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Naproxen Reduces Excitotoxic Neurodegeneration *In Vivo* with an Extended Therapeutic Window[#]

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Abbreviations: COX, cyclooxygenase; MK-801, dizocilpine maleate; NMDA, N-methyl-D-Aspartate; NSAIDs, non-steroidal anti-inflammatory drugs; PPARs, peroxisome proliferator-activated receptors; ROS, reactive oxygen species

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Abstract:

The purpose of this study was to examine the optimal dose and therapeutic window of opportunity of the non-steroidal anti-inflammatory drug naproxen in an animal model of excitotoxic neuronal injury. Injection of N-methyl-D-aspartate (NMDA; 18-20nmol) into the CA1 region of the left hippocampus resulted in significant brain edema as measured by the percentage of total forebrain water content that occurred 24 hr following intrahippocampal microinjection of NMDA with ≈ 50% loss of CA1 neurons assessed 72 hr later. Naproxen pretreatment (20mg/kg) resulted in significantly less brain edema. Ten, 15 or 20 mg/kg naproxen, administered systemically one day (b.i.d.) prior to and for three days after (b.i.d.) NMDA injection, attenuated the neuronal damage by 27.2 ± 7.8%, 39.6 ± 11.1% and 57.0 ± 5.2%, respectively. By comparison, a single dose of MK-801 (2mg/kg, i.p.) given 20 min prior to NMDA injection inhibited subsequent hippocampal injury by 65.6 ± 8.8%. Most importantly, neuroprotection was still evident when naproxen treatment (20mg/kg, i.p.) was initiated six hr after NMDA microinjection. Protection was lost if administration of naproxen was delayed for 20hr. These findings demonstrate that naproxen can prevent excitotoxic neuronal injury *in vivo*, that it is nearly as effective as direct NMDA receptor antagonism, and that it has an extended therapeutic time window. As such, naproxen may be a particularly promising pharmaceutical for the treatment of neurological diseases associated with over-activation of NMDA receptors.

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Brain cells *in situ* contain low concentrations of free fatty acids, such as arachidonic acid, that are released following various pathological insults including those associated with glutamate neurotoxicity (i.e. excitotoxicity) (Bazan, 1970; Wieloch and Siesjo, 1982; Bonventre, 1996). Unesterified arachidonic acid is metabolized via cyclooxygenase and lipooxygenase pathways producing reactive oxygen species (ROS) as by-products (Kukreja et al., 1986). Such ROS formation has been demonstrated in the injured brain (Kontos, 1985) and following NMDA stimulation *in vitro* (Lafon-Cazal et al., 1993; Reynolds and Hastings, 1995). Further, biologically active prostaglandins and other polyunsaturated hydroxy acids – metabolites of arachidonic acid metabolism – may directly contribute to progression of certain neurological injuries (Iwamoto et al., 1989; Aktan et al., 1991; Bezzi et al., 1998; Prasad et al., 1998; Pratico et al., 1998; Rao et al., 1999; Carlson, 2003); although it should be noted that some studies report that certain prostanoids have neuroprotective potential (Cazevieille et al., 1993; Akaike et al., 1994; Cazevieille et al., 1994; Qin et al., 2001).

The idea that inhibition of lipid catabolism can protect brain or spinal cord under various pathological states has been considered for over two decades. With respect to ischemia, particular attention has been given to the possible role of arachidonic acid metabolites in the regulation of post-injury cerebral blood flow (Furlow and Hallenbeck, 1978; Black et al., 1984; Kochanek et al., 1988; Wahl et al., 1993; Zuckerman et al., 1994) and the formation of vasogenic brain edema (Bhakoo et al., 1984; Hall and Travis, 1988; Katayama et al., 1990). However, pre-ischemic administration of non-selective cyclooxygenase inhibitors can also have a positive effect on histological neuronal outcome following ischemic insults (Sasaki et al., 1988; Nakagomi et al., 1989; Costello et al., 1990; Cole et al., 1993; Patel et al., 1993; Antezana et al., 2003). Whether the neuronal protective effect of non-steroidal anti-inflammatory drugs (NSAIDs) observed in the above-mentioned studies was due to a direct cytoprotection or to favorable hemodynamics could

not be ascertained. Recent studies, however, have demonstrated that selective inhibition of the inducible form of cyclooxygenase (COX-2) protected against both global and focal ischemic neuronal injury in rat (Nogawa et al., 1997; Nakayama et al., 1998), in part, through the attenuation of NMDA receptor-mediated excitotoxicity (Iadecola et al., 2001). These observations taken in *toto* support the notion that NSAID administration will be therapeutically useful in neurological diseases associated with excessive NMDA receptor activation. This paper represents a pre-clinical assessment of the ability of naproxen, a commercially available and clinically useful NSAID, to protect against NMDA-induced hippocampal neuronal injury *in vivo*. Optimal drug dosage and therapeutic time window were assessed. We report that naproxen is potently neuroprotective with the added bonus of having an extended therapeutic time window.

MATERIALS AND METHODS:

Materials: N-methyl-D-Aspartate (NMDA), chloral hydrate, (S)-6-Methoxy-α-Methyl-2-Naphthaleneacetic acid ([+]-Naproxen), paraformaldehyde, HPLC Grade 2-methylbutane, and thionin were all obtained from Sigma Chemical Company (St. Louis, MO). Dizocilpine maleate (MK-801) was purchased from RBI (Natick, MA). Pentobarbital sodium was purchased from Abbott Laboratories (North Chicago, IL).

