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Full Title: 5-HT1A Receptor Activation Protects Against NMDA-Induced Apoptotic Cell Death in Striatal and Mesencephalic Cultures.

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Running Title: Neuroprotective effect of 5-HT1A receptor agonists

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Abbreviations: PD, Parkinson's disease; 5-HT1A, 5-Hydroxy-tryptamine-1A; NMDA, N-methyl-d-aspartate; TH, tyrosine hydroxylase; GABA, gamma-aminobutyric acid; Caspase, cysteine-aspartate protease; 8-OH-DPAT, 8-hydroxy-2-(di-n-propylamino)-tetralin; WAY 100635, *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl] ethyl]-*N*-(2-pyridinyl) cyclohexane carboxamide; UH-301, 5-fluoro-8 hydroxy-2-(dipropylamino)-tetralin; MK-801, +(-)5-methyl-10,11-dihydro-5H-dibenzo-cyclohepten-5,10-imine-maleate; Ac-DEVD-AMC, Acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl-coumarin; Z-DEVD-FMK, Z-Asp-Glu-Val-Asp-fluromethyl ketone; EBSS, Earle's Balanced Salt Solution; DMEM,

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Dulbecco's modified Eagle's medium; ELISA, Enzyme linked immunosorbent assay; ABTS, 2,2' –azino-di-(3-ethylbenzthiazoline sulfonate (6)) diammonium salt..

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ABSTRACT:

Apoptosis and glutamate mediated excitotoxicity may play a role in the pathogenesis of many neurodegenerative disorders, including Parkinson's disease (PD). In the present study, we investigated whether stimulation of the 5-hydroxy-tryptamine 1A (5-HT1A) receptor attenuates NMDA and MPP^+ induced apoptotic cell death in cell culture models. A brief exposure (20 min) of M213-2O striatal cells to NMDA and glutamate produced a delayed increase in caspase-3 activity and DNA fragmentation in a dose- and time-dependent manner. NMDA-induced caspase-3 activity and DNA fragmentation were almost completely blocked by the 5-HT1A agonists, 8-OH-DPAT and R-UH-301. Additionally, the protective effects of 8-OH-DPAT and R-UH-301 on NMDA-induced caspase-3 activation and apoptosis were reversed by pretreatment with the 5-HT1A antagonists, WAY 100635 and S-UH-301, respectively. Similarly, dose- and time-dependent increases in caspase-3 activity and DNA fragmentation were observed in rat primary mesencephalic neurons after a brief exposure to NMDA and glutamate. Caspase-3 activation and DNA fragmentation in primary mesencephalic neurons were almost completely inhibited by 8-OH-DPAT. This neuroprotective effect of 8-OH-DPAT was reversed by WAY 100625. Additionally, 8-OH-DPAT blocked tyrosine hydroxylase (TH)-positive cell death following NMDA exposure and also almost completely attenuated the NMDA-induced Ca^{2+}-influx in primary mesencephalic cultures. Furthermore, 8-OH-DPAT and R-UH-301 blocked apoptotic cell death in the primary mesencephalic neurons that were exposed to the Parkinsonian toxin MPP^+. Together, these results suggest that 5-HT1A receptor stimulation may be a promising pharmacological approach in the development of neuroprotective agents for PD.

Parkinson's disease (PD) is a major neurodegenerative disorder with a lifetime incidence of 1-2%. The drug L-DOPA has been the gold standard PD treatment for more than three decades now and can bring considerable relief from the debilitating symptoms of the disease. However, its long-term use has several limitations including severe fluctuations in effectiveness and drug induced involuntary movements (Fahn, 1999; Carlsson, 2002). Importantly, L-DOPA simply compensates for the dopamine deficiency in Parkinson's patients and fails to attenuate the progression of the disease. Hence, novel neuroprotective agents designed to interfere with the basic pathogenic mechanism of cell death in PD are clearly needed.

Excitotoxic mechanisms may contribute to the nigrostriatal degeneration in PD (Greenamyre, 1993; Olanow and Tatton, 1999). Glutamate binds to a variety of excitatory amino acid receptors, especially the NMDA receptor, leading to prolonged and excessive depolarization and neuronal activation resulting in the death of target neurons (Choi, 1988; Lynch and Dawson, 1994). Consequently, NMDA receptor blockade in PD research has been actively pursued over the past decade, but to date has been limited by the side effects of the glutamate antagonists (Nilsson et al., 1997; Li et al., 2002) and their mixed effectiveness (Sonsalla et al., 1992; Engber et al., 1994).

Since the utility of NMDA-receptor antagonists as neuroprotective agents has not been successful, other attenuators of excitotoxicity in PD must be investigated. Stimulation of the 5-HT1A receptor may play a role in neuronal survival (Raymond et al., 2001). 5-HT1A receptor stimulation rescued cultured hippocampal neurons from glutamate mediated excitotoxicity and protected against ischemic neuronal cell death

(Nakata et al., 1997; Harkany et al., 2001). Interestingly, 5-HT1A receptor activation improved the motor complications in rodent and primate models of PD (Bibbiani et al., 2001). Also, a clinical trial evaluating the antidyskinetic efficacy of Sarizotan, a 5-HT1A agonist, in Parkinson patients is underway (Bibbiani et al., 2001; www.brany.com).

Emerging studies emphasize the importance of striatal terminals in PD pathology (Chase et al., 1998; Chase and Oh, 2000). Since the striatum receives massive glutamatergic input from the cortex, it is a likely possible site for neuronal overexcitation in the basal ganglia. The resulting neurophysiological changes due to interactions between dopamine and glutamate in striatal medium spiny neurons appear to contribute to the generation of Parkinsonian symptoms (Chase et al., 1998). The importance of the striatum in the pathogenesis of PD is underscored by the idea that nigral degeneration follows the retrograde degeneration of dopaminergic terminals due to neuronal excitation in the striatum (Lundberg et al., 1994). NMDA sensitization of medium spiny neurons due to changes in the phosphorylation state alters the cortical glutamatergic input to the striatum, modifies the GABAergic output, and alters motor function (Chase and Oh, 2000). Also, striatonigral/striatopallidial GABAergic neuronal dysfunction in PD may increase or redistribute nigral iron to cause substantia nigral neuronal death (Volpe et al., 1998).

Recent advancements in the understanding of PD pathology have revealed that apoptosis is an active cell death process in the degeneration of dopaminergic neurons (Hirsch et al., 1999; Hartmann et al., 2000). Interestingly, apoptosis has been shown to occur in substantia nigral dopaminergic neurons following developmental striatal injury (Macaya et al., 1994). In the present study, we investigated whether 5-HT1A receptor

stimulation protects against NMDA mediated apoptotic cell death in both striatal and nigral neurons. Immortalized striatal M213-2O cells and primary mesencephalic cultures were used as models of striatal and nigral degeneration, respectively. We report herein that stimulation of the 5-HT1A receptor protects striatal cells from NMDA induced apoptosis and mesencephalic neurons from NMDA as well as MPP^+ induced cell death.

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METHODS:

Chemicals: NMDA, glutamate, 8-OH-DPAT, R- and S-UH-301, WAY 100635, MPP^+ and cytosine arabinofuranoside were obtained from Sigma-RBI (Natick, MA); Z-Asp-Glu-Val-Asp-fluoromethyl ketone (Z-DEVD-FMK) was obtained from Alexis Biochemicals; Acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) was obtained from Bachem (King of Prussia, PA); fluorescein isothiocyanate conjugated to VAD-FMK (FITC-VAD-FMK) was purchased from Promega (Madison, WI); antibodies to NMDA and 5-HT1A were purchased from PharMingen (San Diego, CA), and TH from Calbiochem (San Diego, CA); the ECL chemiluminecence kit was purchased from Amersham Pharmacia Biotech (Piscataway, NJ); Hoechst 33342 was bought from Molecular probes (Eugene, OR); and the Cell Death Detection ELISA plus assay kit was purchased from Roche Molecular Biochemicals (Indianapolis, IN). Cell culture supplies included DMEM, Neurobasal medium, gentomycin, and penicillin/streptomycin and were purchased from Invitrogen (Gaithersburg, MD). Fura-2AM and Pluronic were obtained from Molecular Probes (Eugene, OR).

Cell culture: Primary cultures: Cells were cultured from the ventral mesencephalon dissected from gestational 16-18 day old Sprague-Dawley rat embryos and maintained on ice cold calcium free EBSS supplemented with gentomycin (50 mg/ml) and penicillin/streptomycin (200 units/ml). The mesencephalic tissue was digested in EBSS solution containing trypsin (2.5%) for 15 min. Digestion was terminated by the addition of EBSS solution containing trypsin inhibitor (0.25 mg/ml). The tissue was then

mechanically triturated about ten times with a 1 ml pipette to dissociate mesencephalic tissues. After dissociation, the cells were spun down and then resuspended in Neurobasal medium supplemented with the B27 components, L-glutamine (500 μM), L-glutamate (25 μM), penicillin (200 units/ml), and streptomycin (200 units/ml). Cells were plated in 6-well or 24-well poly-l-lysine (1 mg/ml) coated plates. Cells were then maintained in a humidified CO_2 incubator (5% CO_2, 37°C). The second day after plating, the cells were treated with cytosine arabinoside (10 μM) for 24 hr to inhibit glial growth. Half of the culture medium was changed every four days.

M213-2O cells: The M213-2O cells were derived from the rat striatum and immortalized using a temperature sensitive allele of the large SV40 T antigen (Giordano et al., 1993) and are susceptible to apoptosis induction by glutamate (Conejero et al., 1999). These cells have a multipolar and polygonal neuronal phenotype. Cells were grown in DMEM/F12 medium containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 units penicillin, and 50 μg/ml streptomycin. The cells were incubated at 33°C in a humidified atmosphere containing 5% CO_2.

Treatment paradigm: An excitotoxic treatment paradigm developed by Choi (1988) was used. Seven to ten day old primary mesencephalic neurons and three to four day cultures of M213-2O cells were treated with varying concentrations (100 μM, 300 μM, 500 μM, and 1 mM) of NMDA or glutamate for 20 min in EBSS medium containing 5 μM glycine. After this brief exposure to the excitatory amino acids, cell culture wells were washed and replaced with fresh culture medium. After exposure, the cell samples were collected at the appropriate time points and caspase-3 activity and DNA

fragmentation were measured and immunocytochemical studies were conducted in cell lysates. The mesencephalic cultures were treated with MPP^+ for 48 hours and then the cell lysates were assayed for caspase-3 activity and DNA fragmentation. For the inhibitor studies, the cells were pretreated for 20 min with the inhibitors MK-801, Z-DEVD-FMK, WAY 100635, 8-OH-DPAT, R-UH-301, and S-UH-301 and then exposed to NMDA or MPP^+.

