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NMDA attenuates CXCR2-mediated neuroprotection through enhancing the receptor phosphorylation and blocking the receptor recycling

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Abbreviations Used: AD, Alzheimer's disease; NMDA, N-methyl-D-aspartate; PBS, phosphate buffered saline; CNS, central nervous system; MIP2, macrophage inflammatory protein 2; DMEM, Dulbecco's Modification of Eagle's Medium; NG108-15, Neuroblastoma-glioma hybrid cells; LDH, Lactate dehydrogenase; TUNEL, Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay; FACS, Fluorescence-activated cell sorting analysis; PP2A, protein phosphatase 2A; ERK, extracellular signal regulated kinase; ECL, enhanced chemiluminescence.

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Abstract

Abnormal extracellular accumulations of β -amyloid, a major component of the senile plaques, and of the excitatory amino acid glutamate are both thought to be associated with degeneration of nerve cells in the central nervous system of Alzheimer's disease (AD) patients. The chemokine receptor CXCR2 has been shown to play a role in protecting neurons against β -amyloid induced injury *in vitro*, it remains unclear whether CXCR2-mediated neuroprotection is affected by glutamate. We demonstrated that pre-treatment of hippocampal neurons with a sublethal concentration of N-methyl-D-aspartate (NMDA) attenuated the macrophage inflammatory protein 2 (MIP2) induced protection against β -amyloid induced neuronal death. The NMDA induced inhibition was blocked by (+)-5-methyl-10, 11-dihydro-5H-dibenzo [a,d] cyclohepten-5,10-imine maleate (MK-801), a non-competitive NMDA receptor antagonist, indicating involvement of NMDA receptors in this process. A sublethal dose of NMDA pre-treatment induced CXCR2 phosphorylation, although in a less extent compared to the receptor phosphorylation induced by MIP2, and differential serine residues were involved in NMDA- and MIP2-induced CXCR2 phosphorylation. Moreover, NMDA-treatment reduced the CXCR2-mediated Ca^{2+} mobilization, suggesting that NMDA induces cross-desensitization of CXCR2. CXCR2 underwent dephosphorylation after removal of the extracellular ligand, but the dephosphorylation rate was significantly reduced in the cells pretreated with NMDA. Treatment of the neuronal cells with NMDA retarded the recycling of CXCR2. In view of the critical role of receptor phosphorylation and recycling in the functional responsiveness of the chemokine receptor, these observations indicate

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a novel pathway through which glutamate may interfere with the neuroprotective function of chemokines.

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Alzheimer's disease (AD) is pathologically characterized by the deposition of a 39-43 amino acid residue peptide, known as β -amyloid, in senile plaques in the brains of affected individuals (Bateman and Chakrabarty 2004). Evidence of the involvement of β -amyloid in the neuronal death in AD came from studies showing that β -amyloid itself was neurotoxic *in vitro* (Manelli and Puttfarcken, 1995) and *in vivo* (Bishop and Robinson, 2003). However, the mechanisms of cell death induced by β -amyloid are not yet fully elucidated. It is proposed that β -amyloid induced neuronal death is associated with the release of excessive glutamate from the reactive microglial cells (McGeer and McGeer, 1998). Glutamate, the major excitatory neurotransmitter in central nervous system (CNS), binds to both ionotropic and metabotropic receptors to mediate effects on synaptic transmission and integrity, and the NMDA subtype of glutamate receptors are among the most widely distributed and abundant receptor-operated ion channels in the CNS (Doraiswamy, 2003; Butterfield and Pocernich, 2003). The effect of glutamate on neurons is concentration-dependent. Physiological concentration of glutamate plays important roles in brain development and in processes such as learning, memory, sensory activity, movement control, and modulation of synaptic transmission (Traven *et al.*, 1993). However, prolonged β -amyloid stimulation of the microglial cells results in release of excessive glutamate, which triggers massive Ca^{2+} influx and the subsequent neuronal damage, although the underlying mechanisms are not fully understood.

Recent studies have revealed that chemokines and their cognate receptors are involved in the pathogenesis of AD. Chemokines are a family of small molecular proteins (8-14 kDa) that play a role in the migration of neutrophils and lymphocytes to

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the inflammation site. Approximately 40 chemokines have been identified, and these proteins are classified into four subfamilies, CC, CXC, CX3C, and XC chemokines, according to the location of the conserved cysteine residues in the protein sequence. The most prominent chemokines associated with β -amyloid stimulation in the CNS are CCL2, CCL3, CCL4, CCL5, CCL8, CXCL1, CXCL8 and CXCL10 (Che *et al.*, 2001; Gitter *et al.*, 1995; Johnstone *et al.*, 1999; Ishizuka *et al.*, 1997; Xia *et al.*, 2000; Hesselgesser *et al.*, 1999). Several chemokine receptors, including CXCR2, CCR3, CCR5, and CCR1, were found associated with AD pathogenesis (Halks-Miller *et al.*, 2003; Xia *et al.*, 1998; Horuk *et al.*, 1997). Although the functions of the chemokines and chemokine receptors in CNS are not well documented, studies have demonstrated that several chemokines protect neurons against different injuries. CCL5, the ligand for the chemokine receptor CCR5, protected neurons from β -amyloid (25-35) induced damage (Bruno *et al.*, 2000). We have demonstrated previously that MIP-2, a specific ligand for CXCR2, protected hippocampal neurons against β -amyloid (1-42) induced apoptosis (Watson and Fan, 2005). In addition, several other chemokines, including CCL5, CXCL12 and CX3CL1, protect hippocampal neurons against gp120 neurotoxicity (Meucci *et al.*, 1998).

Since both chemokines and excessive glutamate are released from the reactive microglia in the brain of AD patients, it is interesting to investigate whether chemokine receptor-mediated neuroprotection against β -amyloid induced neuronal death is affected by glutamate, especially when the concentration of glutamate is not high enough to directly induce neuronal damage. Here we report that CXCR2-mediated

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protection against β -amyloid induced hippocampal neuronal death was significantly reduced by pretreatment with a sublethal concentration of NMDA. Exposure of the neuronal cells to NMDA resulted in phosphorylation and desensitization of CXCR2. Moreover, the dephosphorylation and recycling of CXCR2, which are critical for the re-establishment of the functional responsiveness of the chemokine receptor, was retarded in the presence of NMDA.

Materials and Methods

Generation of CXCR2 mutants. The truncated CXCR2 mutants, S342T and S352T, were generated using polymerase chain reaction. The primer pair for each reaction included a common primer for the 5' end of the open reading frame. Unique primers for the 3' end and were as follows: 342T, GCGAAGCTTTTAAGGCCTGCTGTCTTTGGG; and 352T, GCGAAGCTTTTAAGTGTGCCCTGAAGAAGA. The polymerase chain reaction-generated fragments were isolated, and subcloned into pRc/CMV. Mutagenesis of specific serine to alanine was conducted using the site-directed mutagenesis system (Promega). The following mutations were synthesized using the indicated primers: S346A, TCCTTTGTTGGCGCCTCTTCAGGGC AC; S347A, CTTTGTTGGCTCAGCTTCAGGGCACA; S348A, GTTGGCTCTTCTGCCGGGCACACTTCC. Once the mutations were confirmed, the cDNAs encoding the open reading frame for the CXCR2 mutants were subcloned into the pRc/CMV expression vector.

Cell cultures. Primary hippocampal cell cultures were established from neonatal mice (born within 24 hours). Dissociated cells were seeded onto poly-L-lysine-coated plastic dishes or 22-mm² glass coverslips and incubated in Dulbecco's Modification of Eagle's

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Medium (DMEM) (GIBCO BRL, Gaithersburg, MD) containing 2 mM L-glutamine, 25 mg/ml gentamicin, 1 mM HEPES, and 0.001% gentamicin sulfate. After 2 days *in vitro* (DIV), non-neuronal cell division was halted by a 3-day exposure to 10 μ M β -D-arabinofuranoside. All experiments were performed using 5-7 DIV unless indicated. Our preliminary experiments using microtubule-associated protein (MAP) 2 immunostaining indicated that approximately 95% cells were neurons after β -D-arabinofuranoside treatment for 3 days. Neuroblastoma-glioma hybrid (NG108-15) cells were cultured with DMEM supplemented with 10% fetal calf serum (GIBCO BRL, Gaithersburg, MD), 10% calf serum (GIBCO BRL), 0.1 mM hypoxanthine, 1 μ M aminopterin, and 16 μ M thymidine as described previously (Cai *et al.*, 1996). Cells were transfected with a vector encoding CXCR2 or an enhanced green fluorescent protein (EGFP) vector containing CXCR2 using lipofectAMINE plus reagent (Life Technologies, Inc.). Stably transfected cells were selected with 560 μ g/ml Geneticin (G418).