Intrahippocampal injection of NMDA: Male mice (ND-4; 27-30g; Harlan, Indianapolis) were anesthetized with 400mg/kg, i.p. chloral hydrate and placed in a Kopf 900 Small Animal Stereotaxic Frame with mounted Model 926 Mouse Adaptor (Kopf Instruments; Tujunga, CA). An incision was made in the scalp to expose the skull and a 1 mm diameter hole was drilled. NMDA (18-20nmol in 0.1M phosphate-buffered saline, pH = 7.4) was injected (0.35µl) using a Kopf 5000 microinjection unit equipped with a 5 µl Hamilton syringe and a 26 gauge blunt-tipped needle. The needle was placed into the CA1 region of the left hippocampus at stereotaxic coordinates 1.9 mm posterior to bregma**,** 1.2 mm lateral from the midline, and 1.6 mm ventral from the skull surface (Franklin and Paxinos, 1997). Injections were made over 12 min and the needle left in place for an additional 10 min to minimize back flow. Control animals received intra-hippocampal injections of PBS (0.35µl). After the procedure, the skin wound was sutured and animals were returned to their cages under *ad libitum* conditions. Before, during, and directly after the procedure (up to 1hr), animal temperature was maintained at 35.8-36.2°C using a Harvard Homeothermic Blanket Control Unit for small animals. This protocol was approved by the IACUC of the University of Connecticut Health Center.

Effect of MK801: Sixteen mice were randomly assigned to four treatment groups (4 mice/group) for injection as follows [intrahippocampal (0.35 µl): intraperitoneal (100µl)]: 1) PBS:PBS; 2) NMDA:PBS; 3) PBS:MK-801; 4) NMDA:MK-801. Intraperitoneal injections were delivered 20 min prior to intrahippocampal injections.

Effect of Naproxen: Thirty-one animals were randomly divided into six groups (4-7/group). Control Groups 1 and 2 and received PBS or naproxen (20mg/kg) i.p. one day before (b.i.d.) and for three days after (b.i.d.) intrahippocampal microinjection of PBS. Groups 3 4, 5 and 6 received PBS, 10, 15 or 20mg/kg naproxen i.p., respectively, one day before (b.i.d.) and for three days after (b.i.d.) intrahippocampal microinjection of NMDA (20nmol). The different doses were all dissolved in 100µl of sterile PBS so that the injection volume was the same.

Determination of Therapeutic Time Window: Sixty-five mice (4-8/group) were assigned to the following post-NMDA injection times: 25, 40, and 60 min; 3, 6, and 20hr. At each administration time, animals either received naproxen (20mg/kg, i.p.) or an equal volume of the vehicle PBS. Both naproxen and PBS were administered at the indicated times after injection as well as for the next two days (b.i.d.).

Measurement of Brain Edema: Forty-eight mice were divided into four groups (12 animals/group). Group one received no treatment (naïve). Group 2 received PBS (i.p. 0.1ml), one day before (b.i.d.) and the same day (b.i.d.) as an intrahippocampal microinjection of PBS. Group 3 was treated with PBS the day before and the same day (b.i.d.) as an intrahippocampal microinjection of NMDA (18nmol). Group 4 received naproxen (i.p. 20mg/kg) the day before and the same day (b.i.d.) as an intrahippocampal microinjection of NMDA. Brain water content in the combined hemispheres (minus the cerebellum) as well as in the ipsilateral and contralateral hippocampi (derived from a two mm thick coronal section centered on the mark of injection) was measured 24hr after intrahippocampal microinjections using the wet-dry method (Dempsey et al., 2000).

Assessment of Neuronal Injury: Seventy-two hr following microinjection, mice were sacrificed under general anesthesia (pentobarbital; 100 mg/kg, i.p.) by transcardiac perfusion with ice-cold 0.9% NaCl followed by 4% of PBS-buffered paraformaldehyde. Brains were removed and kept in the same fixative for an additional hour, then placed in 15% sucrose/H_20 follow by transfer to 30% sucrose/H_20 solution for storage (2-3 days; 4°C). The day before sectioning, tissues were frozen in 2-methylbutane at -80°C. Serial coronal sections (30 μm) were cut on a Leica Microsystems CM1900 cryostat at a distance 1.2 mm to 2.7mm posterior to bregma. Every 5^{th} section was mounted and stained with 0.1% thionin, a standard Nissl stain used for the histological verification of lesions (Powers and Clark, 1955). The extent of hippocampal injury was assessed via image analysis using a modification of the procedure developed by Manahan-Vaughan and colleagues (Manahan-Vaughan et al., 1998). Ten coronal sections at 150μm intervals were imaged (both ipsilateral and contralateral sides) using a CRX digital camera (Digital Video Camera Co; Austin, TX) mounted on an Olympus IX50 inverted microscope (2-4x magnification). Digitized images were processed and quantified using *NIH Image* (Scion) Software. An integrated density measurement for 8-12 static, non-overlapping, user-defined pixel volumes was calculated in the areas of interest (entire CA1, CA3 or dentate gyrus). The mean integrated density on the ipsilateral (I) and contralateral (C) sides of the 10 analyzed serial images were calculated and compared. The counts in the contralateral hippocampus served as an internal control for each animal, as injury was restricted to the ipsilateral hippocampus, which was determined by comparison to naïve animals (data not shown). In most cases, data are expressed as the percent difference in mean integrated density [100(C-I)/C].