Caspase-3 activity assay: Caspase-3 activity was measured as previously described (Anantharam et al., 2002). Briefly, after treatment, the cells were spun and the cell pellets were lysed with Tris buffer, pH 7.4 (50 mM Tris HCl, 1 mM EDTA, and 10 mM EGTA) containing 10 μM digitonin for 20 min at 37°C. Lysates were centrifuged at 900 *g* for 3 min, and the resulting supernatants were incubated with the fluorogenic substrate Ac-DEVD-AMC (50 μM) at 37°C for 60 min. Levels of cleaved (active) caspase substrate were monitored at an excitation wavelength of 380 nm and an emission of 460 nm using a fluorescent plate reader. Caspase-3 activity was expressed as fluorescence units/mg protein/hr. The protein concentrations were determined using the Biorad protein assay kit.

DNA fragmentation assay: DNA fragmentation was measured using a recently developed and highly sensitive cell death detection ELISA kit (Roche Biochemicals, Indianapolis, IN, USA). This assay provides a quantitative measurement of histone-associated low molecular weight DNA fragments following apoptotic stimulation (Anantharam et al., 2002). Apoptosis in M213-2O cells was measured using this kit

according to the manufacturer's instructions. Following brief NMDA exposure, the cells were centrifuged and washed once with PBS. Cells were then incubated with lysis buffer (supplied with the kit) for 30 min, centrifuged at 500 rpm for 10 min, and then 20 μL of cell lysate was placed in the streptavidin-coated 96-well multititer plates. The antibody cocktail was a mixture of anti-histone biotin directed against the histones (H1, H2A, H2B, H3, and H4) and anti-DNA peroxidase (POD) directed against both single and double stranded DNA in the nucleosomes. After incubation, the unbound components were removed by washing with the incubation buffer, and the nucleosomes retained by the anti-DNA-POD in the immunocomplex were quantified photometrically with ABTS as a horseradish peroxidase (HRP) substrate (Roche Biochemicals). Measurements were made at 405 nm against an ABTS solution as a blank (reference wavelength 490 nm).

Hoechst staining: The cells were cultured on poly-l-lysine (0.1 mg/ml) coated plates and exposed to NMDA alone or NMDA in combination with 8-OH-DPAT or Z-DEVD-FMK. After exposure, the cells were fixed with 10% buffered formalin for 30 min at room temperature and then washed with PBS. The fixed cells were stained with Hoechst 33342 (10 μg/ml) for 3 min in the dark. Cells were then viewed under a Nikon Diaphot microscope (Tokyo, Japan) using uv illumination and images were photographed with a SPOT digital camera (Diagonostic Instruments, Sterling Heights, MI)

***In situ* caspase-staining:** We used the CasPACE kit (Promega, Madison, WI**)** to detect caspase activation in intact cells following NMDA exposure. FITC-VAD-FMK, an FITC conjugate of the cell permeable caspase probe Z-VAD-FMK, binds to activated caspase

and acts as an *in situ* marker for caspase-like proteases. The experiment was performed according to the manufacturer's protocol, with slight modifications. Briefly, M213-2O cells were grown on poly-l-lysine (0.1 mg/ml) coated coverslips for about two days in a 5% CO_2, 37°C incubator. Cells were then exposed to 500 μM NMDA with or without 1 μM 8-OH-DPAT, according to the treatment paradigm. After exposure, the cells were treated with 10 μM FITC-VAD-FMK for 20 min at 37°C and then rinsed once with 1 X PBS, and fixed in 10% buffered formalin on coverslips. The fixed cells were mounted to slides using mounting medium. The slides were observed under a fluorescent microscope (Nikon Diaphot, Tokyo, Japan) and digital images were taken with a SPOT digital camera (Diagonostic Instruments, Sterling Heights, MI).

Immunocytochemistry: Cells were plated on poly-l-lysine (0.1 mg/ml) coated coverslips. After 24-48 hr, the cells were fixed with 4% formaldehyde and permeabilized with 0.2% triton X-100. The cells were then incubated with antibodies directed against the NMDAR1 subunit (Pharmingen, 1:300), the 5-HT1A receptor protein (Pharmingen, 1:1000), or tyrosine hydroxylase (TH) (Calbiochem, 1:1000) overnight at 4°C followed by incubation with the appropriate Cy3-conjugated secondary antibody (Jackson Laboratories, 1:300) for 90 min at room temperature. The cells were then mounted on slides, viewed, and imaged under a Nikon Diaphot fluorescence microscope (Tokyo, Japan) coupled to a SPOT digital camera (Diagonostic Instruments, Sterling Heights, MI).

To determine the TH-positive cell count, the cells were plated on poly-l-lysine coated coverslips and treated with NMDA alone or NMDA and 8-OH-DPAT.

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Immunocytochemistry, as previously described, using the appropriate anti-TH antibodies was conducted. After staining, the coverslips were mounted on slides and observed under the microscope. About 25 fields were checked and the cells therein were counted. The total number of cells under the light microscope and the number of TH-positive cells under rhodamine fluorescence were counted in every field for each treatment (control, NMDA treatment, and 8-OH-DPAT plus NMDA treatment). The percentage of TH-positive cells in each treatment group was calculated and averaged over the 25 fields.

Western Blotting: M213-2O cells were harvested and then washed once with ice-cold Ca^{2+}-free PBS and resuspended in 2 ml of homogenization buffer (20 mM Tris-HCl (pH 8.0), 10 mM EGTA, 2 mM EDTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml aprotinin, and 10 μg/ml leupeptin). Suspensions were sonicated for 10 s and centrifuged at 100,000 *g* for 1 hr at 4°C. The pellets containing the membrane fraction were washed with ice-cold PBS and resuspended in 200 μL homogenization buffer containing 1% triton X-100 and then sonicated for 10 s. The cell membrane lysates were centrifuged at 15,000 *g* for 15 min and the supernatant was then collected as the membrane fraction. Membrane fractions containing equal amounts of protein were loaded in each lane and separated on a 10% (or 12%) SDS-polyacrylamide gel and proteins were then transferred to a nitrocellulose membrane by electro-blotting. After blocking the non-specific binding sites using blocking reagents, the nitrocellulose membranes were incubated with primary antibodies against the NMDAR1 subunit (1:1000) and the 5-HT1A receptor (1:1000) for 1 hr at room temperature. Subsequently, the membranes were treated with secondary IgG conjugated to horseradish peroxidase

(1:2000) for 1 hr at room temperature. Secondary antibody bound to proteins was detected using Amersham's ECL chemiluminescence kit. Equal protein loading in blots was confirmed by reprobing with a monoclonal β-actin antibody (1:5000).

Calcium Imaging: The effect of NMDA and 8-OH-DPAT on intracellular Ca^{2+} levels in the mesencephalic cultures was evaluated by using the Fura-2AM fluorescence ratio imaging technique. For these studies, cells were harvested and plated on poly-l-lysine coated coverslips in 35×10 mm petri-dishes and maintained in a 37°C, 5% CO_2 incubator. After 7 days in culture, the cells were loaded with 8 μM Fura-2AM (Molecular probes) for 60 min at 24°C. One μL of 25% (w/w) Pluronic F-127 (Molecular probes) was mixed per 8 μM of Fura-2AM to help dissolve the ester into the aqueous medium. After 1 hr, the primary neurons were washed and loaded onto a perfusion chamber and mounted on a microscope (Carl Zeiss Inc, Germany) for visualization. The cells were continuously perfused with Hepes Buffer containing 8.18g/L NaCl, 0.22g/L $CaCl_2$, 0.373 g/L KCl, 2.6 g/L Hepes, and 900 mg/L glucose. NMDA (300 μM), glycine (5 μM), and 8-OH-DPAT (1 μM) were applied to the cells via this perfusion system. After measurement of base line Ca^{2+}, cellular responses to NMDA and NMDA+8-OH-DPAT were recorded. All image processing and analyses were performed using Attofluor software version 4. Background subtracted ratio images (340/380 nm) were used to calculate intracellular Ca^{2+} levels.

Data analyses and statistics: The data are expressed as mean ± SEM, and statistical significance was determined by analyses of variance with Dunnett's post-hoc test for

multiple comparisons with the control or the Bonferoni's multiple comparison test for multiple comparisons between treatment groups. Single comparisons were made using the Student's *t* test or the Welch-corrected unpaired *t* test, where appropriate.

RESULTS:

Identification of the NMDA and 5-HT1A receptors in M213-2O cells.

M213-2O cells are immortalized rat striatal neurons used as an *in vitro* model to examine the effect of 5-HT1A receptor stimulation on NMDA receptor mediated apoptotic cell death in the striatal region. We first determined if M213-2O cells express the NMDA and 5-HT1A receptors using Western blot and immunohistochemical analyses. M213-2O cells had a neuronal phenotype with numerous processes connecting adjacent cells. As shown in Fig 1A, Western blot analysis revealed a 118 kDa band corresponding to the NMDAR1 receptor subunit. Immunohistochemical results depicted the NMDAR1 receptor subunit in the cell membrane (Fig 1A), supporting the Western blot data. Additionally, Western blot analyses and immunocytochemistry also showed the 47 kDa 5-HT1A receptor protein (Fig 1B). Thus, M213-2O cells express both the 5-HT1A and NMDA receptors.

NMDA induces apoptosis in M213-20 cells.

We employed a delayed excitotoxic treatment paradigm (Choi, 1988; Tenneti and Lipton, 2000) to determine if the M213-2O cells undergo apoptotic cell death in response to NMDA and glutamate treatment. The cells were exposed to serum-free medium containing varying concentrations (100 μM, 500 μM, and 1 mM) of NMDA or glutamate for 20 min and then returned to normal growth medium. Morphologically, the M213-2O cells withdrew their processes and decreased in cell size and cell number in a time-dependent manner after the brief exposure to NMDA (Fig 2).

