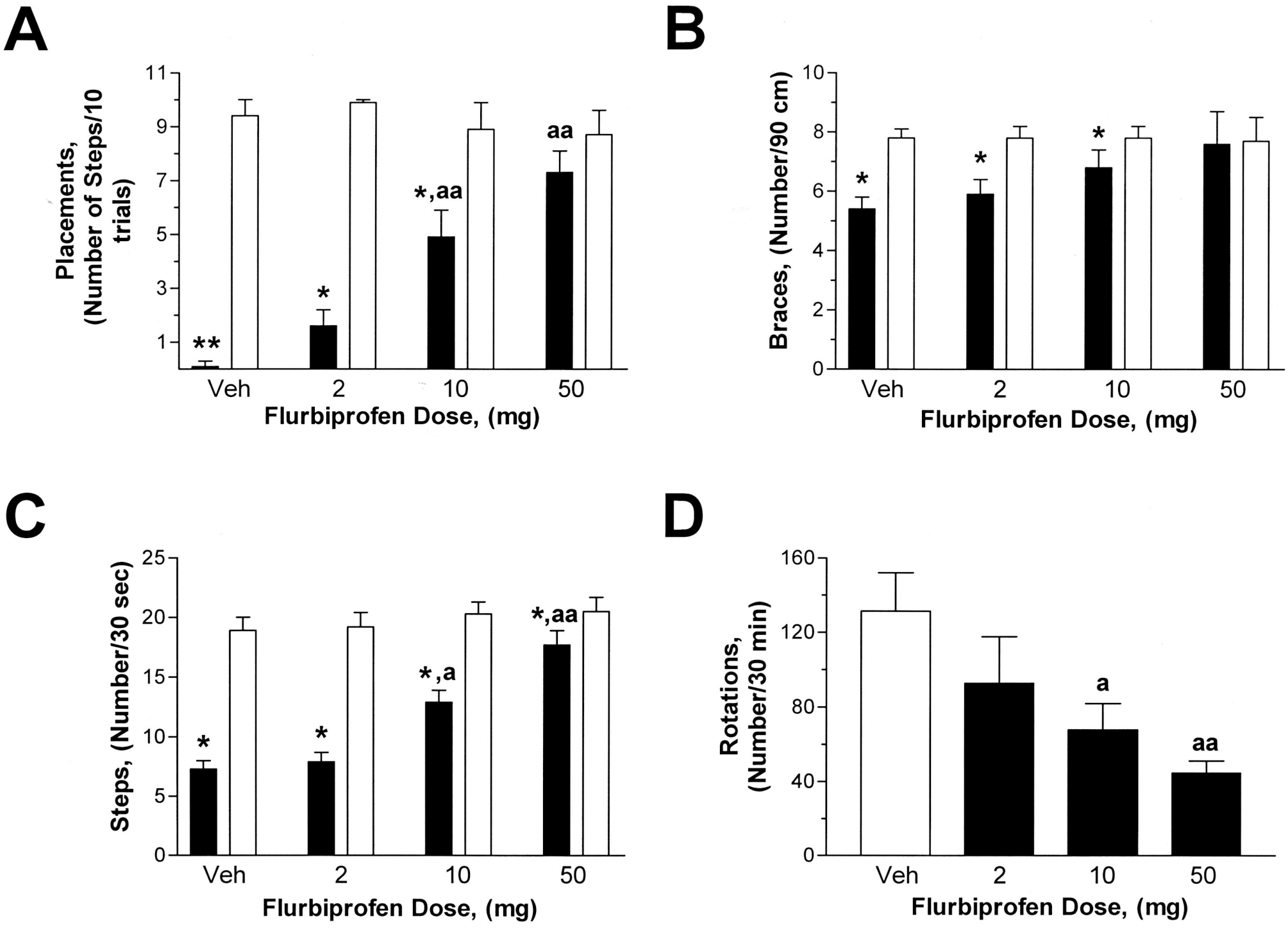
test (C), and apomorphine-induced rotations (D). Performance of the impaired limb (■, contralateral to brain side receiving lesion) is compared to that of the unimpaired limb (□, ipsilateral to lesion) in A, B, and C. Rotation scores are compared to vehicle group in D. *,**: Performance is significantly different from corresponding unimpaired limb, $p<0.05$, 0.01, 2-way ANOVA + Tukey's test.

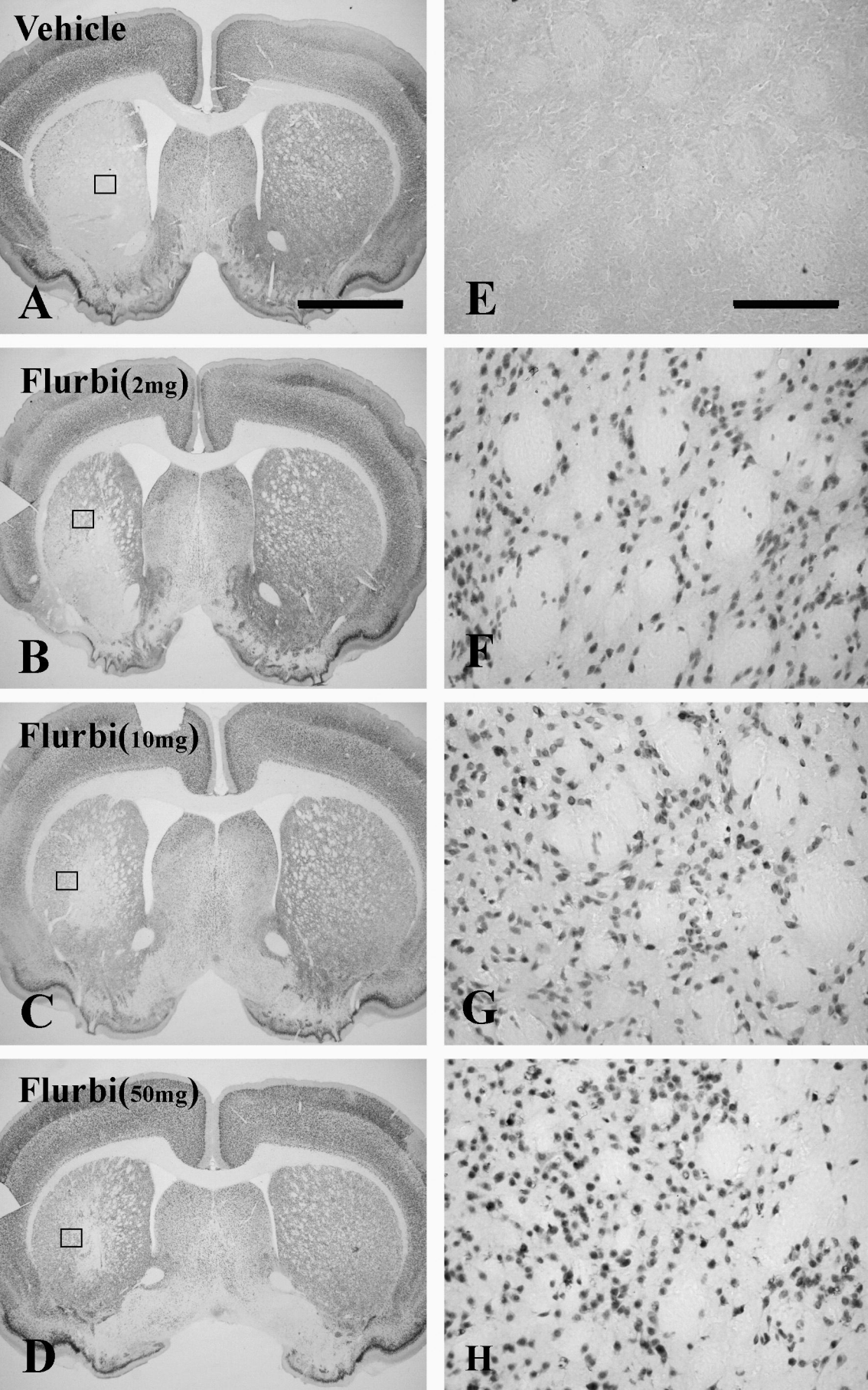
Fig. 9. Orally administered NS-398 improves the motor performance scores of rats receiving QA lesions. Rats received either vehicle (Veh) or NS-398 (2 or 10 mg, PO) 10 min prior to receiving unilateral lesions of the striatum with QA. Data represent the mean ± SEM of motor performance scores of rats (n=8) in the placement test (A), bracing test (B), akinesia test (C), and apomorphine-induced rotations (D). Performance of the impaired limb (■, contralateral to brain side receiving lesion) is compared to that of the unimpaired limb (□, ipsilateral to lesion) in A, B, and C. Rotation scores are compared to vehicle group in D. *,**: Performance is significantly different from corresponding unimpaired limb, $p<0.05$, 0.01, respectively; or [a], vehicle control group, $p<0.01$, 2-way ANOVA + Tukey's test (A-C), 1 way ANOVA + Dunnet's test (D).