Lactate dehydrogenase (LDH) assay. Primary hippocampal neuronal cultures were treated with or without different concentrations of NMDA (0.1 -100 μ M) for 24 h. LDH levels in the culture medium were assayed using a Sigma Diagnostic® LDH kit. Briefly, 10 μ l of media was collected from each well of the 24-well plate and mixed with 250 μ l of Sample Start Reagent at 25 °C. Absorbance at 340 nm was recorded (SPECTRAMax 190, Molecular Devices) at 1, 2 and 3 min. The mean of the absorbance change was calculated for each treatment and expressed as a percentage of the level from control cells. The experiment was repeated three times with four wells per condition per replication.

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Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)

assay. Primary hippocampal neuronal cultures were treated with or without different concentrations of NMDA (0.1 -100 μM) for 24 h, or with β -amyloid (1-42) (50 μM) and NMDA (1 μM) in the absence or presence of macrophage inflammatory protein 2 (MIP2) (10 nM) and/or MK801 (1 μM) (Schelman *et al.*, 2004) for 24 h. The TUNEL assay (In Situ Cell Death Detection Kit; Roche) was performed to detect apoptotic cell death by enzymatic labeling of DNA strand breaks with fluorescein-dUTP and TdT (Gorczyca *et al.*, 1993). Briefly, 1×10^5 cells grown in eight-well Poly-L-Lysine-coated Falcon glass culture slides, were fixed in 4% formaldehyde/PBS (pH 7.4) for 60 min at room temperature, washed in PBS, and then suspended in permeabilization solution (0.1% Triton X-100/0.1% sodium citrate) for 3 min on ice. Cells were washed again, resuspended in 50 μl of TUNEL reaction mixture or in 50 μl of label solution alone (negative control), and incubated in a humidified dark chamber at 37°C, followed by washing in PBS. The number of TUNEL-positive cells was also counted in five different fields.

Ca²⁺ mobilization assay. Primary hippocampal neuronal cells were released by a short exposure (1-2 min) to Versine (trypsin/EDTA) and washed once in culture medium containing 5% FBS. Cells were then washed in Hank's solution without Ca²⁺ or Mg²⁺, then incubated with FURA-2 (2 μM final concentration). After 30 min, the volume of buffer was doubled with the Hank's solution (without Ca²⁺ and Mg²⁺), and the cells were incubated for 10 min. Cells were then centrifuged (300 \times g, 6 min) and washed once (50 ml) in Hank's solution containing Ca²⁺ and Mg²⁺ (1 mM). The cells were finally adjusted

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to 1×10^6 cells/ml. Cells were stimulated with vehicle, 10 nM MIP2, or 1 μ M NMDA in the absence or presence of MK801 (1 μ M) (Schelman *et al.*, 2004) for 5 min before being washed three times with 15 ml of Hank's solution with Ca^{2+} and Mg^{2+} (1 mM). Cells were finally resuspended at (1×10^6 cells/ml) and kept on ice until needed. Cells were allowed to warm to 37 °C for 5 min before the second stimulus of MIP2 (10 nM). The ligand-induced Ca^{2+} mobilization was measured continually for the specific time using a single scanning spectrofluorimeter constructed by the University of Pennsylvania Department of Bioengineering. Data were collected using an IBM model PS-II computer with custom written software provided by the Department of Laboratory Automation, SmithKline Beecham. Data were analyzed using the software program, Igor, which used the following equation to determine free Ca^{2+} : Ca^{2+} nM = $244 \times (F - F_{\min}) / (F_{\max} - F)$. F is the ligand-induced fluorescence. F_{\max} is the maximum fluorescence (in the presence of 1 mM free Ca^{2+}), and F_{\min} is the minimum fluorescence in the presence of EGTA (5 mM). The constant, 224, is the dissociation (K_d) constant between FURA-2 and Ca^{2+} .

Western blot analysis. The hippocampal neuronal cells were lysed in lysis buffer containing 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 150 mM NaCl, 1% NP-40, 1 mM Na_3VO_4 , 10 mM NaF, 1 mM PMS F, pepstatin A (20 μ g/mL), leupeptin (20 μ g/mL) and aprotinin (20 μ g/mL). The lysate was centrifuged at 14 000 g for 10 min at 4 °C and the supernatant was assayed for protein concentration using the Bio-Rad protein assay reagent. Equal amounts of protein extract (20 μ g) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to PVDF

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membranes (Millipore, MA, USA) by a semi-dry transfer method. The membrane was blocked with 2% bovine serum albumin (BSA) for 1 h at room temperature, and then incubated with CXCR2 antibody (1: 1000; Santa Cruz Biotechnology) for 2 h at room temperature. After washing (0.1% Tween 20 in TBS buffer), three times for 10 min each, the membrane was incubated with horseradish peroxidase-conjugated IgG (1: 2000, UBI) in 0.05% TBST containing 2% BSA for 1 h at room temperature. After extensive washes, the peroxidase signals were detected by enhanced chemiluminescence (ECL plus; Amersham, NJ, USA).

Phosphorylation and dephosphorylation assay. Receptor phosphorylation assay was performed as described previously (Fan *et al.*, 2001). In brief, after incubating in serum- and phosphate-free medium for 60 min, cells were labeled by incubating in ^{32}P -orthophosphate (100 $\mu\text{Ci/ml}$) (Dupont NEN) in the same medium at 37 °C for 2 h. Cells were then stimulated with or without MIP2 (10 nM) or NMDA (1 μM) for different lengths of time (0, 5, 10, 30 min). After washing of the cells for three times, cells were lysed and CXCR2 was immunoprecipitated from the cell lysate using a rabbit polyclonal antibody (1:100). Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore, MA, USA). The phosphorylated receptors were then detected by autoradiography. For the dephosphorylation experiment, cells were metabolically labeled with ^{32}P -orthophosphate for 1 h and stimulated with MIP2 for 10 min. The cells were either kept on ice or continued incubation for different lengths of time (0, 10, 20, 30, 60 min) in the absence or presence of NMDA (1 μM) before the cells were lysed in lysis buffer. CXCR2 was immunoprecipitated from the cell lysate and phosphorylation was detected by

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autoradiography as described above. The expression of CXCR2 was determined by Western blotting.

Confocal visualization of agonist-induced internalization of CXCR2. NG108-15 cells stably expressing CXCR2 were pretreated with or without NMDA for 60 min before being treated with MIP2 (10 nM) at 37°C for 60 minutes. The ligand was removed, and the cells either were fixed immediately or were incubated with ligand-free medium for another 60 min before being fixed. Cells were washed with phosphate-buffered saline three times, followed by incubation with a mouse monoclonal CXCR2 antibody (Santa Cruz Biotechnology, Inc.) for 30 min. Cells were washed and incubated with a Cy3-conjugated anti-mouse antibody (Molecular Probes) for 30 min. Confocal microscopy was performed using a Zeiss LSM-510 laser scanning microscope equipped with a Zeiss 63 × 1.3 NA oil immersion lens. Quantification of the internalized CXCR2 in internal vesicles was performed in 30 neuronal cells for each treatment by measuring the density of the fluorescent vesicles using the MetaMorph Imaging System (Universal Imaging, Downingtown, PA).

Fluorescence-activated cell sorting (FACS) analysis. NG108-15 cells stably expressing CXCR2 were pre-treated with or without NMDA (1 μM) for 60 min. Cells were then treated with or without MIP2 (10 nM) at 37°C for 60 min. Cells were washed, followed by continued incubation in ligand-free medium at 37 °C for 60 min. For the staining of CXCR2, cells were incubated with a monoclonal PE-conjugated CXCR2 antibody (PharMingen) at 4 °C for 60 min. Cells were washed and fixed in 2% formaldehyde in phosphate-buffered saline and analyzed in FACScan equipped with CellQuest software (Becton Dickinson).