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We used an ELISA method to quantify DNA fragmentation, a hallmark of apoptosis (Anantharam et al., 2002). Exposure to varying concentrations (100 μM, 500 μM, and 1 mM) of NMDA or glutamate dose- and time-dependently increased DNA fragmentation (Fig 3A and 3B). DNA fragmentation increased two to eight fold over the levels in the untreated groups, during a 12 hr NMDA post-exposure. Pretreatment with 10 μM MK-801, a non-competitive NMDA antagonist, almost completely blocked both the NMDA and glutamate induced DNA fragmentation, indicating that NMDA-mediated apoptotic cell death is primarily mediated by the NMDA receptor.

NMDA induces caspase-3 activation in M213-2O cells.

After confirming that exposure to NMDA induces DNA fragmentation and cell death in M213-2O cells, we examined the involvement of a key upstream mediator of the apoptotic process, namely caspase-3. We first examined caspase activation in M213-2O cells using an *in situ* labeling technique (CasPACE-FITC-VAD-FMK, Promega). As shown in Fig 4A, the number of caspase-activated cells treated with 500 μM NMDA, as determined by the increase in green fluorescence positive cells, increased in a time dependent manner, whereas virtually no activation of caspase-3 occurred in the untreated cells. Next, we quantified caspase-3 enzyme activity 6 and 12 hr after NMDA and glutamate exposures by using a caspase-3 specific peptide substrate, namely Ac-DEVD-AMC. The cells responded to varying concentrations (100 μM, 500 μM, and 1 mM) of both NMDA and glutamate, in a robust fashion. As shown in Fig 4B and 4C, there was a dose-dependent (100 μM, 500 μM, and 1 mM) and time-dependent (6 and 12 hr) three to 20 fold increase in caspase-3 activity post NMDA and glutamate exposure. Pretreatment

with MK-801 almost completely ($p<0.001$) inhibited caspase-3 activity at both 6 and 12 hr after 500 μM NMDA and 1 mM glutamate exposures, confirming caspase-3 activation is primarily mediated by the NMDA receptor.

Stimulation of the 5-HT1A receptor prevents the NMDA-induced increase in caspase-3 activation

In the next series of experiments, we examined the effect of 5-HT1A receptor stimulation on NMDA receptor induced apoptotic cell death. Since we previously established the presence of the 5-HT1A receptor in M213-2O cells, we tested the ability of 8-OH-DPAT, a known 5-HT1A agonist, to block NMDA induced caspase-3 activity in these cells. Caspase-3 activity was increased 700% and 1250% over the control groups at 6 hr and 12 hr post-NMDA exposure, respectively (Fig 5A). Treatment with 8-OH-DPAT (1 μM) dramatically attenuated NMDA induced caspase-3 activation by 300% and 800% at 6 and 12 hr post-exposure, respectively. Further, pretreatment with WAY 100635 (4 μM), a 5-HT1A antagonist, almost completely abolished the 8-OH-DPAT mediated inhibition of NMDA induced caspase-3 activation (Fig 5A), indicating that the observed pharmacological effect was mediated by the 5-HT1A receptor. These results were further confirmed by *in situ* experiments demonstrating caspase-3 activation. As shown in Fig 6, the 8-OH-DPAT plus NMDA treated cells showed very little caspase activation (as indicated by green fluorescence), as compared to the NMDA only treated cells.

To further substantiate the protective role of 5-HT1A receptor stimulation against NMDA induced caspase-3 activation, we used the recently available UH-301

enantiomers; the R-UH-301 enantiomer acts as a 5-HT1A agonist and the S-UH-301 isomer is a 5-HT1A antagonist (Bjork et al., 1992). As shown in Fig 5B, R-UH-301 decreased the NMDA-induced caspase-3 activation in a dose- and time- dependent fashion. The 5-HT1A antagonist S-UH-301 (50 μM) blocked the protective effect of R-UH-301 so that caspase-3 activity nearly reached the levels resulting from NMDA stimulation.

Neuroprotective effect of 5-HT1A receptor agonists against NMDA-induced apoptosis

Since 5-HT1A agonists profoundly reduced NMDA induced caspase-3 activity, we further investigated their potential to protect M213-2O cells from NMDA induced apoptotic cell death. A significant ($p<0.001$) decrease in DNA fragmentation in M213-2O cells resulted from combined 8-OH-DPAT plus NMDA treatment, as compared to NMDA treatment alone (Fig 7A). 8-OH-DPAT decreased NMDA-induced DNA fragmentation to 200% and 400% of control levels at 6 and 12 hr post-treatment, respectively. In addition, when the cells were pretreated with the 5-HT1A antagonist WAY 100635 (4 μM), the protective effect of 8-OH-DPAT on NMDA-induced DNA fragmentation was almost completely reversed. WAY 100635 treatment alone did not alter baseline DNA fragmentation in untreated cells, further confirming the specific role of 5-HT1A receptor activation in protecting against NMDA mediated apoptosis in M213-2O cells. These data also corroborate our results on caspase-3-activity, as discussed in the previous section.

We also assessed the effects of R-UH-301 and S-UH-301 (Fig 7B) on NMDA induced DNA fragmentation. R-UH-301, a 5-HT1A agonist, significantly decreased the

DNA fragmentation induced by NMDA in a dose- and time- dependent fashion (Fig 7B). In contrast, the 5-HT1A antagonist S-UH-301 reversed the effect of R-UH-301 on NMDA induced DNA fragmentation. Neither R-UH-301 nor S-UH-301 treatment alone altered the basal level of DNA fragmentation.

Further, pretreatment with the caspase-3 specific inhibitor Z-DEVD-FMK almost completely blocked NMDA induced DNA fragmentation, suggesting that DNA fragmentation is mediated by caspase-3 (Fig 7A). The above quantitative determination of the effect of 8-OH-DPAT on DNA fragmentation was reconfirmed with Hoechst 33342 staining of nuclear condensation in M213-2O cells. As shown in the series of panels in Fig 8, chromatin condensation in the 8-OH-DPAT plus NMDA or Z-DEVD-FMK plus NMDA treated cells was completely attenuated as compared to NMDA treatment alone. These results confirm the protective action of 8-OH-DPAT in caspase-3 mediated apoptosis in M213-2O cells following NMDA receptor activation.

8-OH-DPAT protects against NMDA-induced apoptosis in primary mesencephalic cultures.

In the next series of experiments, we sought to determine the effects of 8-OH-DPAT on NMDA-mediated excitotoxicity in a primary mesencephalic neuronal culture, a classic *in vitro* PD model. NMDA (300 μM) and glutamate (500 μM) significantly ($p<0.001$) increased caspase-3 activation (Fig 9A) and DNA fragmentation (Fig 9B) 6 hr after a brief exposure (20 min) to NMDA or glutamate. We chose a 6 hr time point in these studies because caspase-3 activation peaked at this time point (data not shown). Pretreatment with MK-801 (10 μM) significantly ($p<0.001$) blocked the caspase-3

activity induced by NMDA, confirming that this effect is NMDA receptor mediated. Similar to the effects in M213-2O cells, 8-OH-DPAT (1 μM) completely inhibited the NMDA-stimulated caspase-3 activity (Fig 9A). 8-OH-DPAT pretreatment decreased NMDA-stimulated caspase-3 activity by 350%, as compared to NMDA treatment. Also, WAY 100635 almost completely antagonized the protective effect of 8-OH-DPAT on NMDA induced caspase-3 activity. These results demonstrate the role of 5-HT1A receptor stimulation in interfering with the NMDA mediated apoptotic cascade.

Caspase-3 activation and DNA fragmentation following NMDA receptor activation were closely correlated in primary mesencephalic cultures. DNA fragmentation analysis revealed a 750% and 650% increase in response to NMDA and glutamate, respectively (Fig 9B). MK-801 blocked the NMDA-induced DNA fragmentation by 600%, reconfirming that apoptotic cell death is mediated via NMDA receptor stimulation. Consistent with the observation in M213-2O cells, 8-OH-DPAT treatment significantly ($p<0.01$) decreased the NMDA-induced DNA fragmentation in primary mesencephalic neurons. Treatment with WAY 100635 blocked the antiapoptotic effect of 8-OH-DPAT, confirming that 5-HT1A receptor stimulation reduces excitotoxic apoptosis in mesencephalic neurons. Additionally, Z-DEVD-FMK, a caspase-3 specific inhibitor, blocked NMDA induced DNA fragmentation, suggesting that apoptosis in mesencephalic cells is caspase-3 mediated.

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8-OH-DPAT protects TH-positive cells against NMDA-mediated excitotoxicity in primary mesencephalic cultures.

The primary mesencephalic cultures contained about 10-15% of TH-positive cells. Therefore, we determined whether 8-OH-DPAT specifically protects dopaminergic neurons. After treatment with NMDA (300 μM) or NMDA plus 8-OH-DPAT (1 μM), the primary cultures were subjected to TH immunocytochemical staining. The TH-positive cells were counted in each treatment group. As shown in Fig 10, the number of TH-positive cells was significantly ($p<0.001$) decreased from 12% in the control cells to 5.5% in the NMDA treated cells. In contrast, the TH-positive cell count was similar to control levels in cultures pretreated with 8-OH-DPAT followed by NMDA treatment. Thus, the results demonstrate the neuroprotective effect of 5-HT1A receptor stimulation against NMDA-induced degeneration of TH-immunoreactive cells.

5-HT1A receptor activation protects against MPP^+ induced apoptosis in primary mesencephalic cultures.

In this set of experiments we studied the ability of 5-HT1A receptor activation to protect against apoptosis induced by the Parkinsonian toxin MPP^+ in primary mesencephalic cultures.

MPP^+ (10 μM) exposure for 48 hrs significantly ($p<0.01$) increased caspase-3 activation and DNA fragmentation in the primary mesencephalic neurons. 8-OH-DPAT (1 μM) significantly ($p<0.01$) inhibited the MPP^+ induced caspase-3 activity (Fig. 11A). There was a nearly 300% decrease in the MPP^+ induced caspase-3 activity following 8-OH-DPAT pretreatment, as compared to MPP^+ treatment alone. Also, WAY 100635

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almost completely antagonized the protective effect of 8-OH-DPAT on MPP^+ induced caspase-3 activity. In addition, R-UH-301 (50 μM) decreased the MPP^+ induced caspase-3 activity by almost 320%. This protective effect of R-UH-301 against MPP^+ induced caspase-3 activation was almost completely reversed by the 5-HT1A antagonist S-UH-301.