Fig. 10 Photomicrographs of NeuN-immunoreactive neurons in the rat striatum. The high magnification photomicrographs in panels E-H were taken from the areas enclosed by the squares illustrated in the corresponding panels A-D. Note the almost complete loss of NeuN-positive neurons in the section from a rat treated with vehicle prior to receiving a unilateral QA injection (A, D). In contrast, almost complete neuroprotection

is seen in QA-lesioned rats receiving 2 (B, E) or 10 (C, F) mg of oral NS-398. Scale bar in panel D = 2200 µm for panels A, B and C; 110 µm for panels B, D, and F.

Fig. 11 Histological indices of QA-induced neurodegeneration are reduced in rats treated with orally administered NS-398. Data represent the mean ± SEM of lesion volume (A) and DARPP-32 immunoreactivity (B) of rats (n=10) administered either vehicle or NS-398 (2, 10 mg) 10 min prior to receiving unilateral lesions of the striatum with QA. *,**: Indices are significantly different from the vehicle control group, $p<0.05$, 0.01, 2-way ANOVA + Dunnet's test. Dashed line equals control level.

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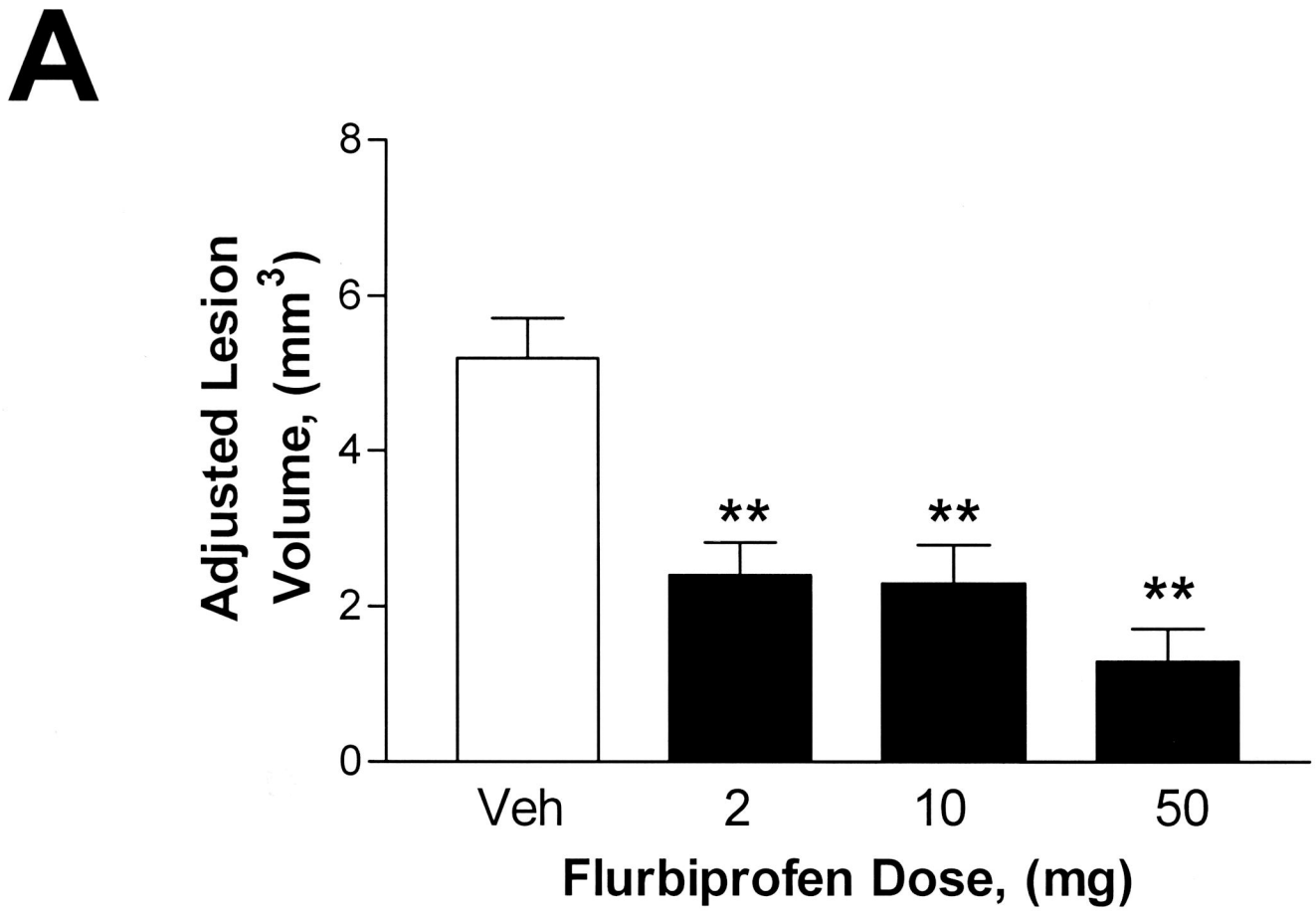