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Statistical Analysis. Data are presented as the means \pm S.E. The means of numbers of cells undergoing apoptosis were subjected to analysis of variance for multiple comparisons. Paired analysis between two groups was performed by Student's *t* test.

Results

Sublethal concentration of NMDA reduced CXCR2-mediated neuroprotection. It has been shown previously that the effect of glutamate on neuronal viability is concentration-dependent, and high concentrations induce neuronal apoptosis and necrosis (Jing *et al.*, 2004; Doraiswamy, 2003; Butterfield and Pocernich, 2003). Our initial studies were performed to evaluate the dose-response effect of NMDA on neuronal viability. Primary hippocampal neuronal cultures were treated with different concentrations of NMDA (0.1 -100 μ M) for 24 h. Neuronal death was measured by LDH release assay. Treatment of the hippocampal neurons with NMDA for 24 h resulted in a dose-dependent increase in LDH release. Significant neuronal death was observed at the concentration of 50 μ M (Fig. 1A). However, the neuronal viability was not affected by NMDA at the concentration of 1 μ M (Fig. 1A). We confirmed that treatment of the hippocampal neuronal cells with 1 μ M of NMDA did not induce neuronal apoptosis as assessed by TUNEL assay (Fig. 1B). Therefore, a concentration of 1 μ M was chosen as a sublethal concentration to explore the effect of NMDA on MIP2 neuroprotection.

We have demonstrated that 50 μ M concentration of β -amyloid (1-42) induced remarkable hippocampal neuronal apoptosis, which was significantly reduced by co-administration of MIP2 (Watson and Fan, 2005). To examine whether a sublethal concentration of NMDA affects MIP2 induced neuroprotection, hippocampal neuronal cultures were pre-treated with NMDA (1 μ M) for 1 h in the presence or absence of the

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NMDA receptor antagonist, MK801 (1 μ M) (Schelman *et al.*, 2004), followed by the treatment of β -amyloid (1-42) (50 μ M) with or without MIP2 (10 nM) (Watson and Fan, 2005). Neuronal apoptosis was assessed by TUNEL assay. As shown in Fig. 1C, pretreatment of NMDA significantly reduced the neuroprotective effect of MIP2 against β -amyloid (1-42) neurotoxicity. However, the inhibitory effect of NMDA was blocked by the co-administration of MK801, a non-competitive NMDA receptor antagonist, suggesting involvement of NMDA receptors in this process. It is known that β -amyloid stimulation results in activation of microglia and release of glutamate (Noda *et al.*, 1999), which may exacerbate the β -amyloid induced neuronal injury. However, co-administration of MK801 with β -amyloid did not reduce the β -amyloid induced neuronal death (Fig. 1C). We propose that the percentage of glial cells (<5%) in the hippocampal neuronal cultures was too low to produce enough glutamate to induce remarkable neuronal injury, as demonstrated in the present study that the neurotoxic effect of glutamate is concentration-dependent (Fig. 1A and B). We also examined whether the MIP2-induced neuroprotection against other neurotoxic agents is attenuated by NMDA. Treatment of the hippocampal neurons with staurosporine (0.5 μ M) significantly induce neuronal apoptosis, consistent with the previous report (Tremblay *et al.*, 2000). The staurosporine-induced neuronal apoptosis was significantly reduced by the co-administration of MIP2 (10 nM). However, pretreatment of the neuronal cells with NMDA (1 μ M) significantly prevented the MIP2-induced neuroprotection (Fig. 1D).

NMDA induced phosphorylation of CXCR2. It is known that the functional response of CXCR2 is quickly attenuated after ligand-induced receptor phosphorylation (Mueller

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et al., 1997; Fan *et al.*, 2001). Stimulation of NMDA receptors has been shown to induce the phosphorylation of several membrane and intracellular proteins including ERK and G protein (Finkbeiner and Greenberg, 1996; Kornhauser and Greenberg, 1997; Fan *et al.*, 1998). We therefore examined whether NMDA treatment of CXCR2 expressing cells results in CXCR2 phosphorylation. Hippocampal neuronal cultures were metabolically labeled with ^{32}P -orthophosphate. Cells were treated with carrier buffer (negative control), MIP2 (10 nM) (positive control), or NMDA (1 μM) for different lengths of time (0, 5, 10, 30 min). CXCR2 was immunoprecipitated from the cell lysate and phosphorylation of the receptor was detected by autoradiography. As shown in Fig. 2, in the untreated cells, CXCR2 exhibited basal phosphorylated state, and treatment of MIP2 induced remarkable phosphorylation of the receptor, consistent with the previous results (Mueller *et al.*, 1997). Interestingly, treatment of the cells with NMDA (1 μM) also resulted in CXCR2 phosphorylation, although to a lesser extent compared to the phosphorylation induced by MIP2. We next examined whether different serine residues are involved in NMDA- and MIP2-induced CXCR2 phosphorylation. As shown in Fig. 3A and B, truncation of the C-terminal 352-355 did not affect NMDA- and MIP2-induced receptor phosphorylation. However, truncation of the C-terminal 342-355 prevented both NMDA- and MIP2-induced CXCR2 phosphorylation, suggesting the three serine residues Ser346, Ser347, Ser348, in the C-terminal domain of CXCR2 are the potential major phosphorylation sites for both NMDA- and MIP2-induced phosphorylation. To determine the involvement of the individual serine residues in the phosphorylation, the CXCR2 mutants with each of the Ser346, Ser347, and Ser348 residues replaced with Ala, and NMDA- or MIP2-induced receptor phosphorylation was determined. As shown

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in Fig. 3C and D, replacing the Ser348 to Ala (S348A) remarkably prevented MIP2-induced phosphorylation, although replacing Ser346 or Ser347 to Ala (S346 A or S347A) also reduced the phosphorylation to a less extent. In contrast, replacing either S346 or Ser348 to Ala did not significantly reduce NMDA-induced CXCR2 phosphorylation, but replacing the Ser347 to Ala remarkably prevented NMDA-induced phosphorylation. These data suggest Ser348 is the major residue, but the Ser346 and Ser347 also play a role, in MIP2-induced CXCR2 phosphorylation, whereas Ser347 is the major residue in NMDA-induced CXCR2 phosphorylation.

NMDA induced cross-desensitization of CXCR2. Like other GPCRs, ligand induced phosphorylation of CXCR2 plays a key role in the receptor desensitization. The induction of CXCR2 phosphorylation by NMDA suggests that NMDA pretreatment may induce CXCR2 desensitization. To test this hypothesis, hippocampal neuronal cultures were pre-incubated with vehicle (control), MIP2 (10 nM, positive control), or NMDA (1 μ M) in the absence or presence of 1 μ M MK801 (Schelman *et al.*, 2004) for 30 min. Cells were loaded with fluo-3 for 30 min before being stimulated with MIP2 (10 nM), and Ca^{2+} mobilization was measured. As shown in Fig. 4, MIP2 stimulation resulted in a remarkable increase in the intracellular Ca^{2+} mobilization in the control cells. As a positive control, pretreatment of the neuronal cells with MIP2 completely abolished the ligand-induced Ca^{2+} mobilization. Interestingly, in the cells pretreated with NMDA (1 μ M), MIP2 induced Ca^{2+} mobilization was reduced. The inhibitory effect of NMDA on MIP2 induced Ca^{2+} mobilization was abolished by MK801 (1 μ M). These data suggest that activation of NMDA receptors resulted in cross-desensitization of CXCR2.

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NMDA blocked CXCR2 dephosphorylation. We have previously shown that CXCR2 undergoes dephosphorylation in the protein phosphatase 2A-dependent manner after removal of the extracellular ligands (Fan *et al.*, 2001). Receptor dephosphorylation plays an important role in the re-establishment of the functional responsiveness of the receptor-expressing cells. It is interesting to examine whether the dephosphorylation of CXCR2 is affected by NMDA. Hippocampal neuronal cultures were metabolically labeled with ^{32}P -orthophosphate. Cells were treated with MIP2 (10 nM) for 10 min, then the ligand was removed and the cells were incubated in phosphate-free medium for different lengths of time in the absence or presence of NMDA (1 μM). CXCR2 was immunoprecipitated from the cell lysate and phosphorylation of the receptor was detected as described above. As shown in Fig. 5, CXCR2 receptors were phosphorylated after the ligand stimulation, and the receptors were time-dependently dephosphorylated after removal of the ligand. However, the dephosphorylation of the receptor was blocked in the presence of NMDA. These data suggest that CXCR2 undergoes constitutive phosphorylation in neuronal cells exposed to excessive glutamates under pathological conditions.