Furthermore, MPP^+ induced DNA fragmentation was significantly ($p<0.001$) blocked by pretreatment with either 8-OH-DPAT or R-UH-301 in the primary mesencephalic neurons (Fig. 11B). Additionally, when the cells were pretreated with the 5-HT1A antagonists WAY 100635 (4 μM) and S-UH-301, the protective effects of the respective agonists 8-OH-DPAT and R-UH-301 on MPP^+ induced DNA fragmentation were almost completely reversed, further confirming the specific role of 5-HT1A receptor activation in protecting against MPP^+ induced apoptosis. Treatment with the antagonists alone did not alter the baseline levels of DNA fragmentation in untreated cells (data not shown). Together, these results demonstrate that 5-HT1A receptor stimulation can inhibit the MPP^+ induced apoptotic cascade.

8-OH-DPAT inhibits NMDA induced Ca^{2+}-influx in primary mesencephalic cultures.

Finally, we examined whether 8-OH-DPAT blocks NMDA-induced Ca^{2+}-influx in primary mesencephalic cultures, as a possible mechanism for its neuroprotective effect against NMDA mediated excitotoxicity. NMDA (300 μM) exposure resulted in almost a three fold increase in intracellular Ca^{2+}-levels, as shown in Fig. 12A. Pretreatment with 8-OH-DPAT for 2 min significantly ($p<0.01$) attenuated the NMDA induced Ca^{2+}-influx. The images in Fig. 12B qualitatively depict the intracellular Ca^{2+}-changes in

response to NMDA and NMDA+8-OH-DPAT. The increase in intracellular Ca^{2+} is indicated by the progressive change from blue to red in the color-calibrated scale. These results suggest that the observed neuroprotective effect of 5-HT1A agonists may be due to attenuation of NMDA induced Ca^{2+} -overload.

DISCUSSION:

We demonstrate in the present study that activation of the 5-HT1A receptor blocks NMDA induced caspase-3 activation and DNA fragmentation in immortalized striatal cells and primary mesencephalic dopaminergic neurons. The dose-response effect of the 5-HT1A agonists on NMDA induced apoptosis and the blockade by specific 5-HT1A antagonists suggest receptor-mediated neuroprotection via 5-HT1A receptor stimulation. To our knowledge, this is the first study demonstrating the antiapoptotic properties of 5-HT1A receptor stimulation and the resulting protection against excitotoxic insult in nigrostriatal neuronal cells.

NMDA receptor mediated excitotoxicity may play a role in the etiopathogenesis of PD (Choi, 1988; Olanow and Tatton, 1999). Hence, development of NMDA receptor antagonists for therapeutic use in PD has been pursued actively during the past few years. But these glutamate antagonists have very low effectiveness, and a plethora of side effects which have limited their clinical utility as neuroprotectants (Sonsalla et al., 1992; Engber et al., 1994; Nilsson et al., 1997; Li et al., 2002). An alternate strategy is to attenuate the excitotoxic response in the basal ganglia by indirectly modulating other interacting neurotransmitter systems. The serotonergic system is a major neurotransmitter system that interacts with the key neurotransmitters of the basal ganglia including glutamate, dopamine, and GABA (Hornykiewicz, 2001). Also, pharmacological modulation of the serotonergic system has yielded more successful neuropharmacological drugs than modulation of any other neurotransmitter system in the CNS. Among the different 5-HT receptors, the 5-HT1A receptor subtype appears to

attenuate excitotoxicity. 5-HT1A agonists have been used to rescue cultured hippocampal neurons from glutamate induced excitotoxicity and are also protective in ischemic neuronal cell death (Nakata et al., 1997; Harkany et al., 2001). Our results indicate that 5-HT1A receptor stimulation rescues both striatal and nigral neurons from the NMDA-induced degenerative process.

Many *in vivo* and *in vitro* data support apoptotic involvement in neurodegenerative processes, including PD (Hartmann et al., 2000; Hirsch et al., 1999), however, the mode of cell death in PD remains controversial (Jellinger, 2001). Caspase-3 is a critical effector caspase (Cohen, 1997), which activates a host of downstream events leading to fragmentation of genomic DNA, a hallmark of apoptotic cell death. Caspase-3 is a critical effector cysteine protease (Cohen, 1997) that activates a host of downstream events leading to fragmentation of genomic DNA, a hallmark of apoptotic cell death. Caspase-3 activation is involved in apoptotic cell death in cell culture models and animal models of PD (Dodel et al., 1998; Turmel et al., 2001). Recent studies have identified pro-apoptotic molecules such as cytochrome c and caspase-3 in the Lewy bodies in post-mortem brains of PD patients (Hirsch et al., 1999; Hartmann et al., 2000). In the present study, we show that both striatal-derived cells and nigral neurons undergo apoptosis by caspase-3 activation in a dose- and time-dependent manner after a brief exposure to NMDA and glutamate.

In support of our results, previous studies have shown that intrastriatal injection of NMDA induces apoptosis in rats (Qin et al., 1996). Stimulation of the 5-HT1A receptor attenuates neuronal excitation (Oosterink et al., 1998; Harkany et al., 2001). Serotonin in the basal ganglia modulates dopamine related motor activity through selective receptor

subtypes including 5-HT1A (Doherty and Pickel, 2001). Our study demonstrates that pharmacological stimulation of the 5-HT1A receptor negatively modulates the apoptotic cascade activated by excessive neuronal stimulation in cells derived from the basal ganglia. The stereo-specific effect of the UH-301 isomers demonstrates the specificity of the observed neuroprotective action of 5-HT1A receptor stimulation. The data obtained using TH staining suggest that the 5-HT1A receptor also has neuroprotective effects in dopaminergic neurons in the substantia nigra. Additionally, our results indicate that 5-HT1A receptor stimulation can effectively protect against MPP^+ toxicity in primary mesencephalic neurons. Together, our data suggest that the 5-HT1A receptor appears to be a viable target for development of a neuroprotective pharmacological agent designed to interfere with the apoptotic cell death process in PD neurodegeneration.

The cellular mechanisms underlying the antiapoptotic effect of 5-HT1A receptor activation are not completely understood. The 5-HT1A receptors are G-protein coupled receptors known to couple with Gi/G0 proteins to mediate a range of biological effects (Raymond et al., 2001). The Gα subunit of the 5-HT1A receptor negatively modulates cAMP signaling, which subsequently reduces the phosphorylation of ion channels and thereby reduces neuronal excitation (Raymond et al., 2001; Carr et al., 2002). Our data from the Ca^{2+}-imaging studies suggest that one of the possible mechanisms involved in the protective effect of 5-HT1A receptor agonists against NMDA mediated excitotoxicity is the attenuation of NMDA induced Ca^{2+}-influx. In addition, previous studies have shown that 5-HT1A receptor stimulation can modulate NMDA receptor induced Ca^{2+}-influx (Strosznajder et al., 1996; Matsuyama et al., 1997). Other mechanisms, in addition to inhibition of NMDA induced Ca^{2+}-influx, may be important in the neuroprotection

resulting from 5-HT1A receptor stimulation. Emerging studies indicate that the Gβγ subunit of the 5-HT1A receptor transduces important signaling pathways via PLCβ and MAPK, leading to cell proliferation and transformation (Raymond et al., 2001). The 5-HT1A induced MAPK activation is inhibited by blockers of several signaling molecules, such as PI-3-K, RAS-MAPK pathway inhibitors, Ca^{2+} chelation, Gβγ blockers, and reactive oxygen species blockers (Cowen et al., 1996; Garnovskaya et al., 1996; Mukhin et al., 2000). Further clarification of other signaling molecules involved in the neuroprotective pathway mediated by 5-HT1A receptor stimulation against NMDA induced apoptosis in striatal neurons and primary cultures will provide insight into the cellular mechanisms underlying the neuroprotective effects of 5-HT1A agonists against excitotoxic apoptosis.

In conclusion, our study indicates that 5-HT1A receptor stimulation could serve as a promising therapeutic approach for modulating excitotoxin-induced degenerative processes. Also, our results indicate that M213-2O cells appear to be a suitable model for studies concerning striatal degeneration via NMDA-induced cell death. Forthcoming studies will attempt to further delineate signaling pathways and molecular events which are involved in 5-HT1A receptor-mediated neuroprotection.

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LEGENDS:

Fig 1. Identification of the NMDA and the 5-HT1A receptor proteins in M213-2O cells. **A**. Immunocytochemical staining of M213-2O cells for the NMDAR1 receptor protein. Western blot analysis detected a 118 kDa band corresponding to the molecular weight of the NMDAR1 receptor subunit. **B.** Immunocytochemical staining for the 5-HT1A receptor protein showed positive 5-HT1A receptor expression. Western blot analysis revealed a 47 kDa band corresponding to the 5-HT1A protein. Magnification 600X. The calibration bar in the figure corresponds to 20 microns.

Fig 2. NMDA induced morphological changes in M213-2O cells. The upper panel shows a picture of untreated M213-2O cells. The cells show a polygonal, multipolar, phenotype with well-developed processes. The lower panels show M213-2O cells at 6 hr and 12 hr following treatment with 500 μM NMDA. The cells were decreased in number and in size and had shrunken processes. Magnification 200X. The calibration bar in the figure corresponds to 20 microns.

Fig 3. NMDA treatment induces apoptosis in M213-2O cells. DNA fragmentation was measured by DNA ELISA assay. **A.** The M213-2O cells showed a dose- and time-dependent increase in DNA fragmentation following brief exposure to NMDA (NM) (OD values/30,000 cells: Control-0.098, 1 mM NMDA-0.539). The NMDA response was blocked by 10 μM MK-801. **B.** The cells also responded to glutamate (Glu) exposure and showed a dose- and time-dependent increase in DNA fragmentation (OD/30,000

cells: Control-0.091, 1 mM glutamate -0.333)as compared to the control cells. The glutamate response was blocked by 10 μM MK-801 pretreatment. Data are expressed as the means ± SEM of n of at least 6 from three separate experiments. *p<0.05, **p<0.01, ***p<0.001 as compared to control. ##p<0.01, ###p<0.001 as compared to 500 μM NMDA treatment.

Fig 4. NMDA induces caspase-3 activation in M213-2O cells. A. *In situ* labeling with 10 μM FITC-VAD-FMK showed an increase in caspase-3 activation (green fluorescent positive cells) in a time dependent (6 and 12 hr) manner. Magnification 400X. The calibration bar in the figure corresponds to 20 microns. **B.** 500 μM NMDA (NM) treatment showed a dose- and time-dependent increase in caspase-3 enzyme activity. The caspase-3 activity was measured using the caspase-3 specific substrate, Ac-DEVD-AMC. The NMDA-induced caspase-3 activity was blocked by 10 μM MK-801. **C.** Glutamate (Glu; 1mM) treatment produced a dose- and time- dependent increase in caspase-3 activation as compared to the control. Glutamate-induced caspase-3 activation was blocked by 10 μM MK-801. Data are expressed as means ± SEM of n of at least 6 from three separate experiments. *p<0.05, p<0.01, ***p<0.001 as compared to control, ###p<0.001 as compared to 500 μM NMDA treatment.