Statistical Analysis: Percentage data is by nature non-normally distributed. Thus, the arcsine square root of the percentage data (Steel and Torrie, 1980) was analyzed via one or two way ANOVA followed by the appropriate post-hoc test as described in each figure legend. Values of zero or less were set at $1X10^{-10}$ prior to transformation. Significance was set at $p < 0.05$.

Results:

Unilateral injection of NMDA (20nmol in 0.35µl PBS) into the left hippocampus resulted in degeneration of areas CA1 (≅ 50%), CA3 (≅ 40%) and dentate gyrus (≅ 35%) extending at least 1 mm (rostral to caudal) around the site of injection as assessed histologically three days after the injection (Figure 1). Neuronal degeneration occurs exclusively on the ipsilateral side with no neuronal loss detected in the contralateral hippocampus (Figure 2). Systemic administration of naproxen partially but potently prevented the injury. With respect to the CA1, naproxen given one day prior and for three days after NMDA injection, attenuated neuronal damage in a dose-dependent manner with administration of 10, 15, and 20mg/kg resulting in a 27.2 ± 3.5%, 39.6 ± 5.0% and 57.0 ± 2.4% diminution, respectively (Figure 3). By comparison, a single dose of MK-801 (2mg/kg), given 20 min prior to NMDA injection attenuated CA1 neuronal injury by 65.6 ± 8.8% (Figure 4). Pretreatment with naproxen (20mg/kg) also resulted in significantly less brain edema as measured by the percentage of total forebrain water content that occurred 24 hr following intrahippocampal microinjection of NMDA (Figure 5A). Interestingly, this increase in water content and its subsequent attenuation by naproxen occurred on both the ipsilateral and contralateral sides (Figure 5B). Finally, a detailed study of the therapeutic time window was performed with the first dose of naproxen (20mg/kg) being administered to animals at increasing time intervals after NMDA microinjection and continuing for 72hr. Strikingly, significant protection in all three areas of the hippocampus was still evident even when the drug was given up to six hr after NMDA injection with injury in the CA1, CA3 and dentate gyrus decreased by 38.9 ± 12.5%, 63.6 ± 22.2%, and 52.3 ± 18% respectively (Figure 6). Interestingly, the CA3 and dentate gyrus were more amenable to the neuroprotective effects of naproxen than the CA1 with near complete prevention of injury demonstrated out to 3 hr (Figure 6). No protection was observed in any area if the administration of naproxen was delayed for 20 hr (Figure 6).

Discussion:

The resulting neurodegeneration in animal models of ischemia can be ameliorated by glutamate receptor antagonism (Gill et al., 1991; Katsuta et al., 1995). However, human clinical trials utilizing glutamate receptor antagonists have proven disappointing (Doble, 1999; Lee et al., 1999). While reasons for these failures may be varied (Berge and Barer, 2002; Furlan, 2002; Muir, 2002), it is likely that compounds that prevent glutamate neurotoxicity *after* initial receptor binding has occurred may actually be more clinically practical. Of interest, cyclooxygenase activation/induction occurs secondary to NMDA receptor stimulation (Yamagata et al., 1993; Miettinen et al., 1997; Hewett et al., 2000) and pre-ischemic administration of NSAIDs effectively preserves neuronal integrity (Cole et al., 1993; Nogawa et al., 1997; Nakayama et al., 1998), in part, through attenuation of NMDA receptor-mediated excitotoxicity (Iadecola et al., 2001). Thus, the ability of naproxen to ameliorate excitotoxic neuronal injury *in vivo* over a *delayed* time frame was tested herein. Naproxen was chosen because it is a commercially available and clinical useful NSAID that inhibits both the constitutive (COX-1) and inducible (COX-2) forms of cyclooxygenase (Laneuville et al., 1994), although they are actually both constitutively expressed in murine forebrain neurons (Breder et al., 1995; Hewett et al., 2000); it decreases prostaglandin biosynthesis in rodent brain with an ED_{50} of 2mg/kg (Abdel-Halim et al., 1978); and it also has a long half-life (≈16 hr) (Tomson et al., 1981). Doses herein were chosen to exceed the ED_{50} by up to 10 fold to ensure block of brain COX activity but did not exceed 20mg/kg in order to minimize untoward gastrointestinal or hematological effects. In addition, this represents an acceptable range of doses used to treat inflammatory conditions in humans (MICROMEDEX). Results from the present study demonstrate that systemic administration of naproxen can attenuate CNS parenchymal cell death mediated by excessive activation of neuronal NMDA receptors *in vivo*, nearly as effectively as direct NMDA receptor antagonism. Moreover, it has an extended

therapeutic time window. This *in vivo* data complements and extends our recent *in vitro* studies (Hewett et al., 2000).