NMDA blocked CXCR2 recycling. It has been postulated that receptor dephosphorylation plays an important role in the recycling of CXCR2 and the reestablishment of the functional responsiveness of the receptor (Fan *et al.*, 2001). We sought to examine whether treatment of NMDA affects CXCR2 recycling. This experiment was performed using NG108-15 cells in which CXCR2 was overexpressed. NG108-15 cells stably expressing CXCR2 were pre-incubated with or without MIP2 (10 nM) for 60 min. The ligand was removed and the cells were recovered in ligand-free

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medium for 60 min in the presence of vehicle (control) or NMDA (1 μ M), and receptor recycling was examined by FACS analysis. As shown in Fig. 6, ligand treatment induced internalization of CXCR2 in the cell lines. After removing the agonist and recovering the cells for 60 min, 70% CXCR2 receptors were re-expressed on the cell surface of the control cells, but only 27% CXCR2 receptors were recycled to the cell surface of the cells treated with NMDA.

The inhibitory effect of NMDA on the recycling of CXCR2 was confirmed by confocal microscopy. NG108-15 cells stably expressing CXCR2 were pretreated with or without NMDA (1 μ M) before being incubated with MIP2 at 37°C for 60 min. The ligand was removed and the cells were fixed either immediately or after incubation in ligand-free medium at 37°C for another 60 min. CXCR2 was immunostained with a specific antibody, and the intracellular localization of CXCR2 was visualized by confocal microscopy. As shown in Fig. 7, prior to ligand stimulation, CXCR2 was localized on the cell membrane. After treatment of the cells with MIP2, most of the receptors were internalized into internal vesicles. Following removal of the ligand and recovery of the cells for 60 min, a large proportion of CXCR2 became visible on the cell surface in the untreated cells. However, in the cells treated with NMDA, CXCR2 was retained in the internal vesicles. Quantification and statistical analysis demonstrated that in the cell pretreated with NMDA, CXCR2 recycling was significantly retarded (Fig. 7G).

Discussion

The present studies provided evidence that sublethal levels of NMDA interfere with the CXCR2-mediated neuroprotection. Treatment of hippocampal neurons with a concentration of NMDA, which did not affect the neuronal viability, resulted in a marked

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suppression of MIP2 induced protection against β -amyloid (1-42) and staurosporine neurotoxicity. Exposure of the neurons to NMDA induced CXCR2 phosphorylation and cross-desensitization, and retarded the receptor dephosphorylation and recycling, a process required for the re-establishment of the functional responsiveness of CXCR2 (Fan *et al.*, 2001; Fan *et al.*, 2004). These findings suggest that sublethal levels of glutamate together with β -amyloid may synergistically initiate early lesions of neurons by inhibiting the neuroprotective machinery, and contribute to the propagation of the neurodegeneration in later stages of AD as β -amyloid continues to accumulate.

β -amyloid peptide is known to play a central role in neurodegeneration by directly inducing neuronal lesion, and by activating glial cells (Meda *et al.*, 2001). Reactive glial cells have dual functions. They produce excessive glutamate and other neurotoxic factors that induce neurodegeneration, and also produce certain chemokines that induce neuroprotection through their cognate receptors on the neuronal cell surface. Glutamates, acting through the NMDA type of glutamate receptors, affect neuronal viability in a concentration-dependent manner. *In vitro* studies in our and several other laboratories have shown that high levels of NMDA induce neuronal death via apoptosis and necrosis, whereas lower concentrations of NMDA (1-10 μ M) do not induce neuronal death (Ikegaya *et al.*, 2001). It is conceivable that in the initial stage of AD, the concentration of glutamate in CNS may not be high enough to directly induce neurotoxicity. Then the question arises: do the sublethal doses of glutamate potentiate β -amyloid (1-42)-mediated neurodegeneration in the presence of neuroprotective chemokines? This question was answered by the present result showing that MIP2

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induced protection against β -amyloid (1-42) neurotoxicity was remarkably attenuated in the cells pretreated with a sublethal concentration of NMDA. These data suggest that sublethal levels of glutamate increase neuronal vulnerability under pathologic conditions.

Like other G protein-coupled receptors, receptor phosphorylation is responsible for the attenuation of the functional responsiveness of CXCR2 (Mueller *et al.*, 1995; Mueller *et al.*, 1997). We provided evidence that CXCR2 was phosphorylated by NMDA treatment of the neuronal cells, and differential serine residues in the C-terminal domain are involved in NMDA-induced phosphorylation compared to MIP2-induced phosphorylation. Although the underlying mechanisms are unclear, it is considered that activation of NMDA receptors may stimulate certain serine/threonine protein kinases that can phosphorylate the receptor. NMDA receptors are one type of ion channel that is highly permeable to Ca^{2+} , and calcium influx through NMDA-receptor-operated channels triggers the mobilization of additional calcium from intracellular stores (Lei *et al.*, 1992). Elevation of intracellular Ca^{2+} can activate a series of kinases including calcium calmodulin-dependent protein kinase II and protein kinase C (Nishizuka *et al.*, 1992), the latter has been shown to phosphorylate CXCR2 (Mueller *et al.*, 1995). Activation of NMDA receptors has been shown to induce phosphorylation of certain G protein-coupled receptors such as metabotropic glutamate receptor 5, and PKC has been postulated to be involved in this process (Alagarsamy *et al.*, 2002). These data suggest that CXCR2 and many other G protein-coupled receptors are phosphorylated by the stimulation of NMDA receptors, resulting in receptor desensitization and blockade of the receptor functions. In addition, we have previously shown that activation

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of NMDA receptor resulted in phosphorylation of G α 2 (Fan *et al.*, 1998), a G protein subunit that is coupled with CXCR2, suggesting that glutamate-induced phosphorylation of G proteins may also play a role in the attenuation of CXCR2-mediated neuroprotection.

It is known that phosphorylated CXCR2 receptors are quickly internalized into internal vesicles, in which the receptors undergo dephosphorylation catalyzed by protein phosphatase 2A (PP2A) (Fan *et al.*, 2001), and the dephosphorylated receptors are recycled back to the cell surface. This process is critical for the re-establishment of the functional responsiveness of the chemokine receptors. However, it becomes a question whether CXCR2 can be dephosphorylated and recycled under neurodegenerative conditions in which high levels of glutamate are sustained due to the increased release by reactive microglia and impairment of uptake by astrocytes (Lauderback *et al.*, 1999). It has been shown that stimulation of NMDA receptors led to the reduction of PP2A activity (Chan and Sucher, 2001), and inhibiting the phosphatase activity resulted in neurodegeneration (Arias *et al.*, 1998). Consistently, the present study demonstrated that CXCR2 dephosphorylation was retarded in the presence of NMDA. These data suggest that blocking PP2A activity by the activation of NMDA receptors may also contribute to the inhibitory effect of NMDA on CXCR2-mediated neuroprotection. It is therefore conceivable that under neurodegenerative conditions such as AD, the neuroprotective functions of chemokine receptors are profoundly reduced because of constitutive phosphorylation of the receptors stimulated by the excessive glutamate.

We have shown that in response to ligand-induced phosphorylation, CXCR2 receptors are internalized into early endosomes, in which they are dephosphorylated

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(Fan *et al.*, 2001). The dephosphorylated receptors are either recycled back to the cell surface after removal of the extracellular ligands, or transported to late endosomes/lysosomes for degradation in the presence of high levels of ligands (Fan *et al.*, 2003; Fan *et al.*, 2004). Because the presence of NMDA resulted in retardation of CXCR2 dephosphorylation, it is proposed that CXCR2 receptors remain in the cytoplasm in the presence of NMDA even though the extracellular ligand is removed. Consistent with the hypothesis, we observed that CXCR2 was recycled back to the cell surface after ligand removal in the control cells, but in the cells pre-treated with NMDA, most of the receptors remained in the internal vesicles. These results implicate that the continued presence of excessive glutamate may interfere with the subcellular localization of chemokine receptors and impair the receptor-mediated neuroprotection.

