Expression of NR1/NR2B *N*-Methyl-D-Aspartate Receptors Enhances Heroin Toxicity in HEK293 Cells

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ABSTRACT: Repeated use of drugs of abuse, namely opiates, has been shown to affect glutamate-releasing neurons. Moreover, blockade of *N*-methyl-D-aspartate (NMDA) receptors (NMDAR) prevents cell death by apoptosis induced by morphine, a heroin metabolite. Thus, in this article we investigated the involvement of different NMDAR subunits in heroin cytotoxicity. Human embryonic kidney (HEK293) cells, which do not express native NMDAR, were transfected with NR1/NR2A or NR1/NR2B subunits. As a control, cells were transfected with NR1 alone, which does not form functional channels. Incubation with heroin for 24 h induced a dose-dependent decrease in cell viability both in NR1-transfected and nontransfected cells. The loss of membrane integrity induced by heroin was more evident in cells transfected with NR1/NR2B than in cells transfected with NR1 alone or NR1/NR2A. This decrease in cell viability was blocked by MK-801, a selective and noncompetitive antagonist of NMDAR. Nevertheless, no significant changes in intracellular adenosine 5′-triphosphate (ATP) were observed in cells treated with heroin. These data implicate NR2B-composed NMDAR as important mediators of heroin neurotoxicity.

KEYWORDS: cytotoxicity; drugs of abuse; HEK203 cells; heroin; NMDA receptors

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INTRODUCTION

The abuse of opioids and other drugs of abuse is associated with severe physical and mental health problems. These drugs interact with the central nervous system, inducing changes in the release of neurotransmitters, namely in the dopaminergic system. Many evidences also suggest the involvement of glutamatergic neurotransmission in the mechanisms of drug dependence involving the dopaminergic reward circuit in the brain.[1] On the other hand, some drugs of abuse, such as methamphetamine, are responsible for neurodegenerative mechanisms that lead to the irreversible loss of neurons, with the involvement of dopaminergic and/or glutamatergic systems.[2] Under this perspective, heroin is able to induce cell death in PC12 cells[3,4] and morphine, a heroin metabolite, induces cell death by apoptosis in the spinal cord [5] and in human cerebrocortical neurons.[6]

Among the processes leading to cell death, the excitotoxic mechanism, which involves the hyperactivation of ionotropic glutamate receptors, has been largely studied due to its involvement in ischemia and several neurodegenerative diseases.[7] *N*-methyl-D-aspartate (NMDA) receptors (NMDAR) have an outstanding role in excitotoxic processes, due to their high permeability to calcium, which is responsible for the activation of several intracellular enzymes, leading to mitochondrial dysfunction and cell death. NMDAR are composed by NR1 subunits which can interact with NR2 (A–D) subunits, and less frequently with NR3 (A, B). NR1 subunits contain the glycine-binding site, whereas the NR2 subunits contain the glutamate-binding site, conferring heterogeneity to these receptors. Previous studies demonstrated that NR2B subunits, which are mainly localized in extrasynaptic sites, are associated to increased toxicity in comparison with NR2A synaptic subunits.[8]

It was previously suggested that glutamatergic neurotransmission involving the NMDAR contributes to opioid dependence in humans.[9] Moreover, the NMDAR antagonist MK-801 was shown to specifically block morphine tolerance and neuronal apoptosis in the spinal cord.[5] In this context, in this work we analyzed the involvement of NR1/NR2A and NR1/NR2B subunits in heroin cytotoxicity.

MATERIAL AND METHODS

Cell Culture and Transfection

Human embryonic kidney (HEK293) cells were obtained from the American Type Culture Collection (Manassas, VA) and were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (Biochrom AG, Berlin, Germany), penicillin (100 U/mL), and streptomycin (100 μg/mL). Cells were grown to

50–75% confluence in poly-L-lysine-coated multiwells and transfected using the superfect transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In cotransfections the NR1:NR2 plasmid ratio was 1:4. Cells were transfected with the DNA-superfect mixture for 4 h and further incubated for 24 h with heroin (provided by the *Instituto da Droga e da Toxicodependência*, Lisbon, Portugal) and/or 10 μM dizocilpine maleate (MK-801), in fresh culture media. The rat NR1 (NR1a), NR2A, and NR2B cDNA clones inserted respectively in pEGFP-N3, pcDNAI, or pDP3 were a generous gift from Dr. John Woodward (Medical University of South Carolina, Charleston, SC). NR1 is expressed as a fusion protein with enhanced green fluorescent protein (EGFP), which is referred throughout the manuscript as NR1-GFP.