Several pharmacological actions other than cyclooxygenase inhibition must be considered when interpreting the neuroprotective actions of naproxen, however. Such potent neuroprotection, as that found in the CA3 and dentate gyrus, rarely has been observed with compounds acting downstream of receptor activation, suggesting that naproxen might directly antagonize receptor activity. However, NMDA-induced calcium flux in cortical cells was unaffected by naproxen indicating that it is not an NMDA receptor antagonist (unpublished observation). Interestingly, COX-2 immunoreactive neurons are primarily observed in the pyramidal layer of the CA3 and in the dentate granule cell layer (Breder et al., 1995), which might explain why these areas enjoy greater protection from naproxen than the area CA1. However, it should be noted that an upregulation of COX-2 expression occurs readily in all three areas following seizures (Yamagata et al., 1993) or global ischemia (Nakayama et al., 1998). Indeed, the delayed window of opportunity reported herein coincides precisely with hippocampal COX-2 protein expression measured via western blot analysis. COX-2 levels from the ipsilateral hippocampus increased between 3-6 hr after NMDA injection, peaked between 9 and 12 hr, and declined to near baseline by 24 hr (JMS, T.F. Uliasz and SJH, unpublished observation). This is strikingly similar to results reported by Graham and colleagues following cessation of global ischemia (Nakayama et al., 1998). In the same study, selective COX-2 inhibition protected the CA1 area when given one hr following ischemia. Confirmation that the cell death in our *in vivo* excitotoxicity model occurs via a COX-2-dependent mechanism awaits study utilizing selective COX-2 inhibitors, although *in vitro* data from our and other laboratories support this hypothesis (Kelley et al., 1999; Hewett et al., 2000; Strauss and Marini, 2002).

This study also demonstrates that our model of injury produces significant brain edema 24 hr post-injury, which can be detrimental because of elevation of intracranial pressure and impairment of

cerebral blood flow. However, adverse hemodynamic effects cannot account solely for the subsequent histological damage mediated by NMDA administration, as significant water elevation in the non-injured contralateral cortex also occurred at 24 hr. Interestingly, glutamate excitotoxicity and oxidative stress can cause edema (Chan et al., 1982; Shapira et al., 1990; MacGregor et al., 2003). Since naproxen is not an NMDA receptor antagonist, nor does it have intrinsic anti-oxidant properties (T.F Uliasz and SJH; unpublished observations), it is intriguing to speculate that naproxen effectively attenuates brain edema via decreasing the cellular oxidative stress that occurs secondary to NMDA receptor-mediated cyclooxygenase activation/induction (Lafon-Cazal et al., 1993; Reynolds and Hastings, 1995). Support for this idea comes from a recent study which demonstrates that the NSAID nimesulide reduces measures of oxidative stress that follow global cerebral ischemia in gerbils (Candelario-Jalil et al., 2003). Of course, metabolism of arachidonic acid via cyclooxygenase can also result in the formation of vasoactive prostanoids (Asano et al., 1987), providing an alternate explanation for the beneficial effect of naproxen with respect to edema formation (Ambrus et al., 1985; Asano et al., 1987).

Other cyclooxygenase-independent actions of some NSAIDs include the ability to reduce highly amyloidogenic Aβ42 peptide production from cultured cells (Weggen et al., 2001), apparently via direct modulation of gamma secretase activity (Weggen et al., 2003). However, it is interesting to note that this effect observed with ibuprofen and indomethacin does not occur with naproxen treatment (Weggen et al., 2001). A similar dichotomy was reported with respect to these NSAIDs' ability, or in the case of naproxen its lack thereof, to directly scavenge nitric oxide radicals (Asanuma et al., 2001). Finally, it should be noted that naproxen has been shown to activate peroxisome proliferator-activated receptors (PPARs) (Jaradat et al., 2001) and PPAR agonists can modulate inflammatory responses in brain (Heneka et al., 2000). Although, activators of PPARs failed to mimic the protective effect of NSAIDs in our *in vitro* model (Hewett et al., 2000), a role in the more complicated *in vivo* environment cannot be dismissed.

Regardless of the precise protective mechanism of action, present data demonstrate that naproxen can ameliorate CNS parenchymal cell death and edema formation mediated by excessive activation of neuronal NMDA receptors *in vivo* with no adverse effects and one of the longest therapeutic windows of opportunity reported to date. Thus, naproxen may be a promising pharmaceutical for the treatment of neurological diseases associated with over-activation of NMDA receptors.

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Figure Legends:

Figure 1. ***Integrated density measurement for the determination of cell density***. Hippocampal injury was quantified by measurement of cell density (CA1 shown) on thionin stained coronal brain sections via image analysis. Sections of 30 μM thickness taken at 150 μM intervals were imaged (both left and right sides) using a CRX digital camera mounted on an Olympus IX70 inverted microscope at 4x magnification. Digitized images were processed and quantified using *NIH Image* (Scion) Software. An integrated density measurement for 8-12 static, non-overlapping, user-defined pixel volumes was calculated. The mean integrated density ± SEM on the ipsilateral (I) and contralateral (C) sides to the microinjection of 10 analyzed serial images were calculated and compared. Data are expressed as the percent difference in mean integrated density [100(C-I)/C]. Four of the 10 micrographs, including the 1st and the last, from a representative brain shown above were used to derive measurements in the graph (n = 3 measurements/slice).

Figure 2. ***Loss of hippocampal CA1 neurons following NMDA microinjection***. Thionin stained coronal brain section from a representative mouse three days after NMDA (20nmol) microinjection in the left hippocampus. **(A)** 4x magnification of ipsilateral and contralateral side of brain. **(B,C)** 80x magnification of the right (uninjected) and left (NMDA injected) CA1 layer.