In conclusion, we demonstrated that MIP2 induced protection against β -amyloid (1-42) neurotoxicity was attenuated by a sublethal concentration of NMDA pre-treatment. NMDA treatment promoted basal phosphorylation and retarded dephosphorylation and recycling of CXCR2. Our study may provide significant insight into understanding the role of chemokines in neuroprotection under pathological conditions.

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Footnotes

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Figure legends

Fig. 1. Sublethal concentration of NMDA reduced CXCR2-mediated neuroprotection. A, hippocampal neurons were treated with different concentrations of NMDA (0.1-100 μ M) for 24 h. Neuronal death was measured by LDH release assay. Data are mean \pm S.E. from three independent experiments. * P <0.05, compared to the cells treated with vehicle. B, hippocampal neurons were treated with different concentrations of NMDA (0.1-100 μ M) for 24 h. Neuronal apoptosis was assessed by TUNEL assay. Percentage of TUNEL positive cells was calculated from 200 cells in 5 different fields for each treatment. Data are mean \pm S.E. from three independent experiments. * P <0.05, ** P <0.01, compared to the cells treated with vehicle. C, hippocampal neuronal cells were pre-treated with or without NMDA (1 μ M) for 1 h in the absence or presence of MK801 (1 μ M). Cells were incubated with amyloid (50 μ M) in the absence or presence of MIP2 (10 nM) for 24 h. Neuronal apoptosis was assessed by TUNEL assay. D, hippocampal neuronal cells were pre-treated with or without NMDA (1 μ M) for 1 h. Cells were incubated with staurosporine (0.5 μ M) in the absence or presence of MIP2 (10 nM) for 24 h. Neuronal apoptosis was assessed by TUNEL assay. Percentage of TUNEL positive cells was calculated from 200 cells in 5 different fields for each treatment. Data are mean \pm S.E. from three independent experiments. * P <0.05, compared to the cells treated with β -amyloid and MIP2 without NMDA pretreatment.

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Fig. 2. NMDA induced phosphorylation of CXCR2. A, hippocampal neuronal cultures were metabolically labeled with ^{32}P -orthophosphate for 1 h before being incubated with vehicle, NMDA (1 μM) or MIP2 (10 nM) for different lengths of time. The cell lysates were immunoprecipitated with a rabbit polyclonal anti-CXCR2. Proteins were separated using 10% SDS-PAGE and transferred to a nitrocellulose membrane. Phosphorylated proteins were detected by autoradiography. The membrane was blotted with a mouse monoclonal anti-CXCR2 antibody (E2; Santa Cruz Biotechnology) to confirm equal loading. B, quantification of the density of bands representing phosphorylated CXCR2 was determined by densitometric scanning. Data are mean \pm S.E. from three independent experiments.

Fig. 3. Involvement of differential serine residues in MIP2- and NMDA-induced CXCR2 phosphorylation. A, NG108-15 cells stably expressing the full-length CXCR2, 342T or 352T were metabolically labeled with ^{32}P -orthophosphate and stimulated with or without 10 nM MIP2 for 10 min at 37 $^{\circ}\text{C}$, and whole cell lysates were prepared as described under the Materials and Methods. Equal amounts of cell lysate were immunoprecipitated with a CXCR2 antibody (anti-NH2-terminal peptide) in the presence of protein A/G-agarose. Immunoprecipitates were subjected to SDS-PAGE, and the gel was dried and exposed to film for autoradiography. B, the density of the bands representing the phosphorylated full-length or the truncated forms of CXCR2 were quantified by densitometric scanning from three independent experiments, and normalized with the bands representing the phosphorylation of non-stimulated receptors. Data (mean \pm S.E.) are expressed as the percentage of the phosphorylation

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of the full-length CXCR2 (control). C, NG108-15 cells stably expressing the full-length CXCR2, S346A, S347A, or S348A were metabolically labeled with ^{32}P -orthophosphate and stimulated with or without 10 nM MIP2 for 10 min at 37 °C. The wild-type or the mutant forms of CXCR2 were immunoprecipitated, and autoradiography was performed as described above. D, the density of the bands representing the phosphorylated full-length or the mutant forms of CXCR2 were quantified by densitometric scanning from three independent experiments, and normalized with the bands representing the phosphorylation of non-stimulated receptors. Data (mean \pm S.E.) are expressed as the percentage of the phosphorylation of the full-length CXCR2 (control). * $P < 0.05$, ** $P < 0.01$, compared to the phosphorylation of the full-length CXCR2 in each treatment.

Fig. 4. NMDA induced cross-desensitization of CXCR2. Hippocampal neuronal cultures (1×10^6 cells/ml) were incubated with FURA-2 (2 μM final concentration) for 30 min. Cells were stimulated with vehicle (A), 10 nM MIP2 (B), or 1 μM NMDA in the absence (C) or presence (D) of 1 μM MK801 for 5 min before being washed three times with Hank's solution. Cells were stimulated with MIP2 (10 nM) and the ligand-induced Ca^{2+} mobilization was measured using a single scanning spectrofluorimeter as described in *Materials and Methods*. The relative Ca^{2+} mobilization potency (percentage of control) was calculated from three independent experiments to indicate the effect of different pretreatment of the cells with vehicle (control), MIP2, NMDA, or NMDA and MK801 on the ligand-induced Ca^{2+} mobilization (D). ** $P < 0.01$, compared to the cells pretreated with vehicle.

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Fig. 5. NMDA retarded CXCR2 dephosphorylation. A, hippocampal neuronal cultures were metabolically labeled with ^{32}P -orthophosphate for 1 h and then incubated with MIP2 (10 nM) for 10 min. The cells were either kept on ice or continued incubation for different lengths of time in the absence or presence of NMDA (1 μM). The cell lysates were immunoprecipitated with a rabbit polyclonal anti-CXCR2. Proteins were separated using 10% SDS-PAGE and transferred to a nitrocellulose membrane. Phosphorylated proteins were detected by autoradiography. The membrane was blotted (IB) with a mouse monoclonal anti-CXCR2 antibody (E2; Santa Cruz Biotechnology) to confirm equal loading. Data shown represent one of three independent experiments. B, quantification of the density of bands (mean \pm S.E.) representing phosphorylated CXCR2 was determined by densitometric scanning.

Fig. 6. NMDA blocked CXCR2 recycling. A, NG108-15 cells stably expressing CXCR2 were pre-treated with NMDA for 60 min. Cells were then treated with or without MIP2 (10 nM) at 37°C for 60 min. Cells were washed, followed by continued incubation in ligand-free medium at 37 °C for 60 min. For the staining of the cell surface receptor, cells were incubated with a PE-conjugated CXCR2 antibody (1: 100 dilution) at 4°C for 60 min. NG108-15 cells not expressing CXCR2 were used as a control. Cells were washed, fixed in 2% formaldehyde in PBS, and analyzed in FACScan. Shown are representatives of three independent experiments with similar results. B, quantification of the cell surface fluorescence representing the re-expression of CXCR2 in the cells treated with the vehicle (control) or NMDA after recovery for 60 min. Data are mean \pm

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S.E. from three independent experiments. * $P < 0.05$ compared to the cells pre-treated with vehicle (Veh).

Fig. 7. NMDA treatment resulted in sustain of CXCR2 in interval vesicles. NG108-15 cells stably expressing CXCR2 were pretreated without (A, B, C) or with NMDA (D, E, F) for 60 min before being treated with MIP2 (10 nM) at 37°C for 60 minutes. The ligand was removed, and the cells either were fixed immediately (B, E) or were incubated with ligand-free medium for 1 hour (C, F) before being fixed. For the immunostaining of CXCR2, cells were incubated with a mouse monoclonal anti-CXCR2 antibody for 30 minutes, followed by incubation with a rhodamine-conjugated anti-mouse antibody for 30 minutes. Representative confocal micrographs from 4 independent experiments demonstrating the intracellular distribution of CXCR2 were shown. Arrows indicate the internalized receptors. Images were processed using Photoshop software. G, quantification of the internalized receptors in the internal vesicles was performed in 30 cells for each treatment by measuring the density of the fluorescent vesicles using the MetaMorph Imaging System (Universal Imaging, Downingtown, PA). Data (means \pm S.E.) are expressed as fold increase of the fluorescent vesicles in the cells treated with MIP2 (with or without NMDA pretreatment) over the cells without MIP2 treatment (control). * $P < 0.05$, compared to the cells without NMDA pretreatment but treated with MIP2 and recovered for 1 h. Bars, 10 μ m.