Fig 5. 5-HT1A stimulation inhibits NMDA mediated caspase-3 activity. A. Brief exposure to 500 μM NMDA (NM) profoundly increased caspase-3 activity. The caspase-3 activity was measured using the caspase-3 specific substrate, Ac-DEVD-AMC. Pretreatment with 1 μM 8-OH-DPAT (DPT) blocked the NMDA-induced caspase-3

activation in a time-dependent manner. Pretreatment with 4 μM WAY100635 (WAY; 5-HT1A antagonist) reversed the protective effect of 8-OH-DPAT on NMDA-induced caspase-3 activation. 8-OH-DPAT, WAY100635, and NM+WAY1000635 treatment alone did not alter baseline caspase-3 activation in control cells. **B.** R-UH-301 (R; 5-HT1A agonist) attenuated the NMDA-induced caspase-3 activation in a dose- and time-dependent fashion. 50 μM S-UH-301 (S; 5-HT1A antagonist) reversed the inhibitory effect of R-UH-301 on NMDA induced caspase-3 activation. R- and S-UH-301 did not alter basal caspase-3 activity. Data are expressed as the means ± SEM of n of at least 6 from three separate experiments. ***p<0.001 as compared to control, ##p<0.01, ###p<0.001 as compared to 500 μM NMDA treatment.

Fig 6. Attenuation of NMDA-induced caspase activation *in situ* by 8-OH-DPAT. The first panel shows that there was virtually no activation of caspase in untreated M213-2O cells. The second panel shows 500 μM NMDA-induced caspase-3 activation, as indicated by the increase in green fluorescence positive cells. The third panel shows that pretreatment with 1 μM 8-OH-DPAT almost completely prevented NMDA-induced caspase activation. Magnification 600X. The calibration bar in the figure corresponds to 20 microns.

Fig 7. 5-HT1A stimulation attenuates NMDA-mediated DNA fragmentation in M213-2O cells. A. Brief exposure to 500 μM NMDA (NM) caused a significant increase in DNA fragmentation in M213-2O cells (OD values/30,000 cells: Control-0.107, NMDA-0.374). Pretreatment with 1 μM 8-OH-DPAT (DPT) almost completely

inhibited NMDA-induced DNA fragmentation in a time-dependent manner. Pretreatment with 4 μM WAY100635 (WAY; 5HT1A antagonists) reversed the inhibitory effect of 8-OH-DPAT on NMDA-mediated DNA fragmentation . 8-OH-DPAT, WAY100635, and NM+WAY1000635 treatment alone did not significantly increase DNA fragmentation as compared to the control group. **B.** R-UH-301 (R; 5HT1A agonist) attenuated NMDA-mediated DNA fragmentation in a dose- (1, 10, and 50 μM) and time- (6 and 12 hr) dependent fashion. 50 μM S-UH-301 (S; 5HT1A antagonist) reversed the inhibitory effect of R-UH-301 on NMDA-mediated apoptosis. R- and S-UH-301 treatment alone did not alter baseline DNA fragmentation (OD values/30,000 cells: Control-0.089, NMDA-0.334). Data are expressed as means ± SEM of n of at least 6 from three separate experiments. **p<0.01, ***p<0.001 as compared to control. #p<0.05, ##p<0.01, ###p<0.001 as compared to 500 μM NMDA treatment.

Fig 8. Qualitative analysis of 8-OH-DPAT inhibition of DNA fragmentation by Hoechst 33342 nuclear staining. All cells showed nuclear staining by Hoechst 33342. 500 μM NMDA treated cells showed apparent nuclear condensation, as indicated by the arrows. In contrast, cells pretreated with 8-OH-DPAT (1 μM) or Z-DEVD-FMK (50 μM) show virtually no signs of nuclear condensation. Magnification 200X. The calibration bar in the figure corresponds to 20 microns.

Fig 9. 8-OH-DPAT inhibits caspase-3 activation and DNA fragmentation induced by NMDA in primary mesencephalic neurons. A. Brief exposure to 300 μM NMDA (NM) and 500 μM glutamate (Glu) caused a significant increase in caspase-3 activation

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in rat primary mesencephalic neurons. The caspase-3 activity was measured using the caspase-3 specific substrate, Ac-DEVD-AMC. Caspase-3 activation was inhibited significantly ($p<0.001$) by 1 μM 8-OH-DPAT (DPT) and 10 μM MK-801. The protective effect of 8-OH-DPAT against NMDA induced caspase-3 activation was reversed by 4 μM WAY100635 (WAY; 5-HT1A antagonist). **B.** 300 μM NMDA (NM) and 500 μM glutamate (Glu) significantly increased DNA fragmentation in primary mesencephalic neurons (OD values/30,000 cells: Control-0.091, NMDA-0.525). DNA fragmentation was inhibited significantly ($p<0.01$) by 1 μM 8-OH-DPAT and 10 μM MK-801 ($p<0.001$). WAY 100635 reversed the inhibitory effect of 8-OH-DPAT on NMDA induced DNA fragmentation. Data are expressed as means ± SEM of n of at least 6 from three separate experiments. ***$p<0.001$ as compared to control. ##$p<0.01$, ###$p<0.001$ as compared to 500 μM NMDA treatment.

Fig 10. Protection against loss of TH-positive cells by 8-OH-DPAT following NMDA exposure. NMDA (300 μM) reduced the TH-positive cell number by more than 50% in primary mesencephalic cultures. Pretreatment with 1 μM 8-OH-DPAT (DPT) restored the percentage of TH-positive cells to near to control levels. Magnification 400X. The data are the means of 25 different fields of two different sets of cultures. The table below the graph shows the total and TH-positive cell numbers (mean ± SEM) in response to NMDA and NMDA+DPT treatment. ***$p<0.001$ as compared to control. ###$p<0.001$ as compared to 300 μM NMDA treatment.

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Fig. 11. 5-HT1A receptor activation attenuates caspase-3 activation and DNA fragmentation induced by MPP^+ in primary mesencephalic neurons. **A.** Brief exposure to 10 μM MPP^+ caused a significant ($p<0.01$) increase in caspase-3 activation in rat primary mesencephalic neurons. The caspase-3 activity was measured using the caspase-3 specific substrate, Ac-DEVD-AMC. Caspase-3 activation was inhibited significantly ($p<0.001$) by 1 μM 8-OH-DPAT (DPT) and 50 μM R-UH-301. The protective effects of 8-OH-DPAT and R-UH-301 against MPP^+ induced caspase-3 activation were reversed by the 5-HT1A antagonists WAY 100635 (WAY; 4 μM) and S-UH-301 (50 μM), respectively. **B.** 10 μM MPP^+ significantly increased DNA fragmentation in primary mesencephalic neurons (OD values/30,000 cells: Control-0.091, MPP^+-0.448). DNA fragmentation was inhibited significantly ($p<0.001$) by 1 μM 8-OH-DPAT and 50 μM R-UH-301 ($p<0.001$). WAY 100635 and S-UH-301 reversed the inhibitory effect of 8-OH-DPAT and R-UH-301, respectively, on MPP^+ induced DNA fragmentation. Data are expressed as means ± SEM of n of at least 6 from three separate experiments. **$p<0.01$, ***$p<0.001$ as compared to control. ##$p<0.01$, ###$p<0.001$ as compared to 10 μM MPP^+ treatment.

Fig. 12. Effect of 8-OH-DPAT on NMDA-induced Ca^{2+}-influx in primary mesencephalic cultures. **A.** Brief exposure to NMDA (300 μM) resulted in a significant ($p<0.01$) increase in the Ca^{2+}-influx. This increase in intracellular Ca^{2+}-levels was attenuated by pretreatment with 8-OH-DPAT (1 μM). The values are means ± SEM from 3 independent experiments, with a total of 140 regions monitored. **$p<0.01$ as compared

to the baseline Ca^{2+}-concentration; ##p<0.01, as compared to the peak Ca^{2+}-concentration in response to NMDA treatment.

B. The fluorescent images corresponding to the baseline, NMDA-induced, and NMDA+DPT induced Ca^{2+}-influx are shown in the lower panel, just below the graphs.