Figure 3. *Naproxen attenuates NMDA–mediated excitotoxicity in the hippocampus in a dose-dependent manner*. Animals (4-7 per group) were treated with PBS (0.1ml; i.p) or naproxen (0.1ml, i.p.) at the indicated concentrations one day before (b.i.d.) and for three days after (b.i.d.) intrahippocampal microinjection of NMDA or PBS. A) Loss of CA1 cell density was calculated as described in methods. (#) Indicates a significant enhancement of cell loss resulting from NMDA treatment as compared to animals treated with PBS alone, whereas (*) indicates significant naproxen-mediated diminution of NMDA-induced injury as analyzed by one-way ANOVA followed Student-Newman-Keul's multiple comparisons test [$p<0.001$]; NS = non-significant.

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Figure 4. *MK-801 attenuates NMDA–mediated excitotoxicity in the hippocampus*. Animals (4 mice/group) received either vehicle (PBS, 0.1ml) or the non-competitive NMDA antagonist, MK-801 (2mg/kg), intraperitoneally, 20 min prior to intrahippocampal injection of either NMDA (20nmol) or PBS. A) Three days later, CA1 hippocampal cell loss was quantified as described in methods. (#) Indicates a significant increase in cell loss as compared to animals treated with PBS alone ($p < 0.05$), whereas (*) indicates a significant MK-801-induced diminution of NMDA-induced injury ($p< 0.001$) as determined by two way-ANOVA followed Bonferroni's post-hoc test. B-D) Representative photomicrographs of the CA1 area (40X) from animals that received intrahippocampal:intraperitoneal injections of (B) PBS:PBS; (C) NMDA:PBS or (D) NMDA:MK-801.

Figure 5. *Naproxen attenuates injury-induced brain edema*. (A) Total hemispheric and (B) bilateral hippocampi brain water content were measured from vehicle control (PBS: PBS), injured (NMDA: PBS), naproxen-treated (NMDA: NAP 20mg/kg) and naïve animals using the wet/dry method. Data are expressed as mean ± SEM percent water content (12 mice/group). (*) Indicates a significant increase in water content when compared to naïve animals, whereas (#) indicates a significant naproxen-mediated diminution as analyzed by one-way ANOVA followed Student-Newman-Keul's multiple comparisons test ($p <0.01$).

Figure 6. *Naproxen attenuates NMDA–mediated excitotoxicity in the hippocampus with an extended therapeutic window of opportunity*. [Left Panels]: Animals (n = 4-8/group) were randomly assigned to the following post-NMDA injection times: 25, 40, 60, 180, 360 or 1200 min. Animals either received naproxen (20mg/kg) or an equal volume of the vehicle PBS at the indicated times after NMDA injection as well as for the next two days (b.i.d.). Neuronal cell loss in the CA1, CA3 and dentate gyrus (DG) region of the hippocampus was quantitated. Since there was no significant difference in injury between each respective control (PBS-treated) group, values were pooled and are represented as a single group for ease of presentation only. (*) Indicates a significant diminution of the NMDA-induced cell less when

compared to each of their respective controls as determined by two-way ANOVA followed Bonferroni's multiple comparisons test. For the CA1 area, the p values were <0.0001 for the treatment group effect, 0.0937 for the effect of time of drug administration, and 0.1392 for the treatment x time interaction. For the CA3, the p values were <0.0001 for the treatment group effect, 0.0390 for the effect of time of drug administration, and 0.3514 for the treatment x time interaction. For the DG, the p values were <0.0001 for the treatment group effect, 0.0024 for the effect of time of drug administration, and 0.0106 for the treatment x time interaction. [Right Panels]: Representative photomicrographs of animals that received (A) PBS or (B) naproxen beginning 60 min post-NMDA injection: (a,e; 40x) CA1; (b,f; 80x) CA3; (c,g; 40x) CA3; (d,h; 40x) dentate gyrus.