Fig. 1

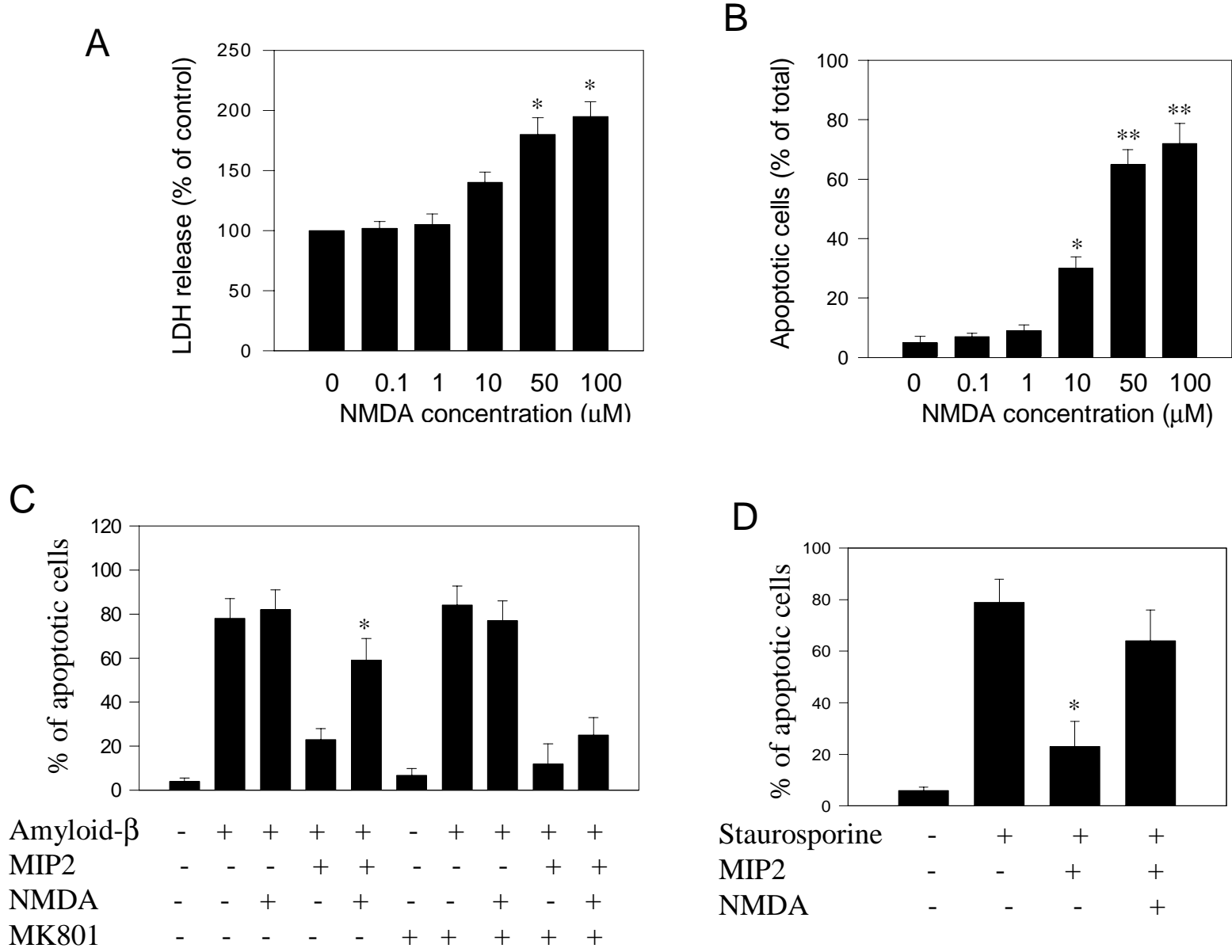


Fig. 2

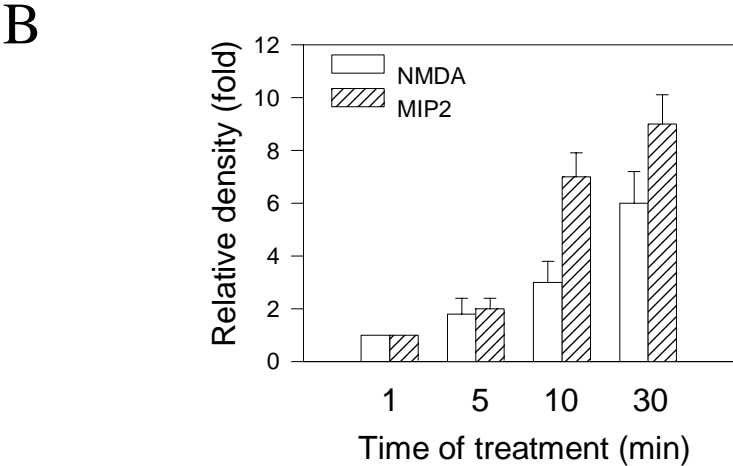
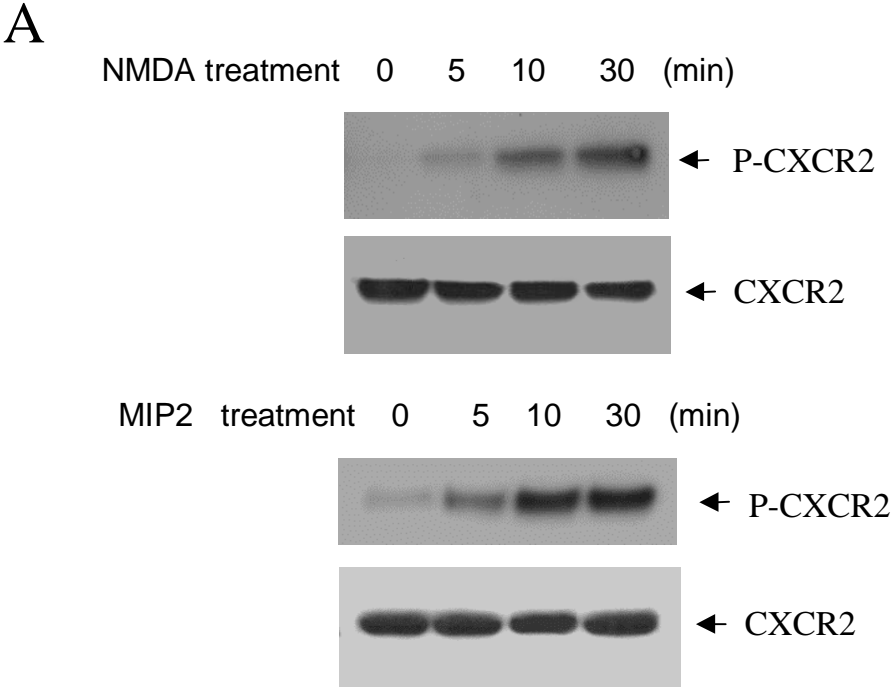