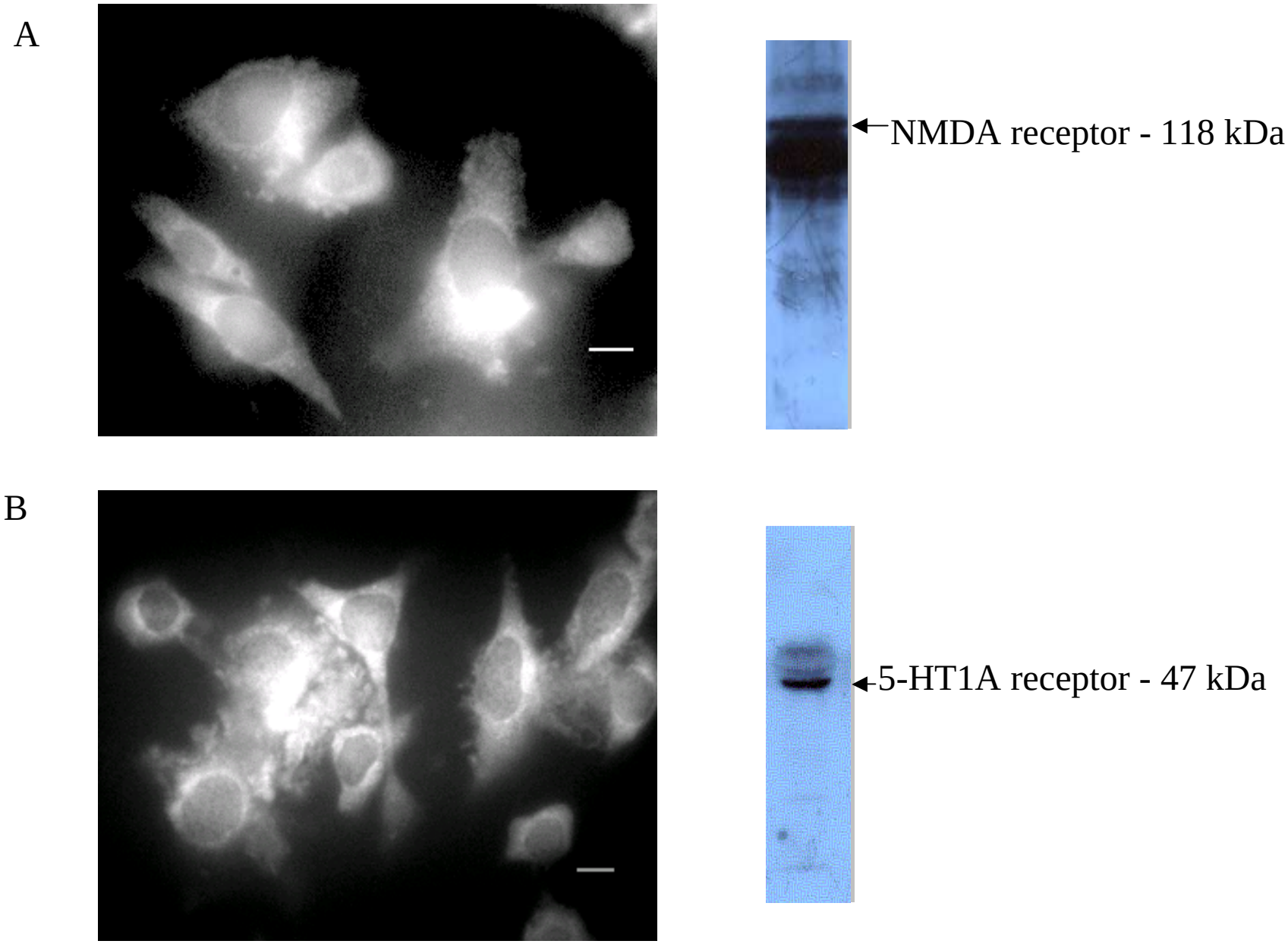


Fig 1

Untreated

6 hr post NMDA treatment

12 hr post NMDA treatment

Fig 2

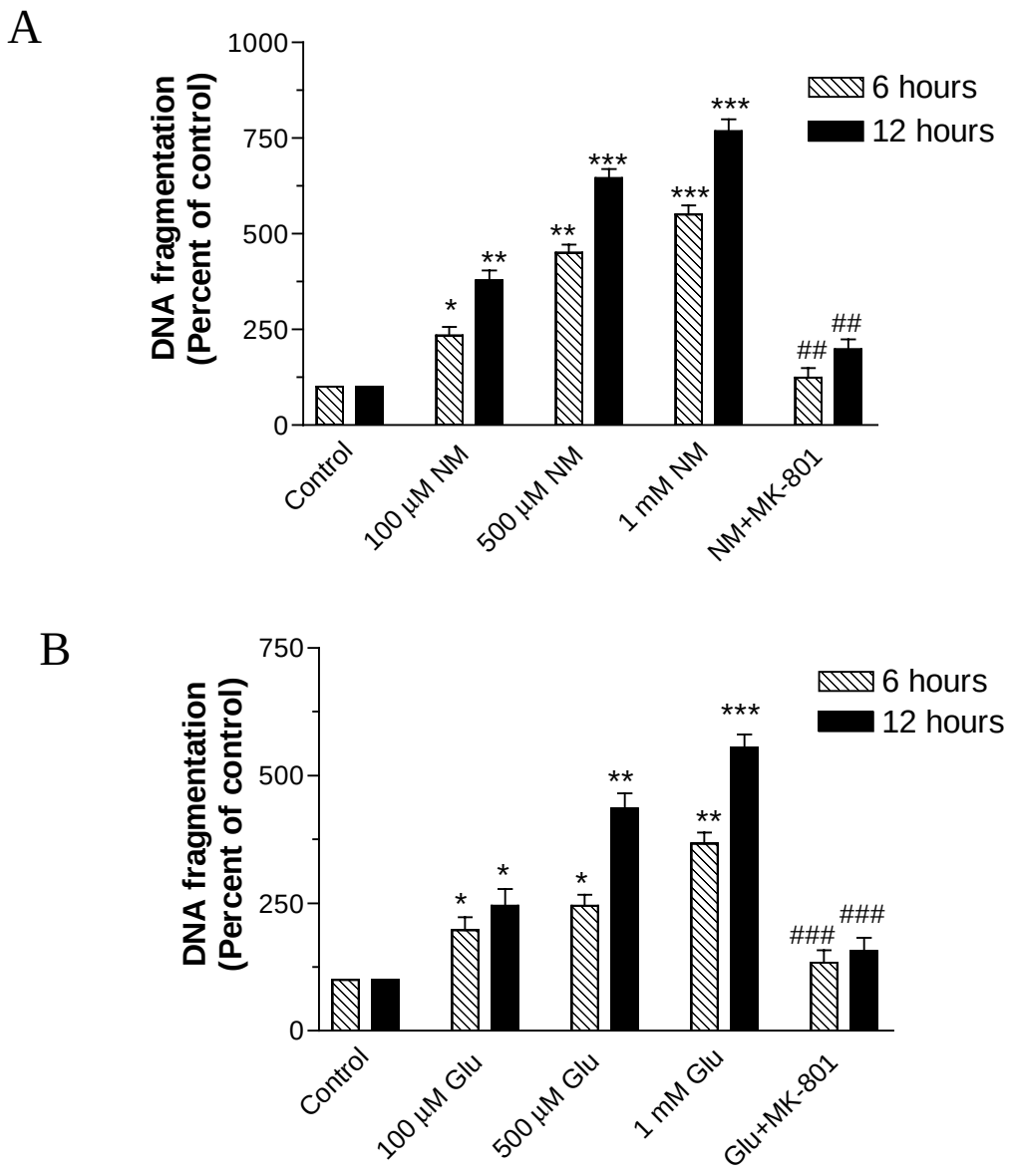


Fig 3

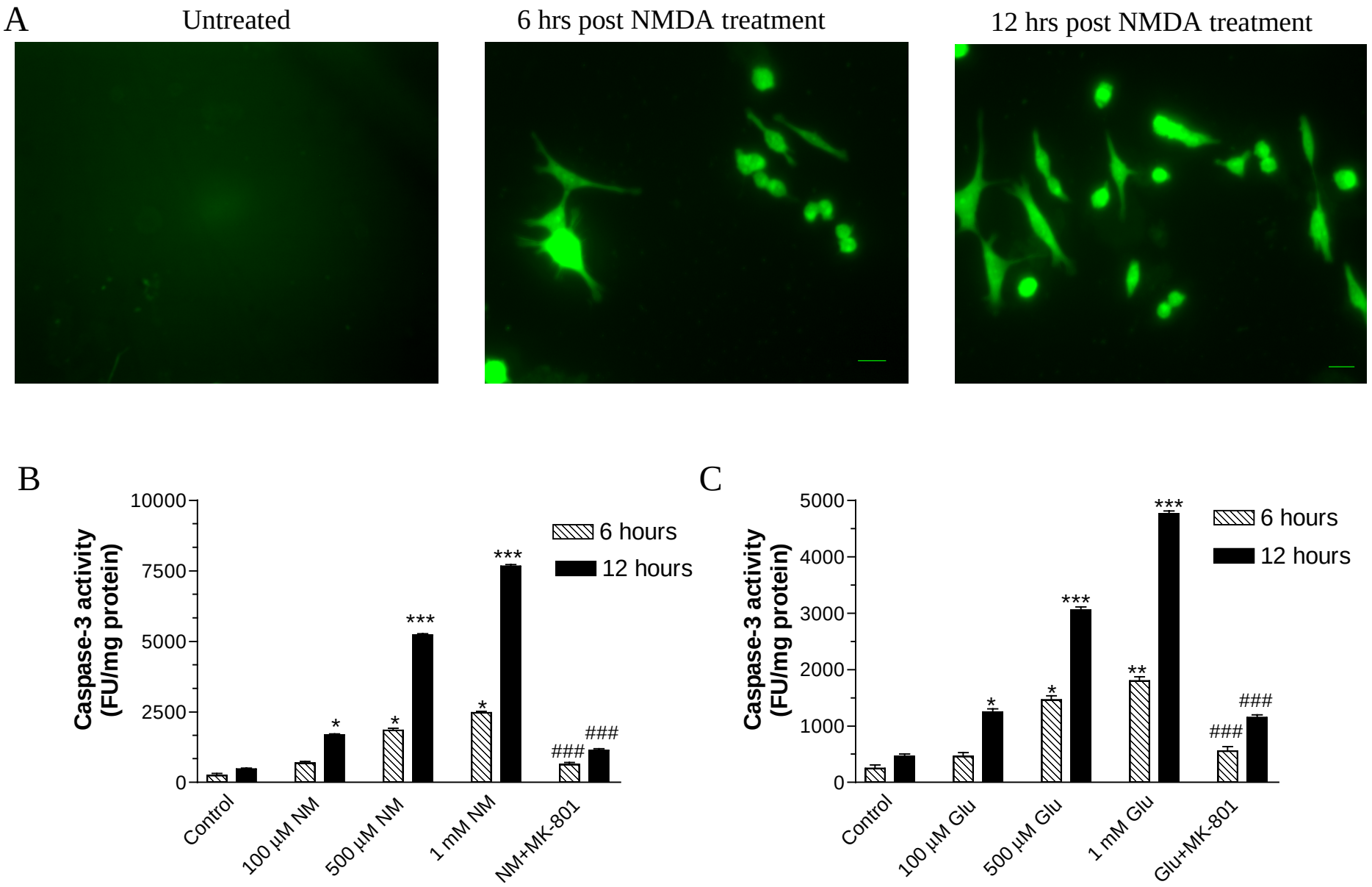
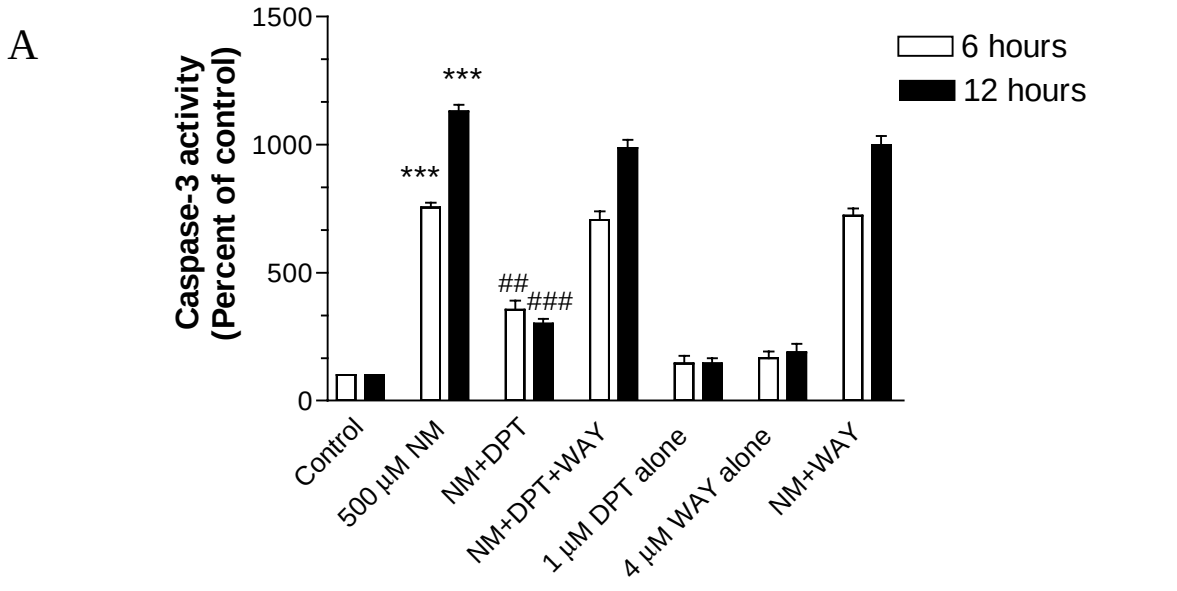