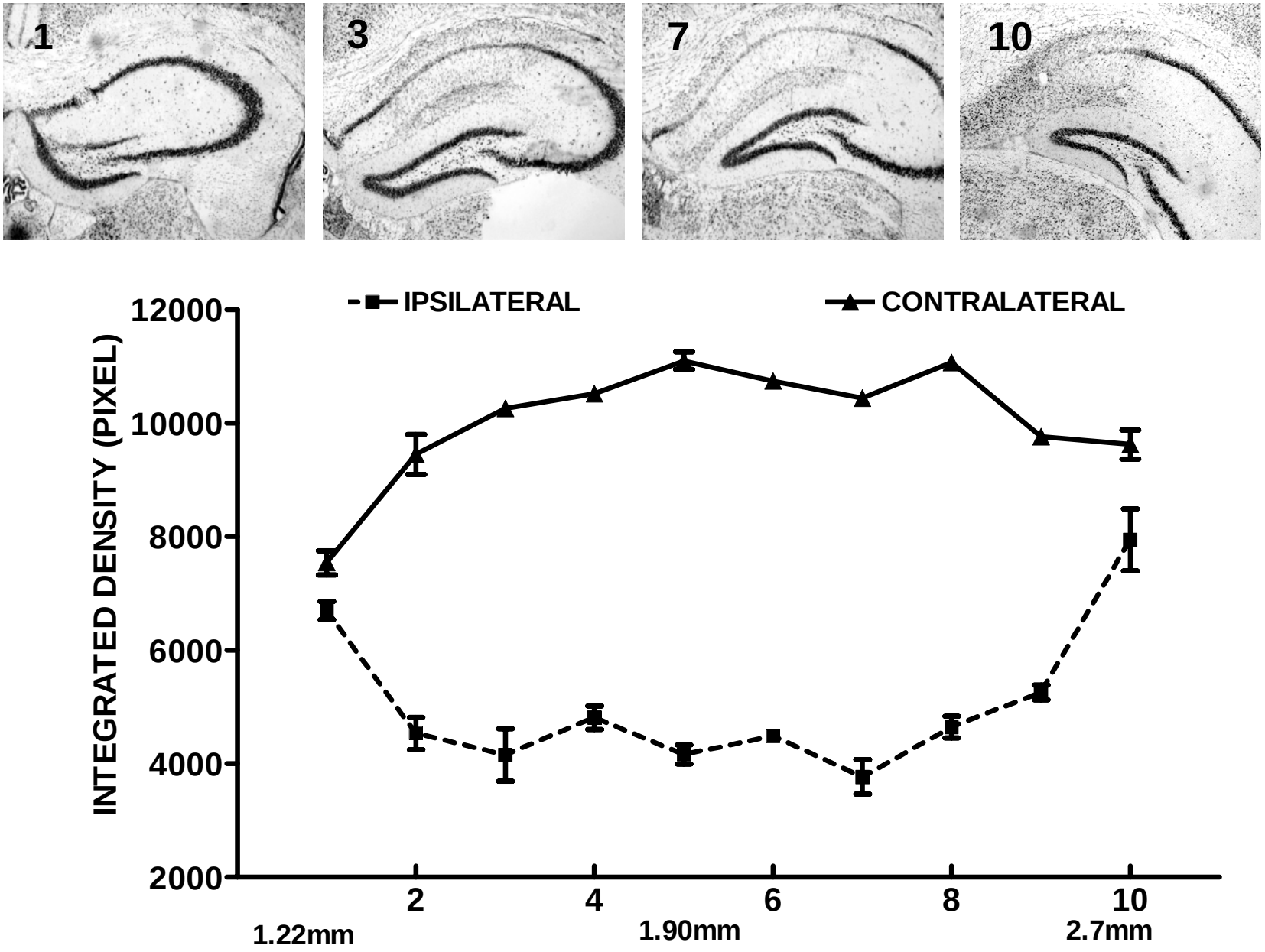
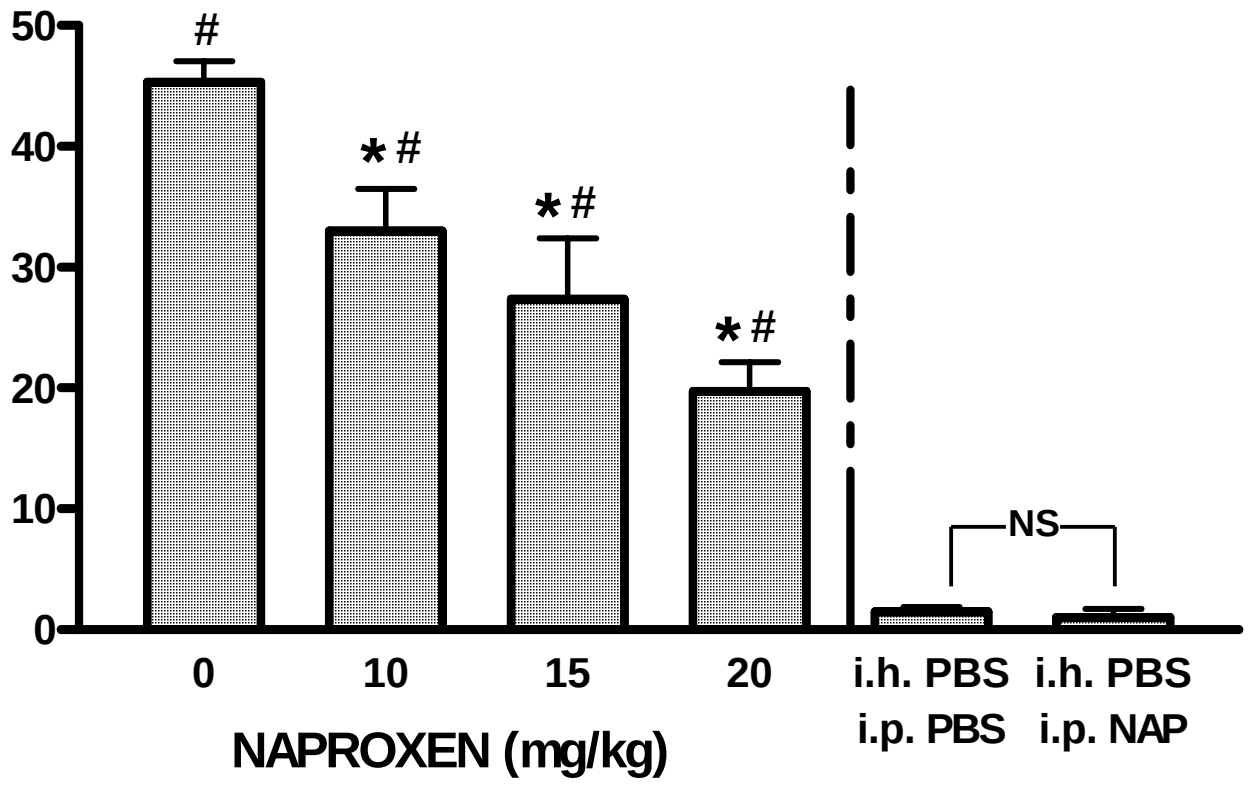