Cell Reducing Capacity Assay

Cell reducing capacity was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay for cell survival. MTT (0.5 mg/mL) in Na^+ medium (in mM: 132 NaCl, 1.2 NaH_2PO4, 1.4 $MgCl_2$, 4 KCl, 10 HEPES, 6 glucose, and 1 $CaCl_2$, pH 7.4) was added to the cultures and incubated for 2 h at 37°C in the dark. When taken up by living cells, MTT is converted to a water-insoluble blue product (formazan). The precipitated dye was dissolved in 0.04 M HCl in isopropanol and was colorimetrically quantified at 570 nm. Data were expressed as the percentage of optical density of untransfected HEK293 cells or NR1-GFP-expressing cells, in the absence of heroin.

Lactate Dehydrogenase Leakage Assay

The integrity of the plasma membrane was determined by monitoring the activity of the cytoplasmic enzyme lactate dehydrogenase (LDH) in the extracellular incubation medium. Intracellular LDH activity was assessed after cell lysis with 10 mM HEPES (pH 7.4) supplemented with 0.01% Triton X-100 (Sigma Chemical Co.), and further freezing at −80°C. Cell debris in both aliquots (intracellular and extracellular) were removed by centrifugation at 20,800 *g*, for 10 min (0–4°C). LDH activity was determined spectrophotometrically, by following the rate of conversion of reduced nicotinamide adenine dinucleotide (NADH) to oxidized NAD^+ at 340 nm. LDH released into the extracellular medium was expressed as a percentage of total LDH activity in the cells (percentage of LDH released = extracellular LDH/[extracellular LDH + intracellular LDH]).

Determination of Adenosine Triphosphate Intracellular Levels

The intracellular content of the adenine nucleotide adenosine 5′-triphosphate (ATP) was determined after cell extraction with 0.3 M perchloric acid (0–4°C). The cells were centrifuged at 20,800 *g* for 5 min, and the pellet was solubilized with 1 M NaOH for total protein analysis using *Bio-Rad Protein Assay Dye* (Bio-Rad Laboratories, Hercules, CA). The supernatants were neutralized with 5 M KOH in 2.5 M Tris and centrifuged at 15,800 *g* for 10 min (0–4°C). The resulting supernatants, stored at −80°C, were assayed for ATP determination, by separation in a reverse phase high-performance liquid chromatography (HPLC) and absorbance was monitored at 254 nm. Peak identity was determined by the comparison with the retention time of an ATP standard.

Statistical Analysis

Data are the mean ± SEM from at least three experiments, performed in duplicate or triplicate. Statistical analysis was performed by one-way ANOVA followed by Tukey post-test (a $P < 0.05$ was considered significant).

RESULTS AND DISCUSSION

Previously, we showed that heroin induces a dose-dependent decrease in cell viability in PC12 cells[3] and in primary cortical neurons (unpublished results). In the present study we observed a dose-dependent decrease in cell reducing capacity after 24 h exposure to heroin in both untransfected and NR1-GFP-transfected HEK293 cells (FIG. 1). The decrease in cell reducing capacity was statistically significant for both populations at a high heroin concentration (1 mM). A decrease in cell viability was also observed in untransfected ($P < 0.05$) and NR1-GFP transfected (nonstatistically significant) HEK293 cells exposed to 100 μM heroin. This concentration of heroin was used in the following experiments, because a moderate decrease in cell viability was observed under these conditions. These results demonstrated that heroin is toxic to HEK293 cells. Furthermore, expression of nonfunctional NR1 did not affect heroin toxicity.

Next we evaluated the effect of functional NMDAR, composed of NR1/NR2A or NR1/NR2B, on heroin-induced toxicity. NMDAR expression in the non-neuronal cell line *per se* led to a nonstatistical increase (approximately 5–10%) in LDH release, indicative of some loss of membrane integrity (FIG. 2). Exposure to heroin (100 μM, for 24 h) did not significantly change membrane integrity of NR1-GFP- or NR1/NR2A-expressing cells (FIG. 2). However, NR1/NR2B-transfected cells showed a significant increase in membrane permeability in the presence of the drug ($P < 0.05$), and this effect was completely prevented by the NMDAR antagonist MK-801 (FIG. 2). The