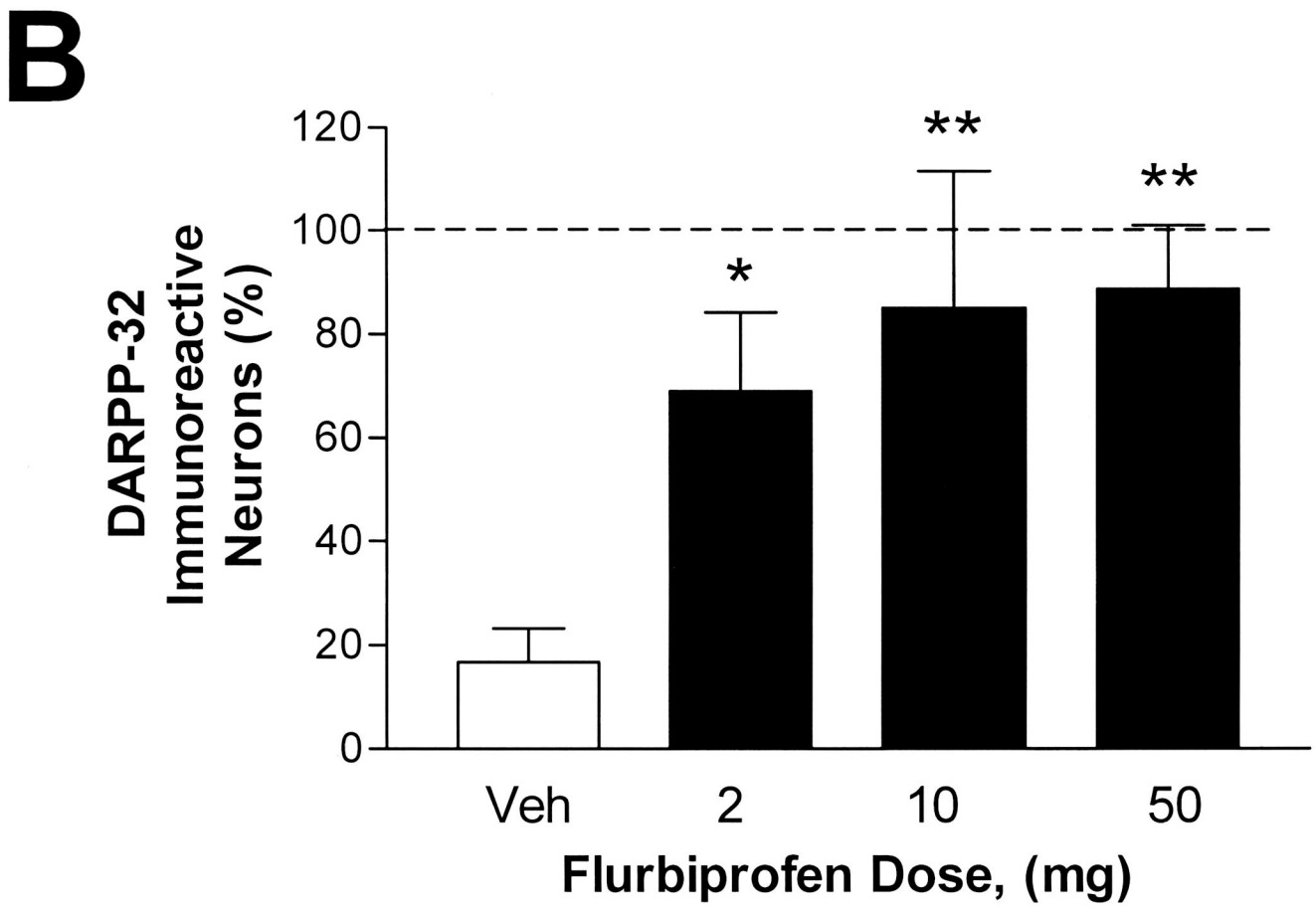

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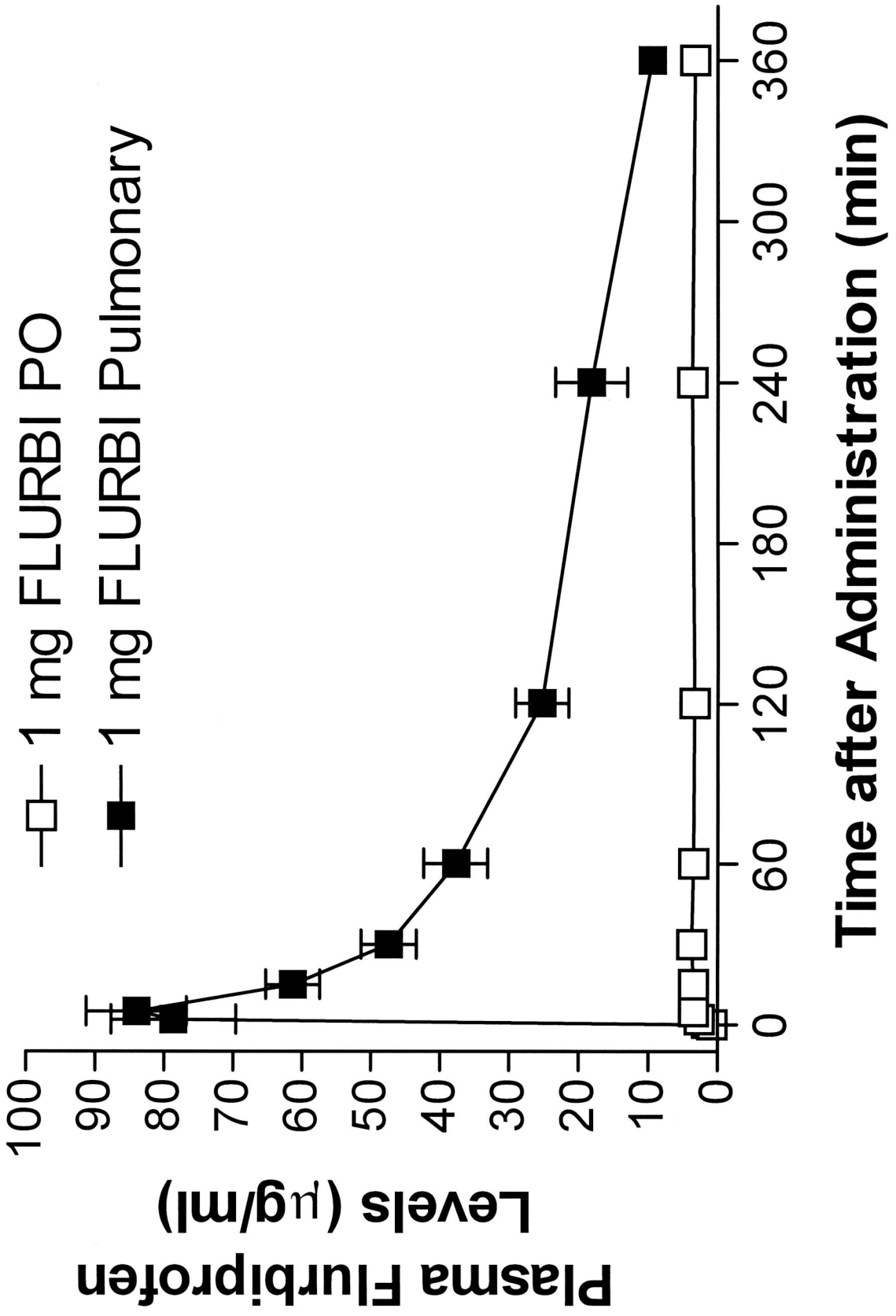