Fig. 3

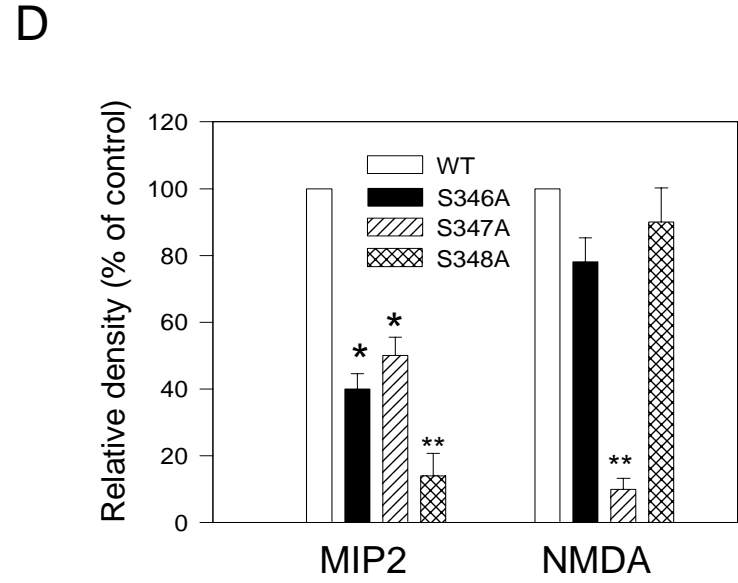
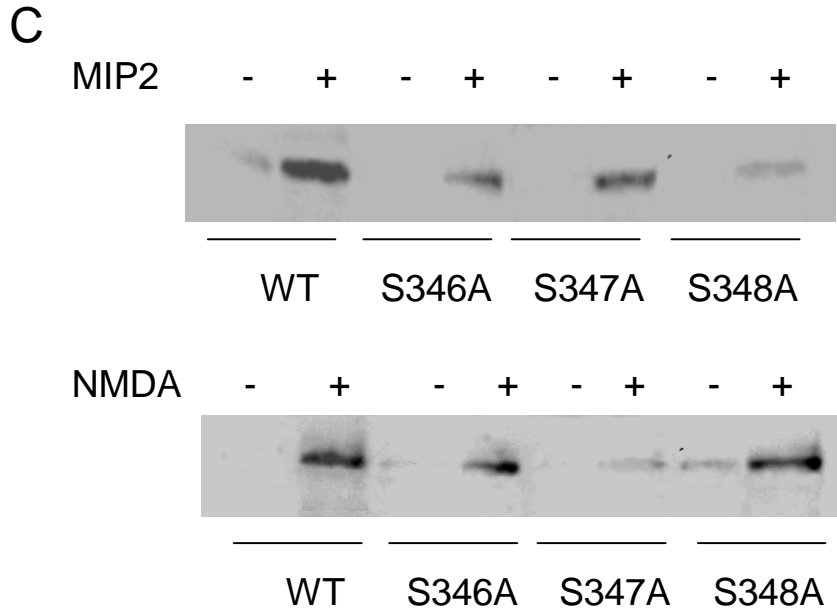
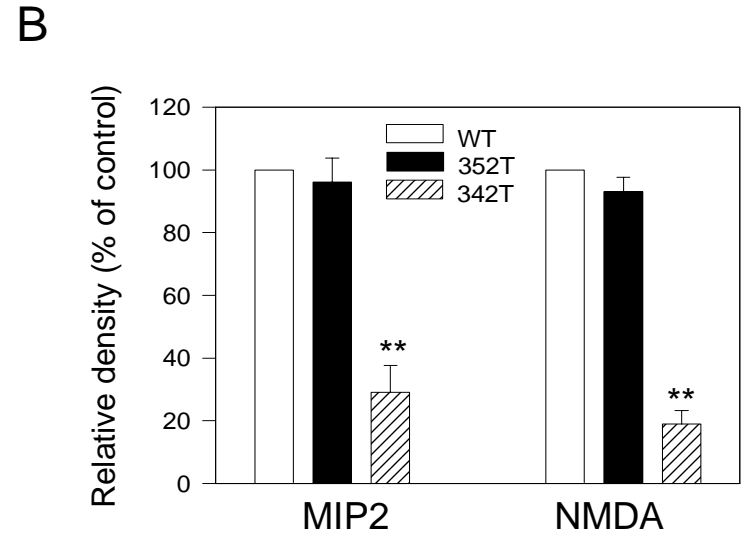
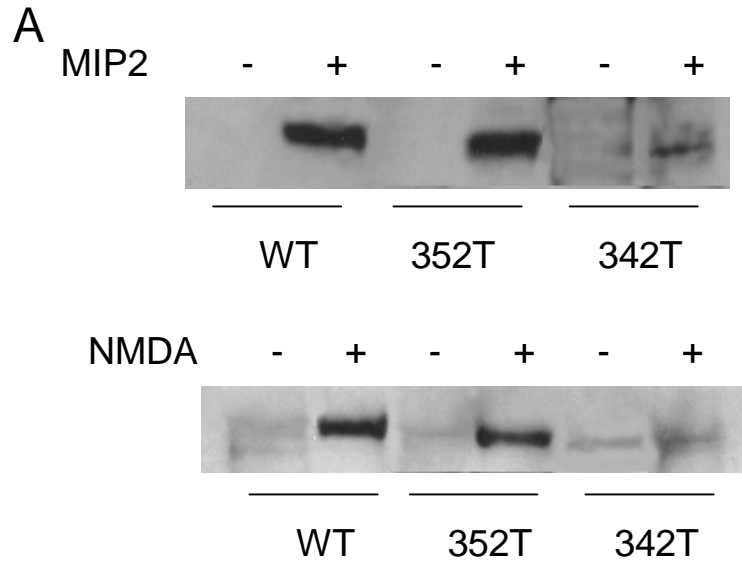