Figure 1. Silakova et al., 2004--JPET/2003/063867

Figure 2. Silakova et al., 2004—JPET #063867



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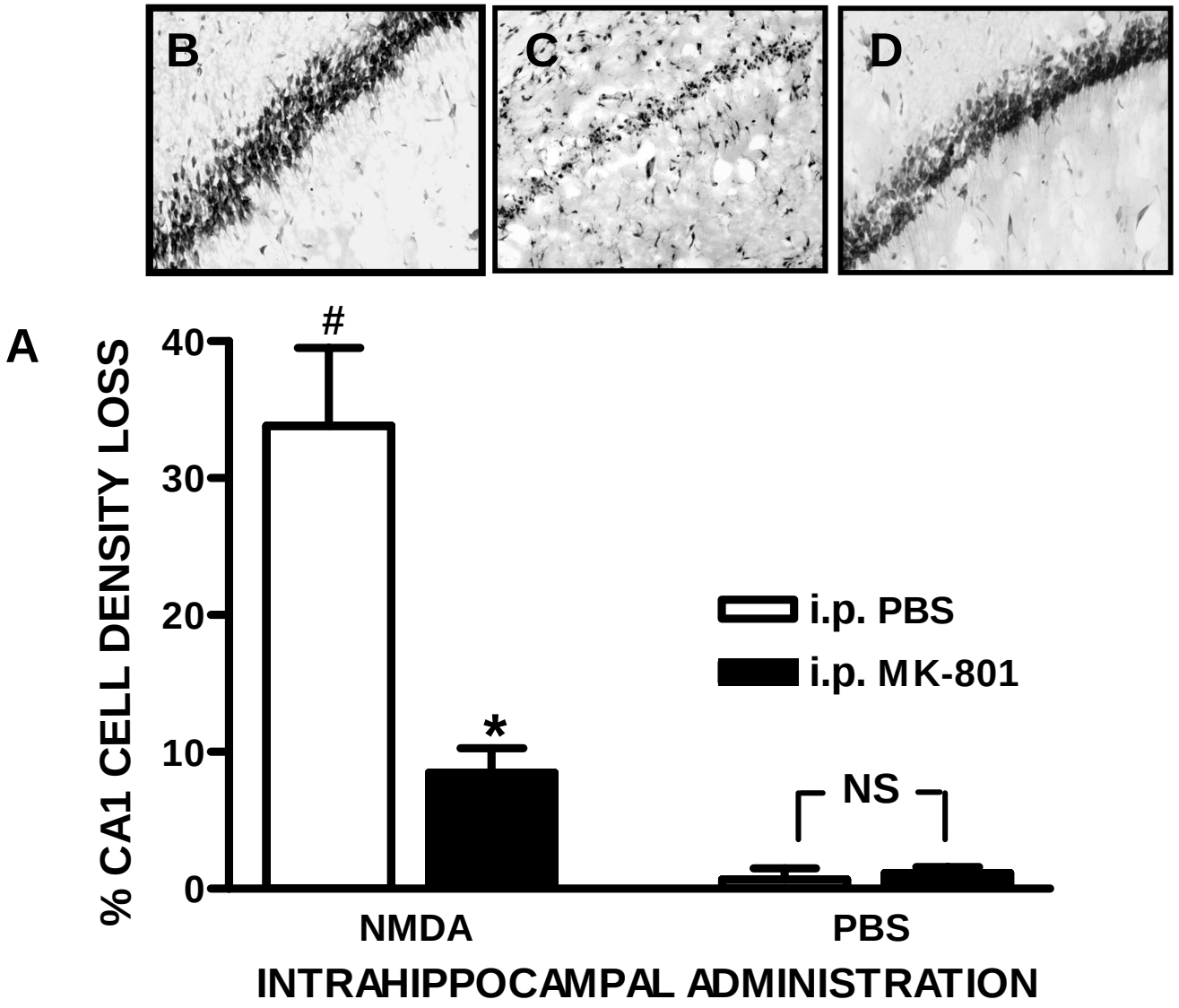
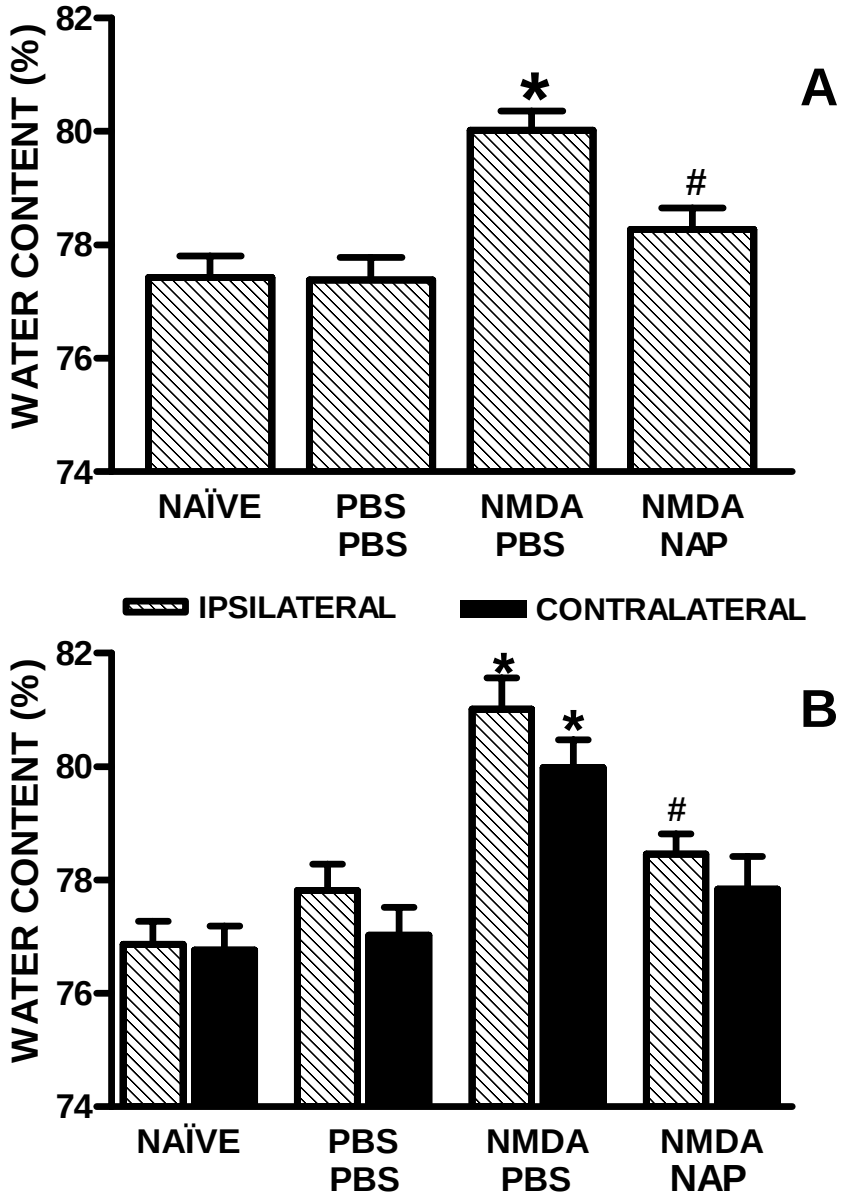