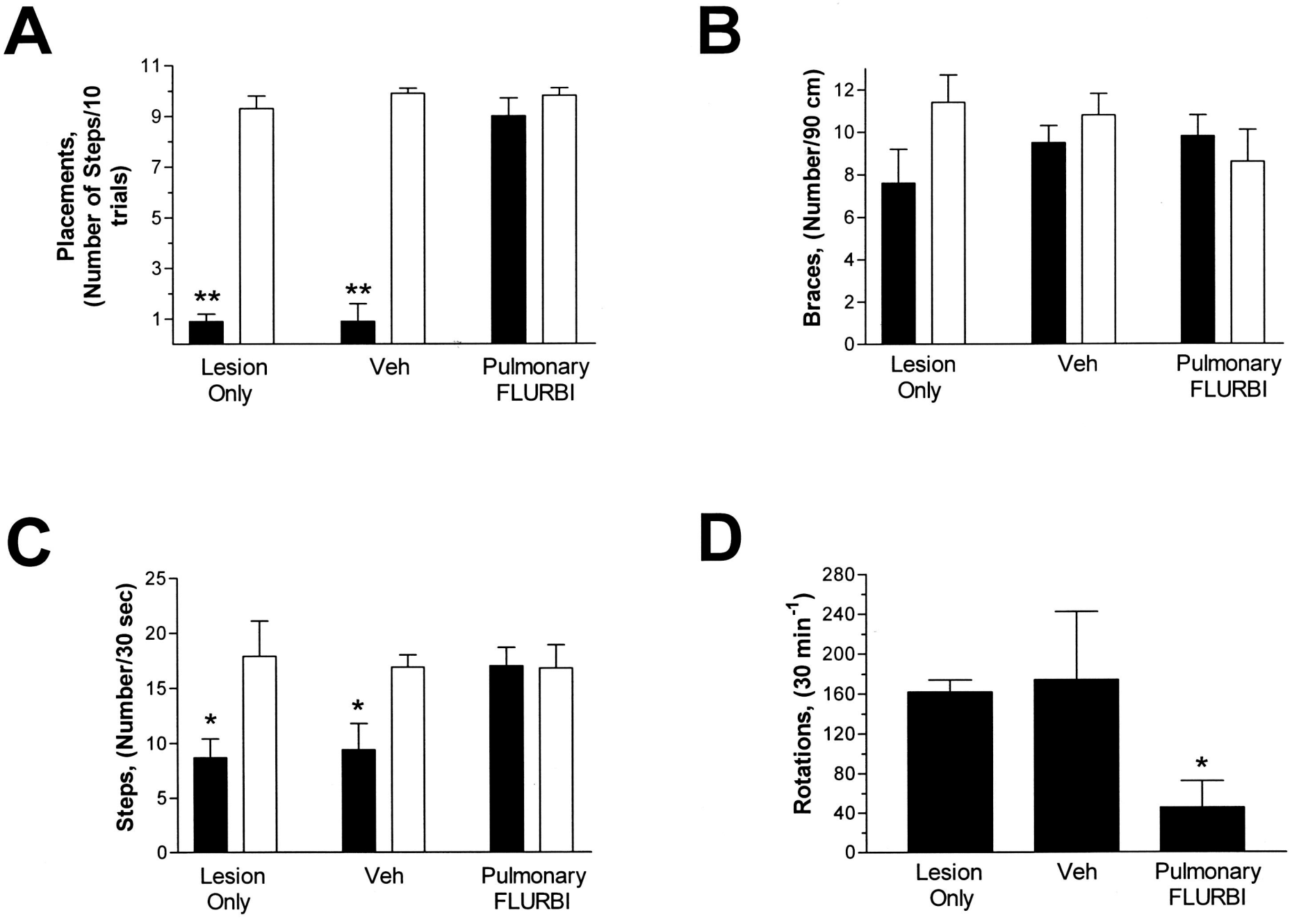

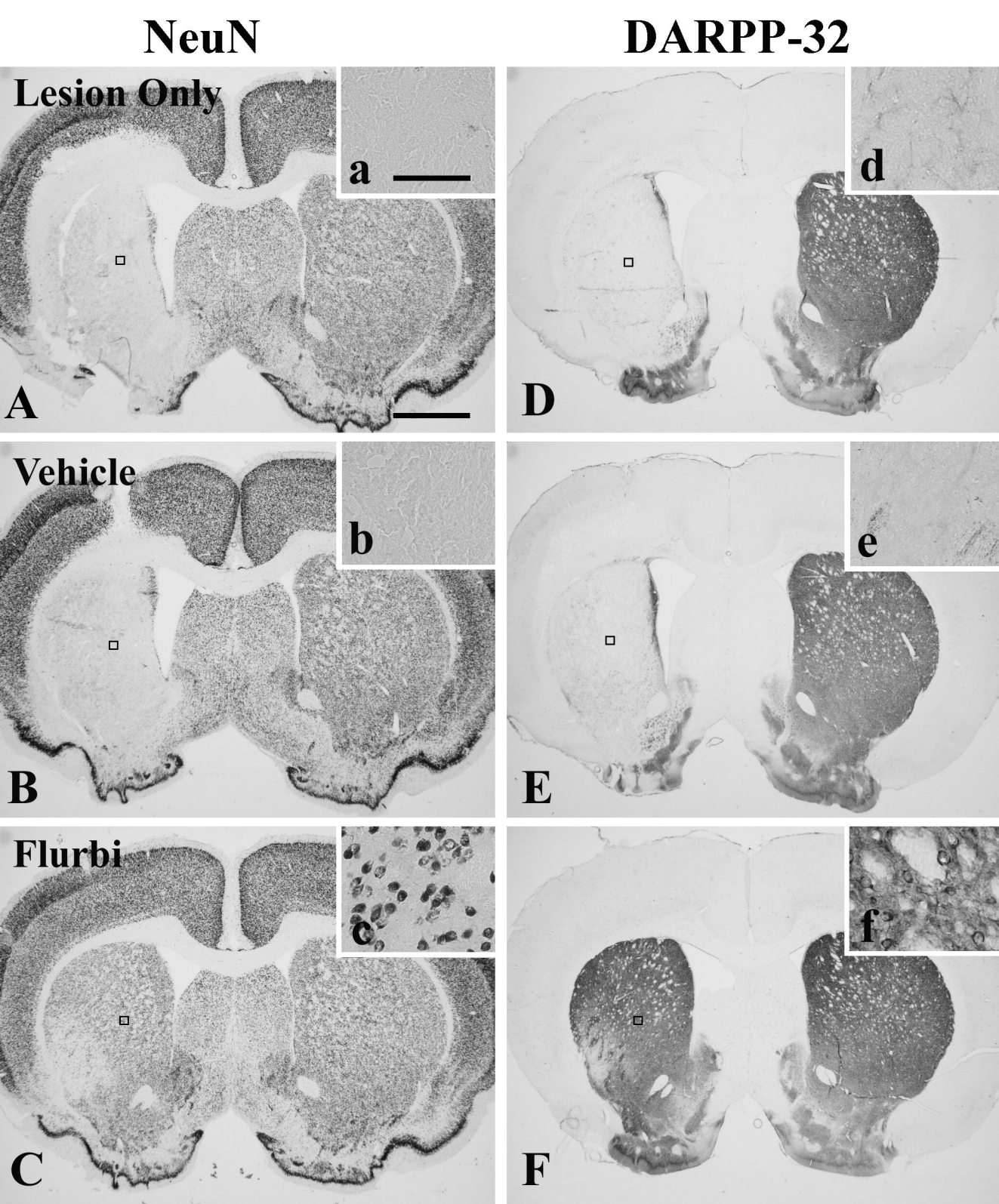