Fig. 4

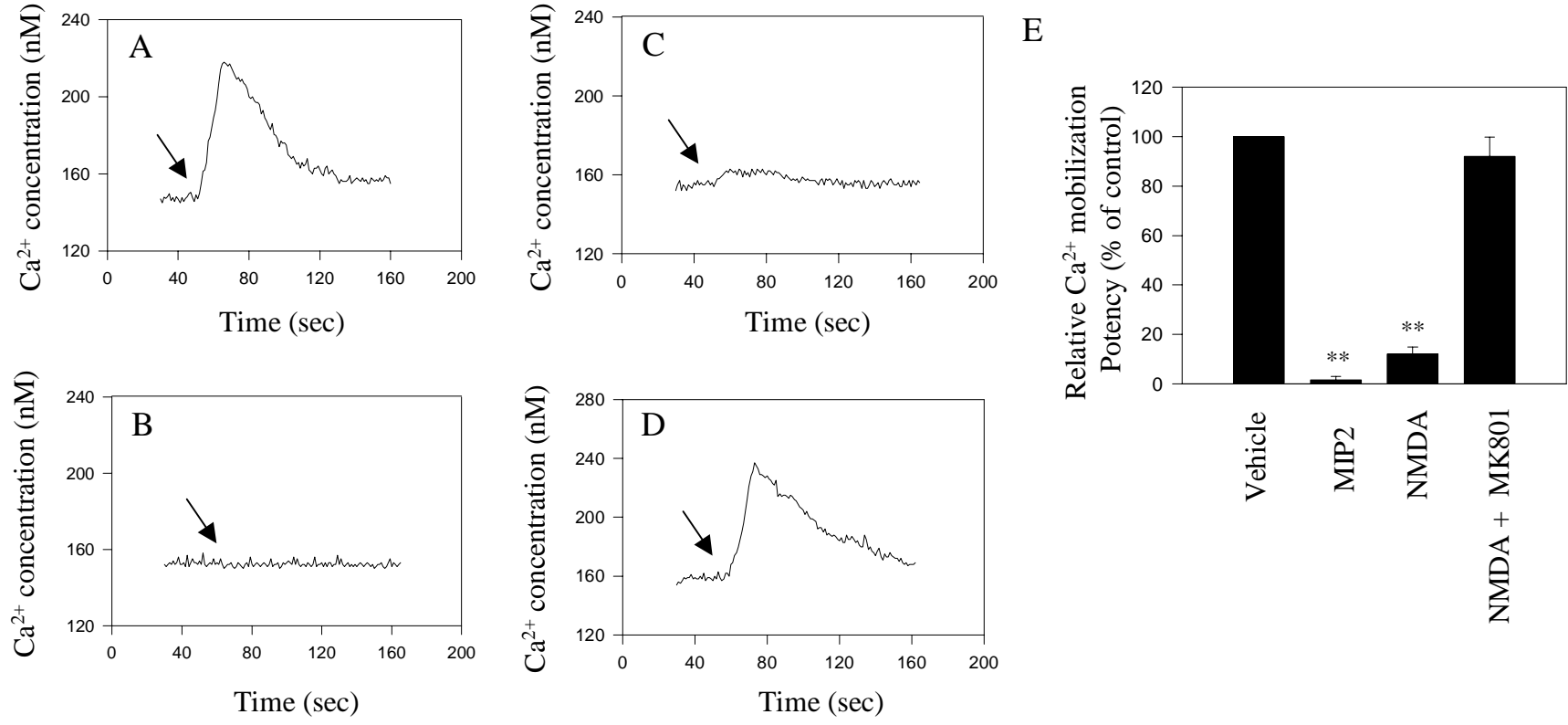


Fig. 5

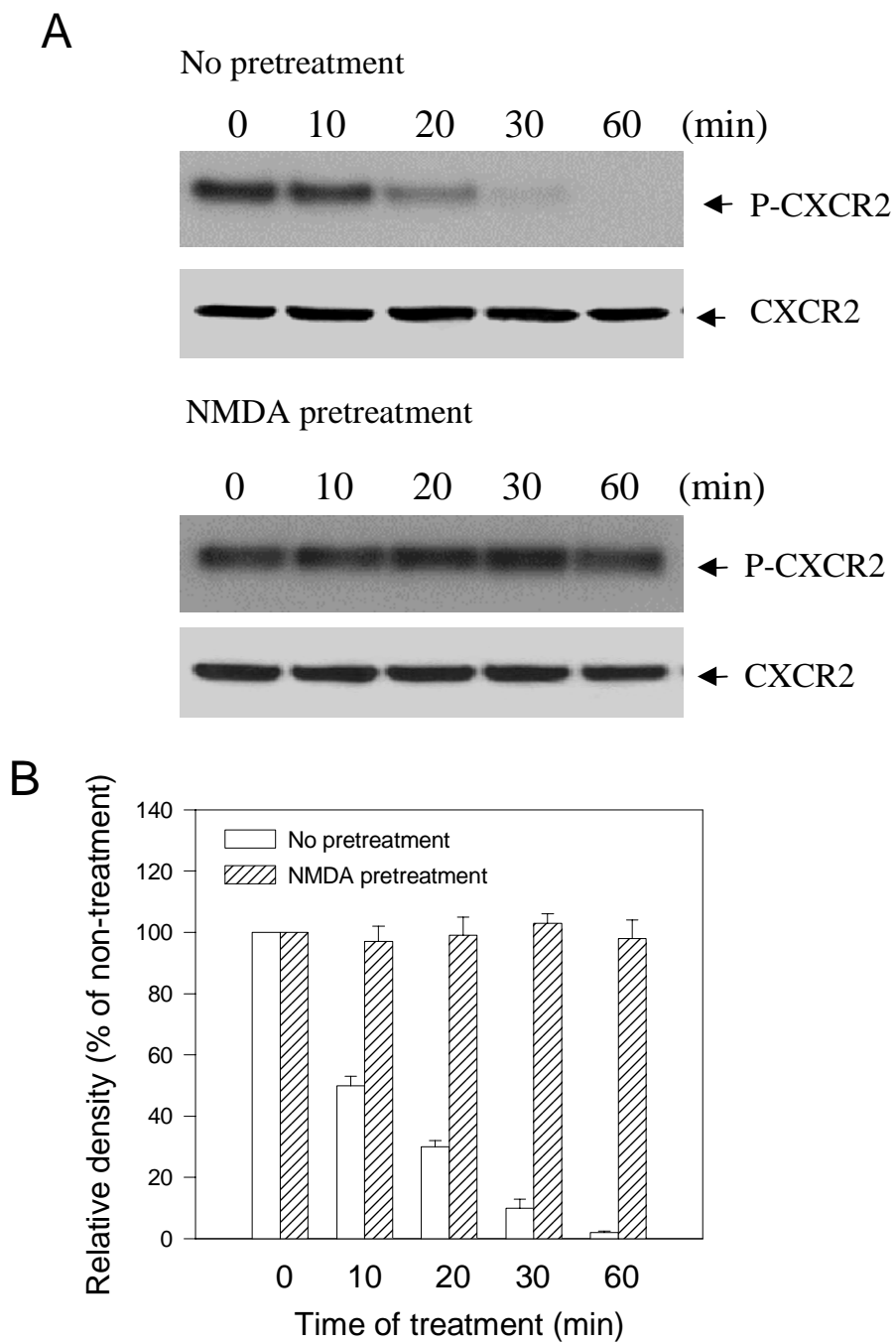
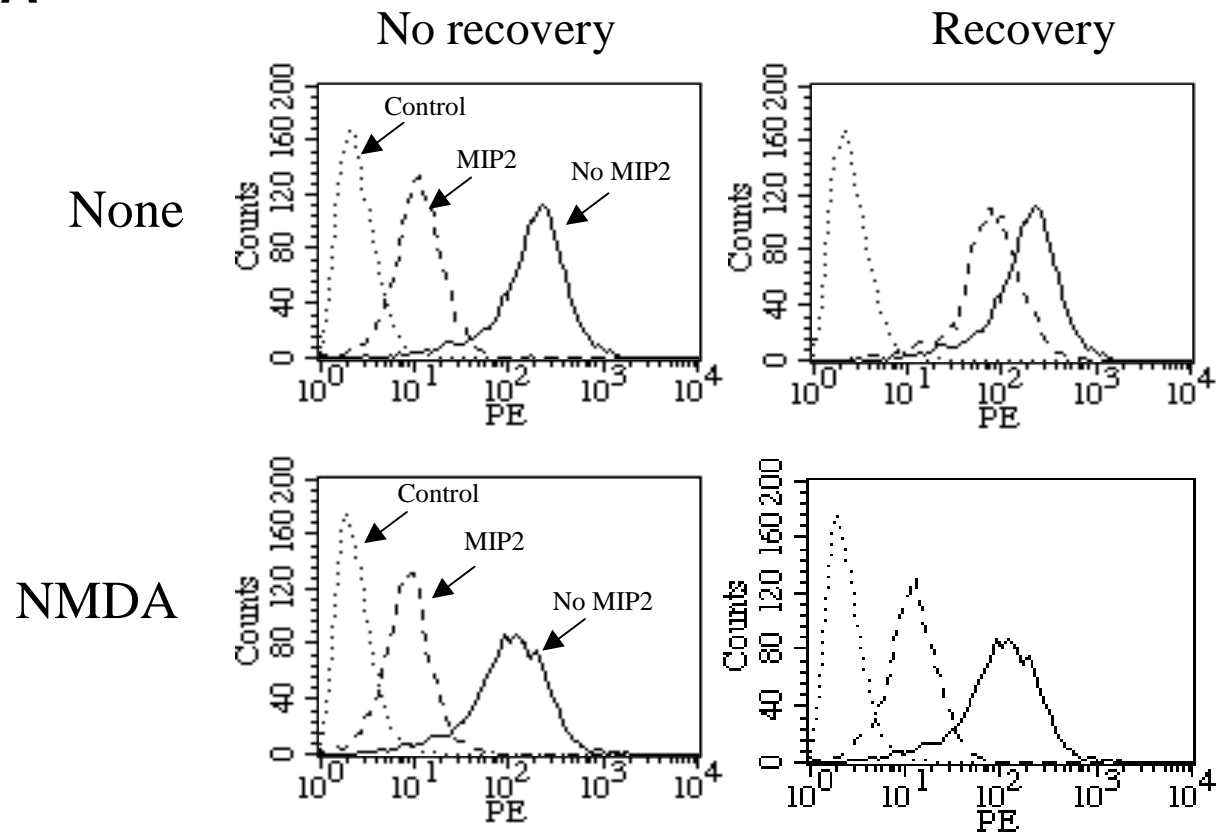


Fig. 6

A



B

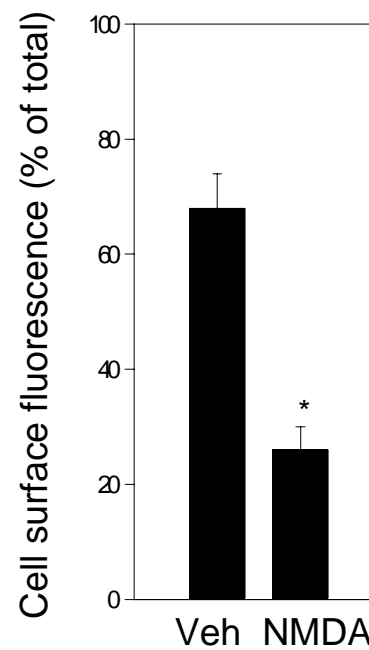


Fig. 7