Figure 4. Silakova et al., 2004--JPET/2003/063867



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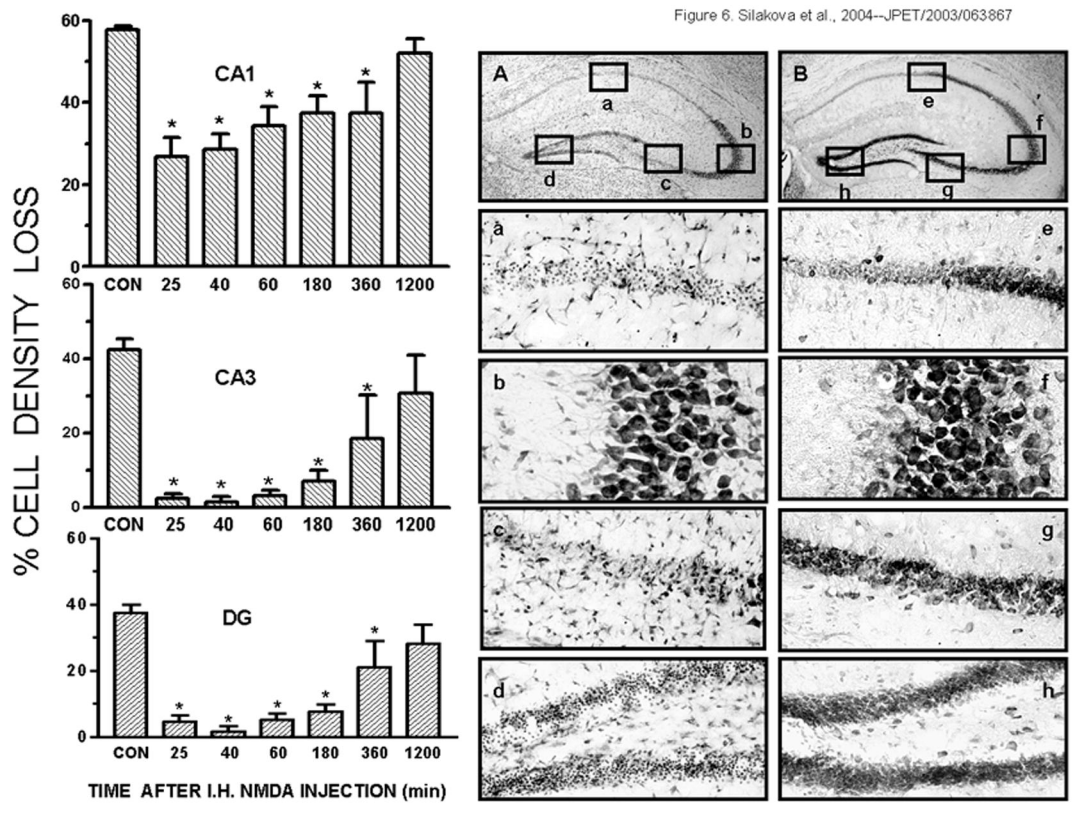
% CELL DENSITY LOSS
CA1
CA3
DG
CON
25
40
60
180
360
1200
TIME AFTER I.H. NMDA INJECTION (min)
A
B
a
b
c
d
e
f
g
h