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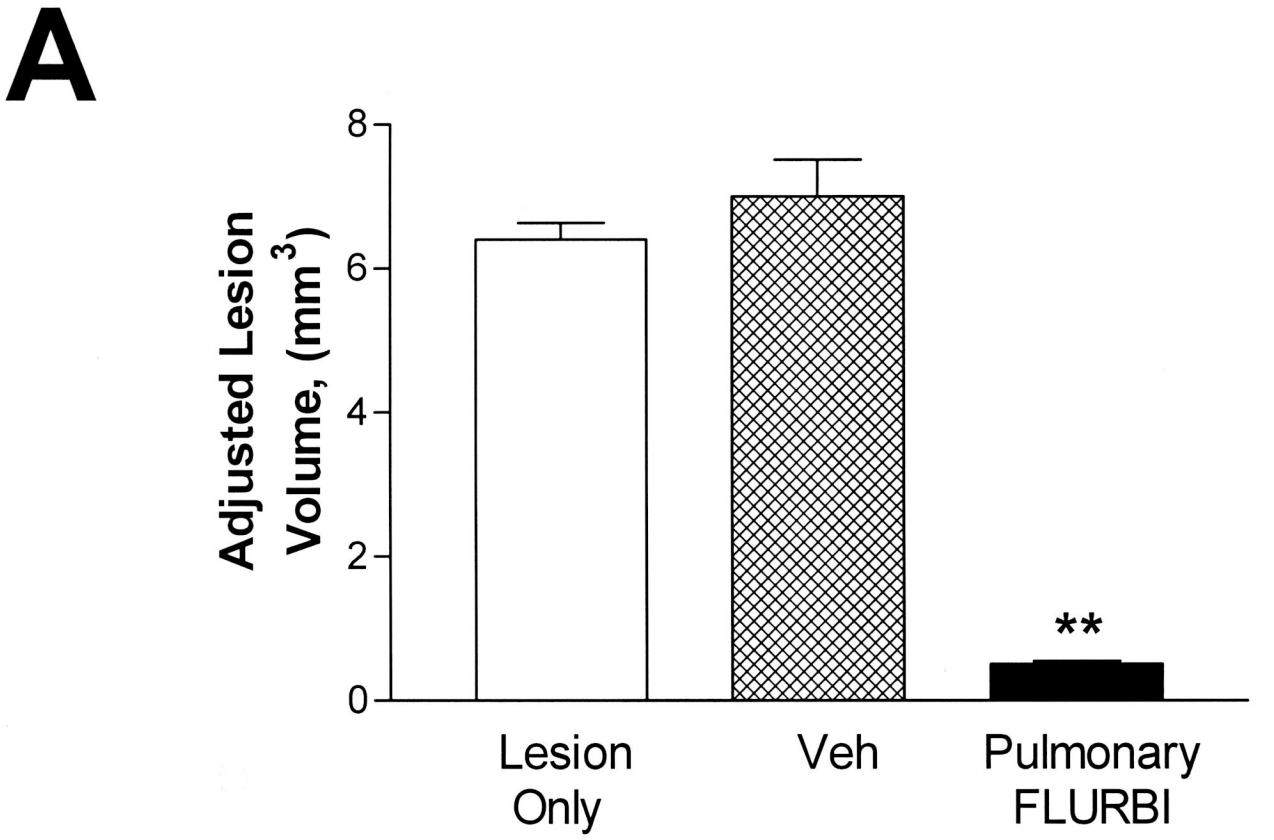