Fig 4



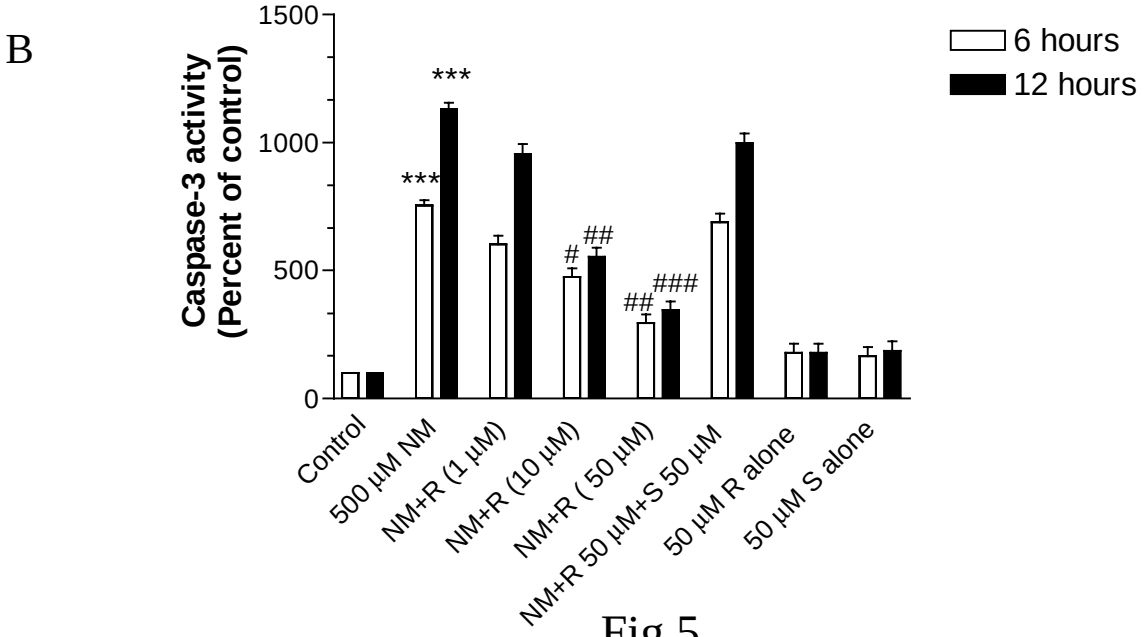


Fig 5

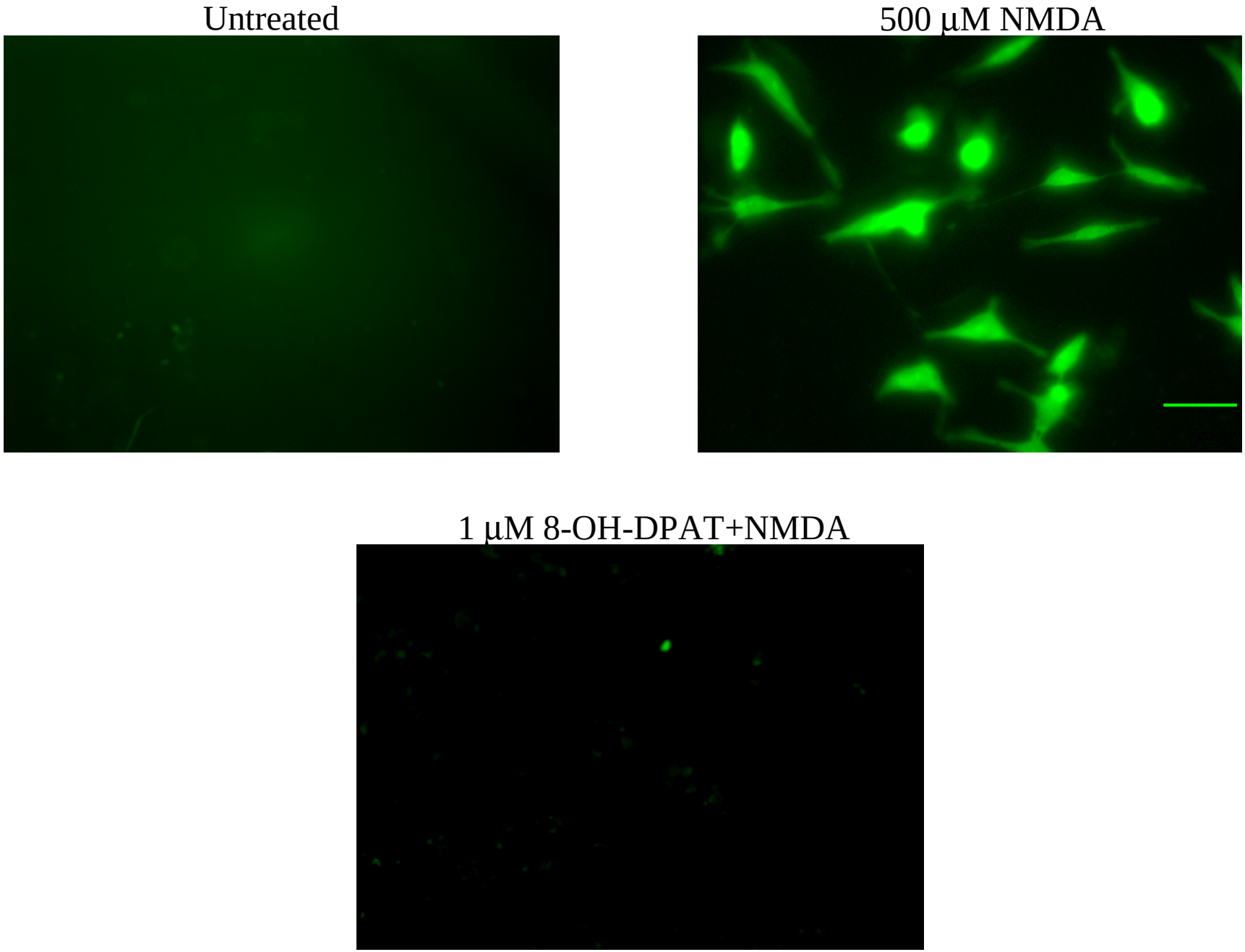


Fig 6

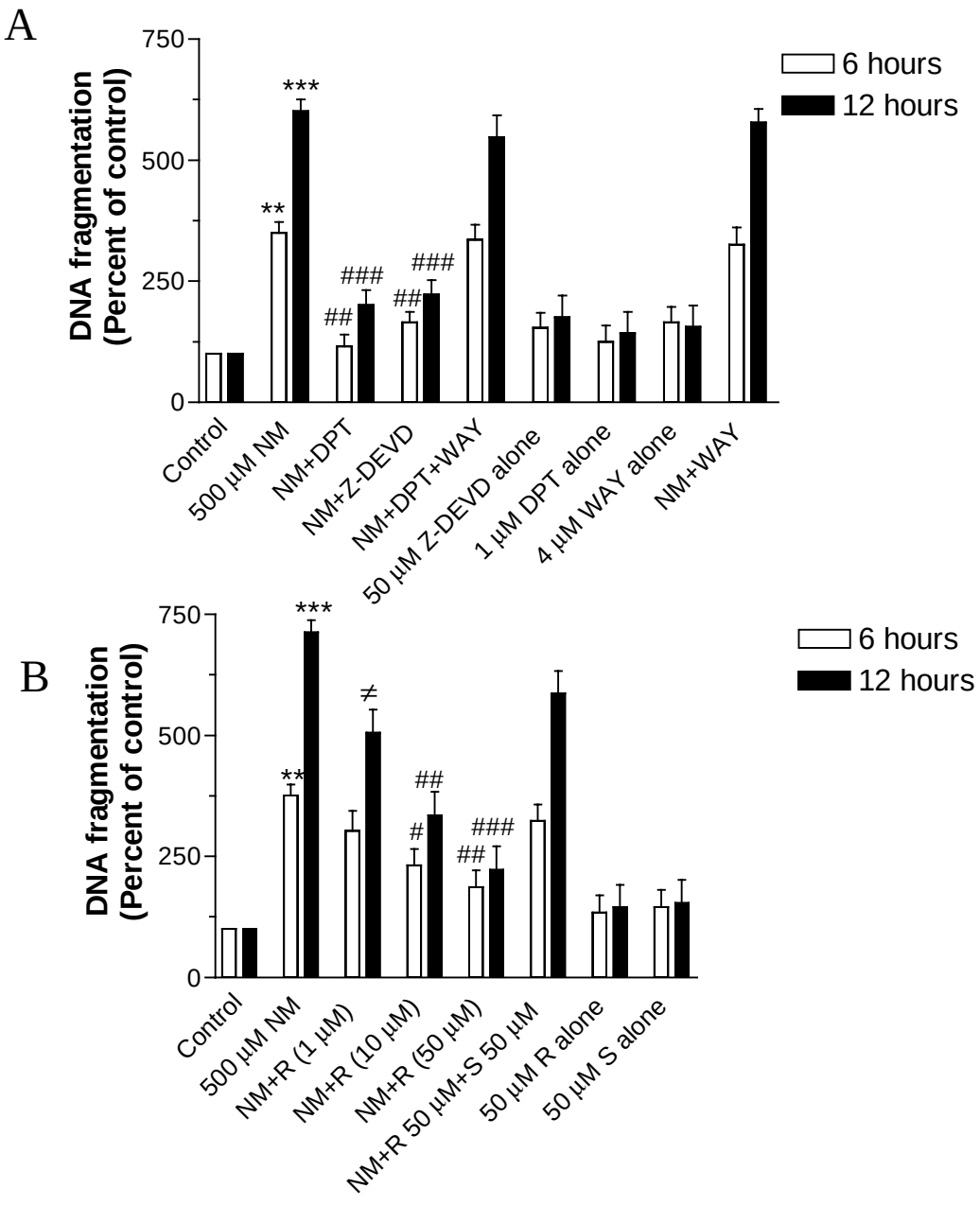


Fig 7

Fig 8

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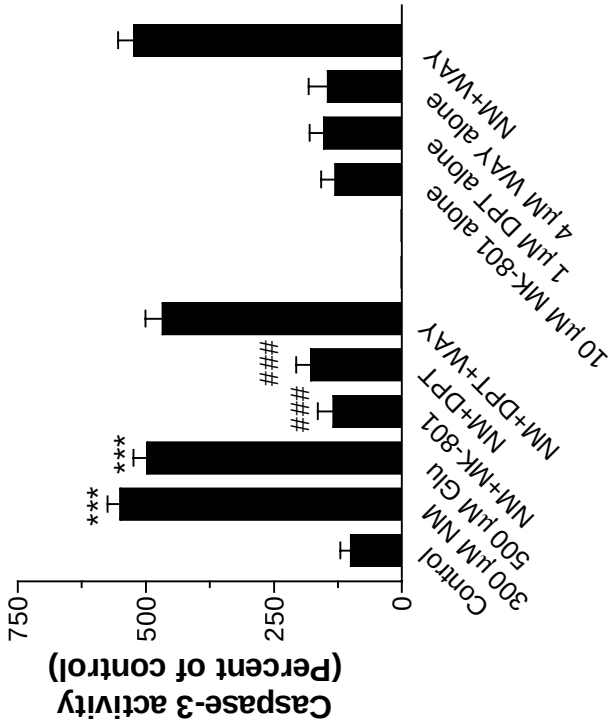

Fig 9A

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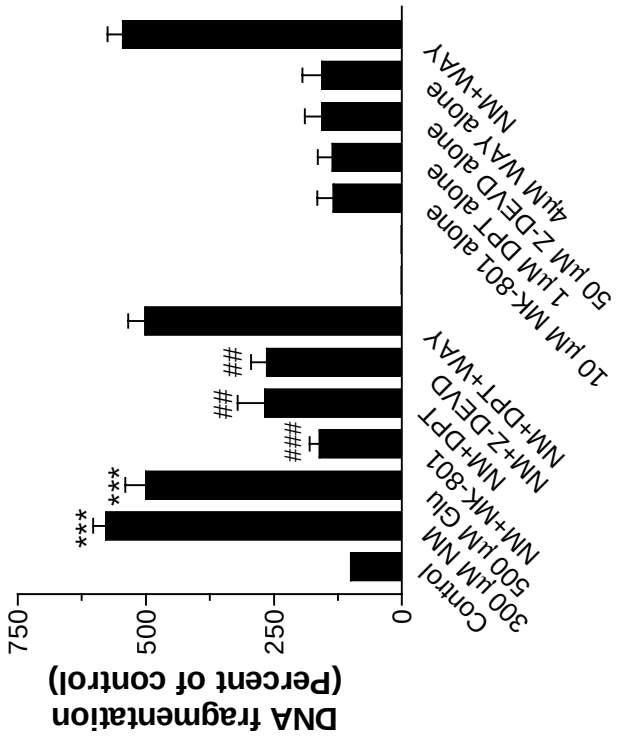
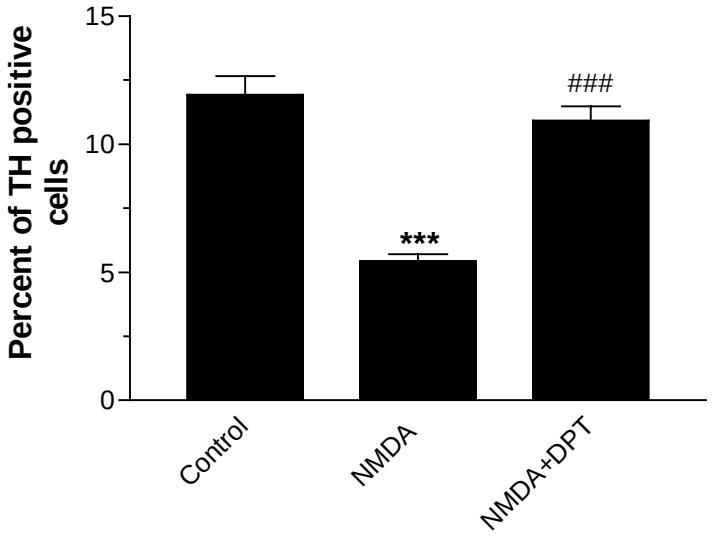


Fig 9B



	Control	NMDA	NMDA+DPT