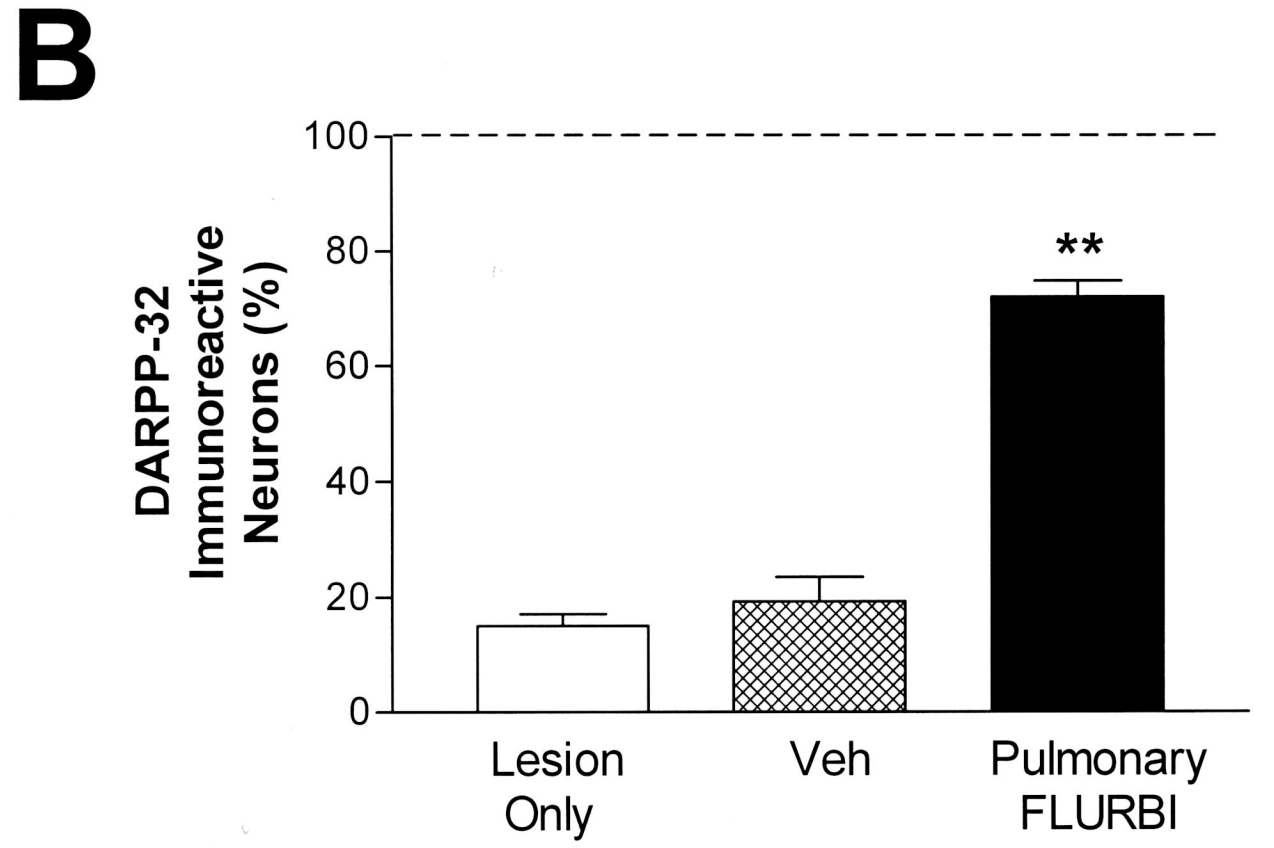

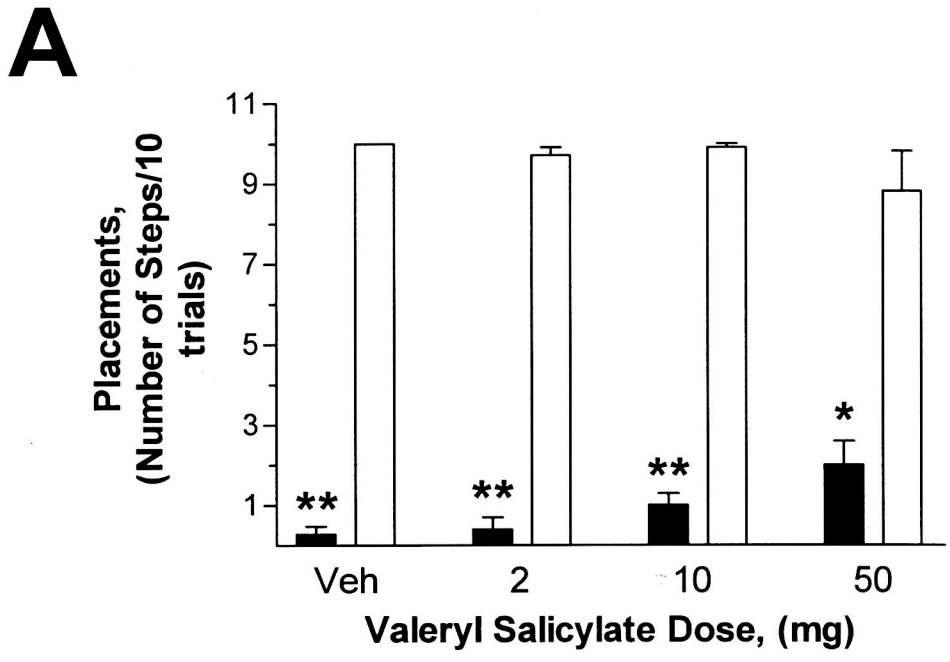

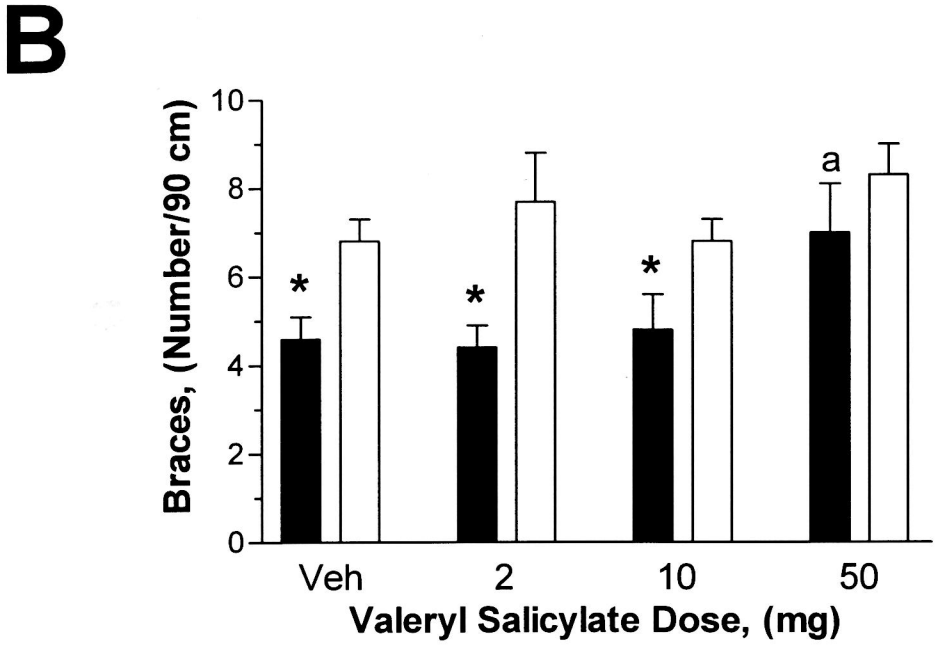

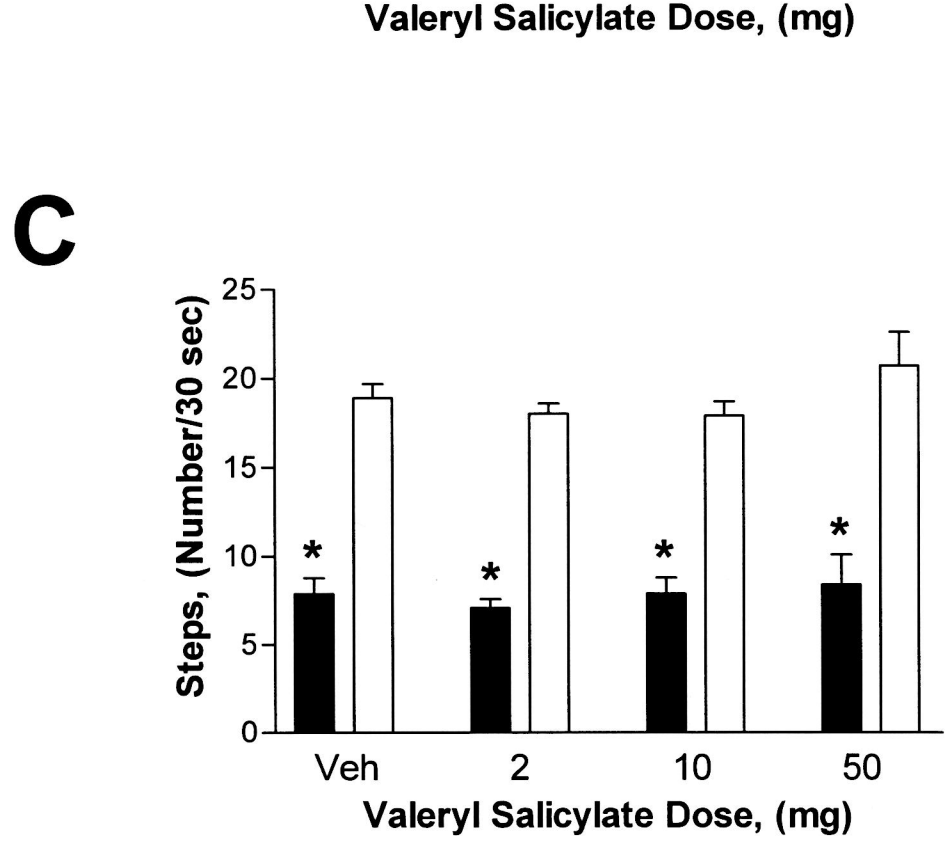

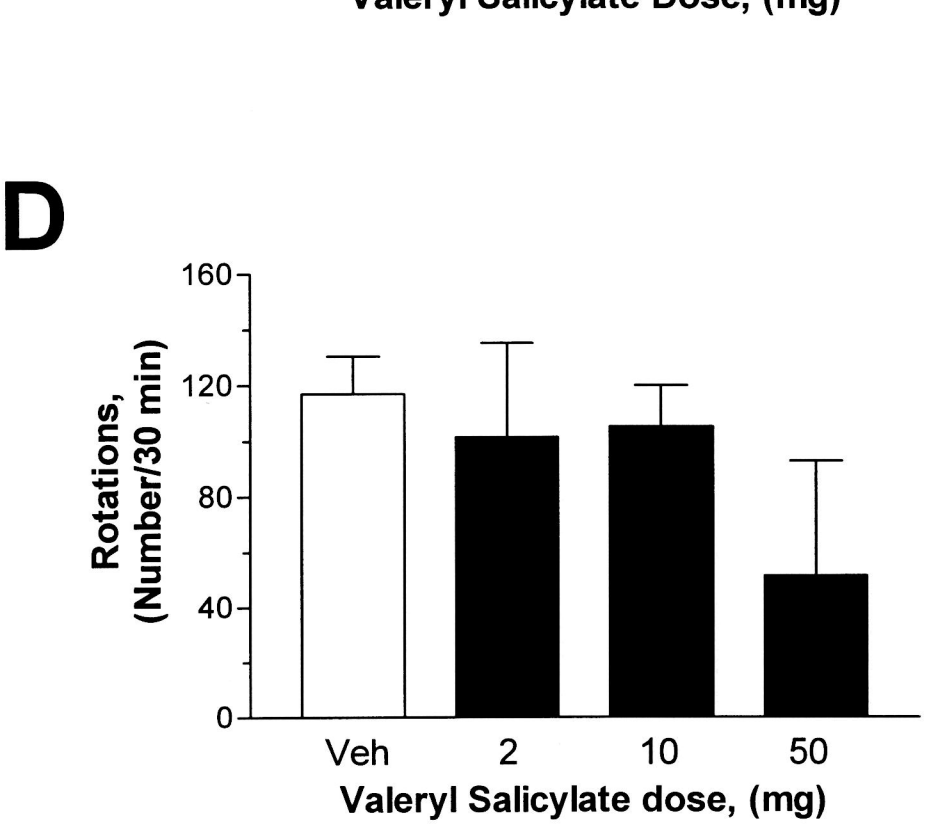

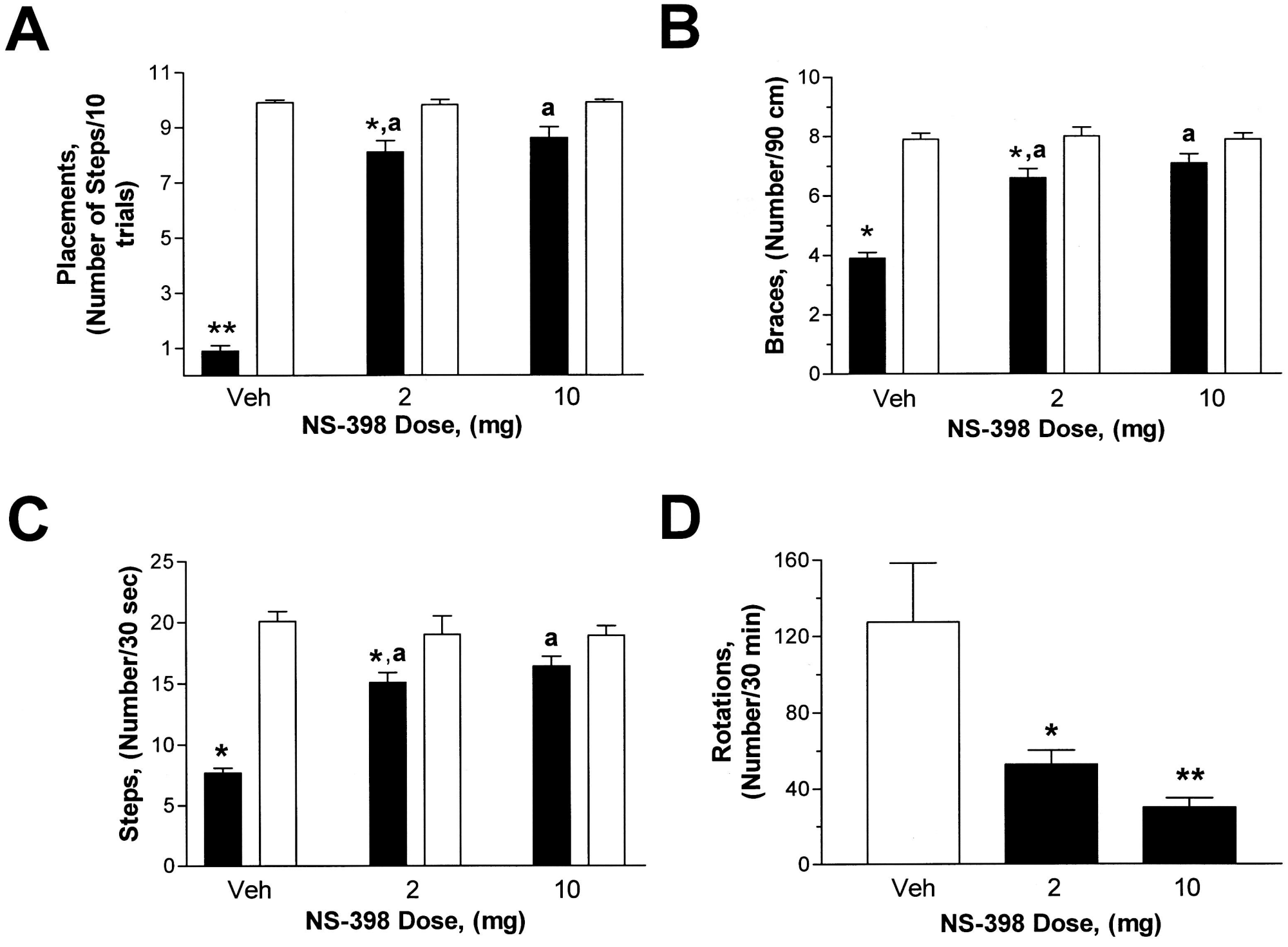