TH+ive cell number	17.29±1.94	7.87±1.15	15.83±1.29
Total cell number	145±15.55	84.8±11.37	110.02±13.35

Fig 10

JPET Fast Forward. Published on November 25, 2002 as DOI: 10.1124/jpet.102.044370
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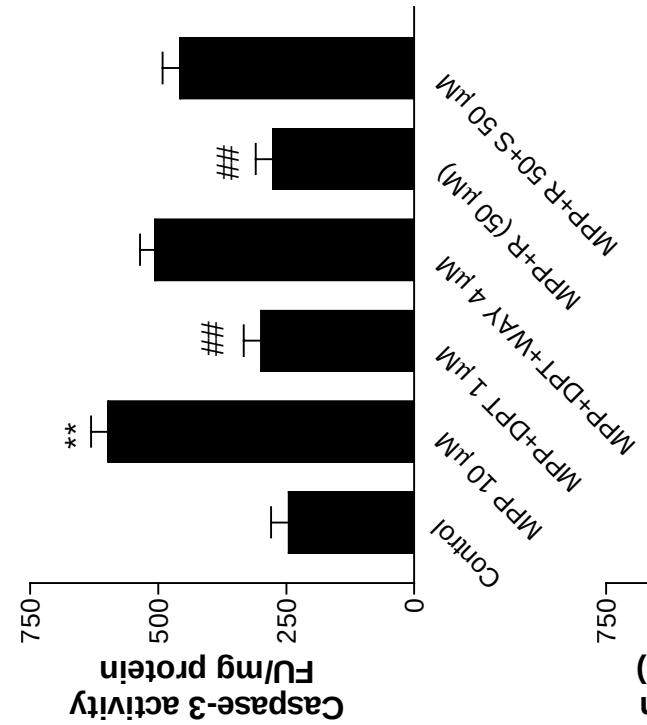
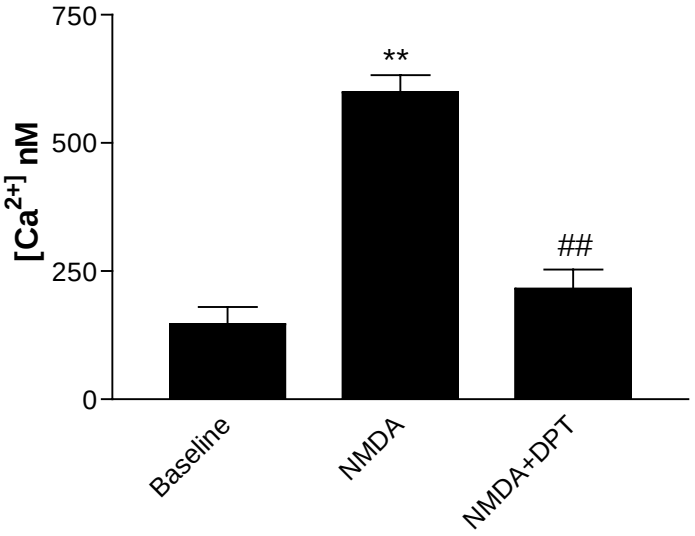
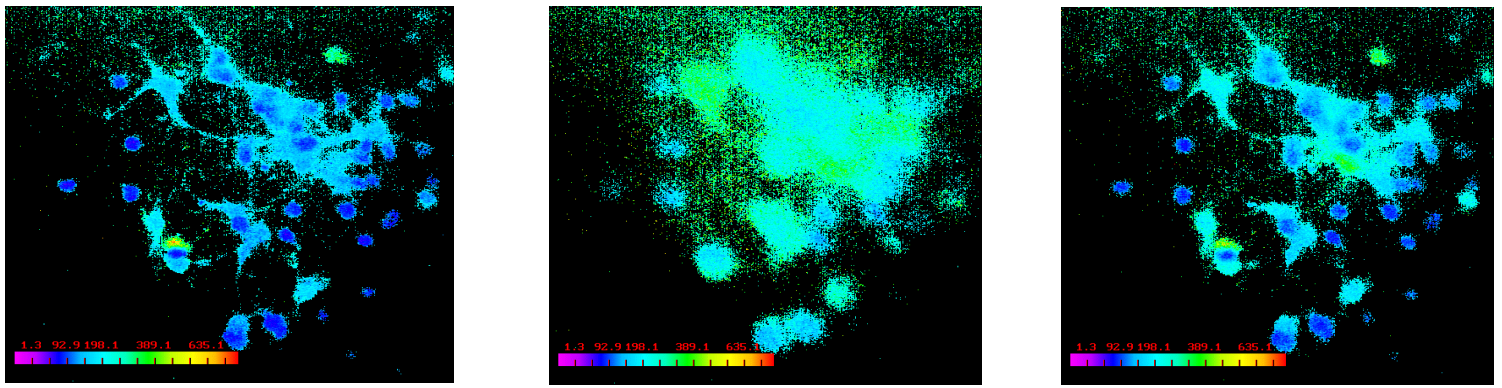


Fig 11

A



B



BASELINE NMDA NMDA+DPT

Fig 12