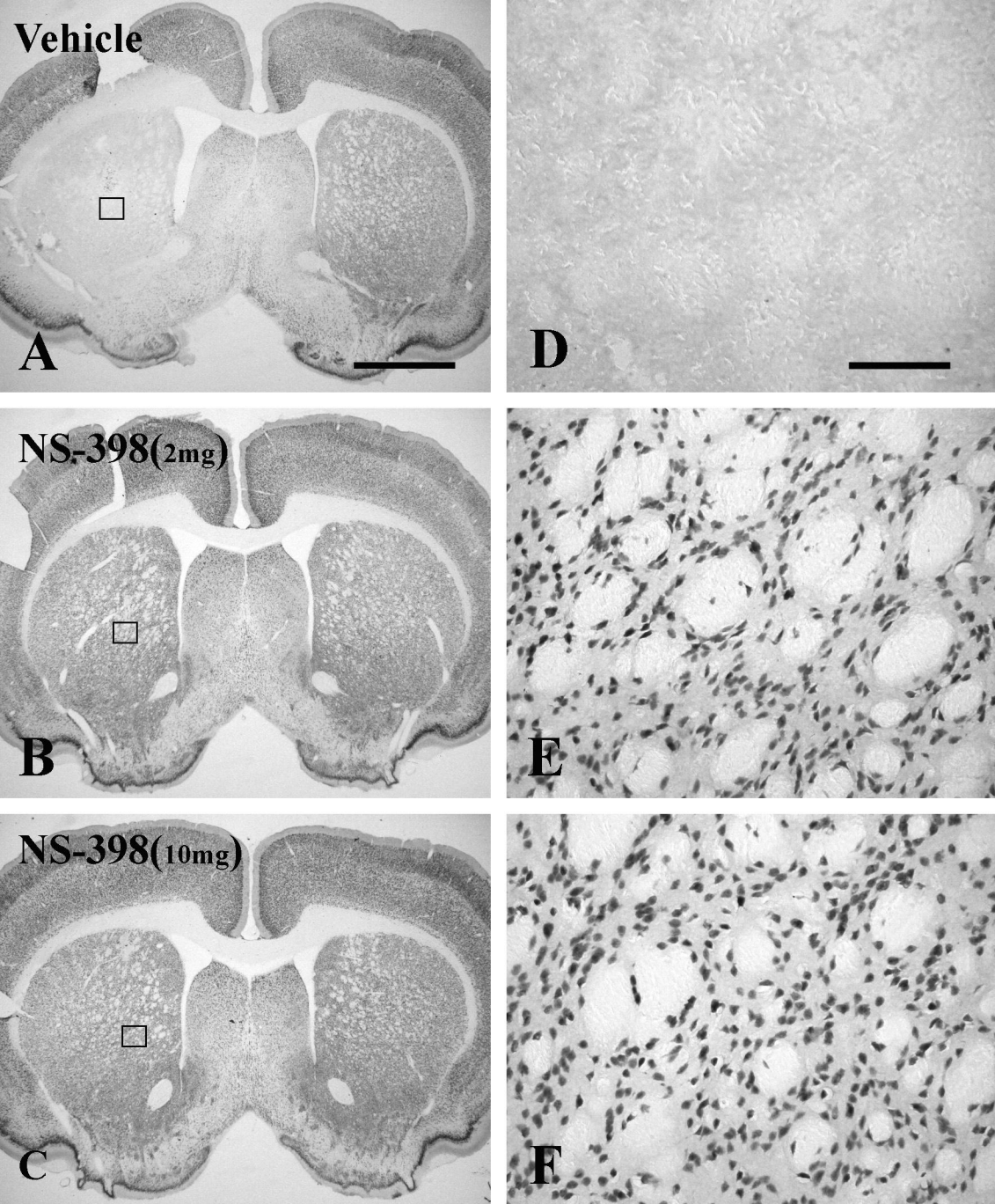
Vehicle
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