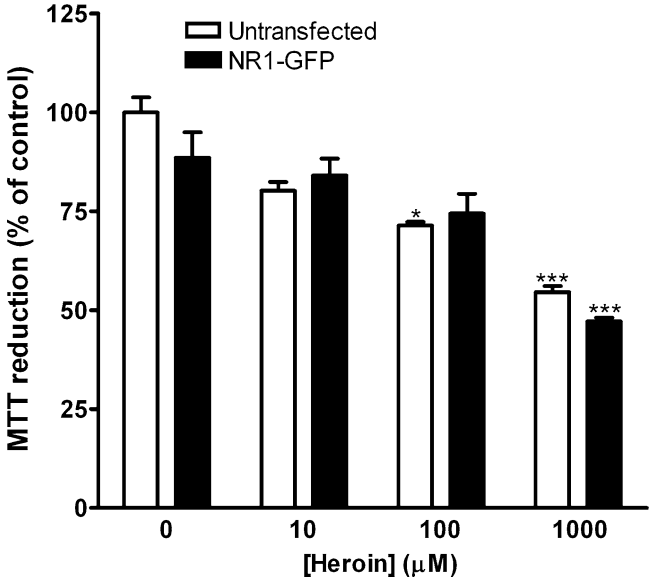

FIGURE 1. Analysis of cell viability in HEK293 cells exposed to street heroin. The viability of untransfected HEK293 cells and cells transfected with NR1-GFP was evaluated upon exposure to increasing concentrations of street heroin (10–1000 μM) for 24 h, by using the MTT reduction assay. Data were presented as mean ± SEM of at least three experiments, performed in triplicate, and were normalized in percentage of nontreated untransfected cells (control). Statistical analysis: $^{*}P < 0.05$ and $^{***}P < 0.001$ compared to the respective untreated cells.

protective effect of MK-801 suggests that activation of NR1/NR2B receptor is necessary for heroin-induced increase in membrane permeability, because MK-801 is an open-channel blocker of NMDAR. Interestingly, NR1/NR2B subtype was previously linked to cellular demise that occurs both in an excitotoxic paradigm[8] and in Huntington´s disease.[10]

Cellular energy deficits are often associated with NMDAR mediated excitotoxicity. Overactivation of NMDAR leads to an excessive increase in intracellular calcium concentration, which is exacerbated by a dysfunction of calcium extrusion mechanisms. This has been suggested to occur as a consequence of mitochondrial dysfunction and resulting decrement in ATP production.[7] Nevertheless, neither the presence of heroin nor the expression of functional NMDAR subtypes, NR1/NR2A or NR1/NR2B, affected ATP intracellular levels significantly (FIG. 3). Because intact cellular energy levels are needed for the prosecution of apoptotic cell death,[11] we hypothesize that NR1/NR2B-mediated increase in heroin cytotoxicity is initially mediated through apoptosis, rather than necrosis. Later stages of apoptosis also involve increase of membrane permeability,[12] explaining the loss of membrane integrity observed in FIGURE 2.

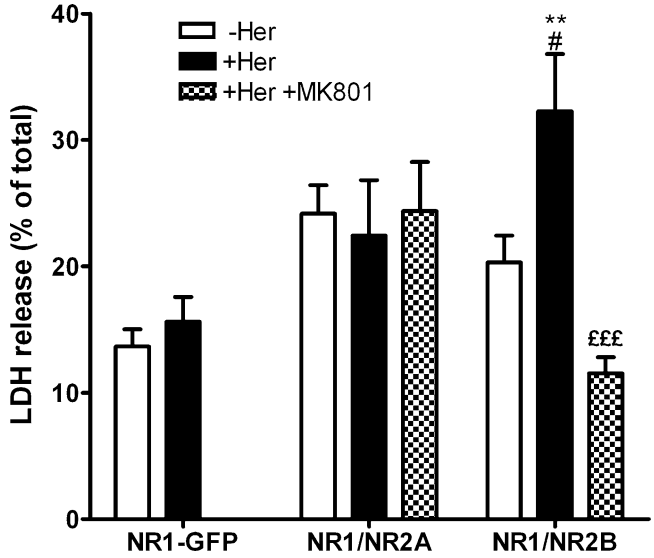

FIGURE 2. Analysis of plasma membrane integrity in HEK293 cells transfected with different subunits of the NMDAR upon acute exposure to street heroin. The cells were transfected with NR1/NR2A or NR1/NR2B subunits of the NMDAR or with NR1-GFP (transfection control) and exposed to 100 μM street heroin and/or 10 μM MK-801 for 24 h. The integrity of the plasma membrane was evaluated by the LDH leakage assay. Data were expressed as a percentage of total LDH and presented as mean ± SEM of at least three experiments, performed in triplicate. Statistical significance: $^{**}P < 0.01$ as compared to NR1-GFP, $\#P < 0.05$ compared to the respective untreated cells, and £££$P <$ 0.001 compared to heroin-treated cells expressing NR1/NR2B.

Taken together our results indicate that the NR1/NR2B subtype of NMDAR is a mediator of heroin-induced cytotoxicity. Previously, chronic exposure to morphine, a heroin metabolite that can co-exist in heroin solutions,[13] was reported to alter NMDAR subunit composition in rat nucleus accumbens neurons. Neuroadaptation to chronic morphine exposure was shown to increase NR2A activity and decrease NR2B activity.[14] In the present work we hypothesize that overactivation of NR2B-composed receptors is responsible for the deleterious cellular effects induced by heroin. It is possible that the decrease in the activity of NR2B-composed NMDAR observed by Martin *et al.*[14] is due to selective cell death of neurons exhibiting higher expression levels of this NMDAR subunit. In this case, these neurons may be more susceptible to toxic insults, similarly to what was reported in Huntington´s disease.[10]

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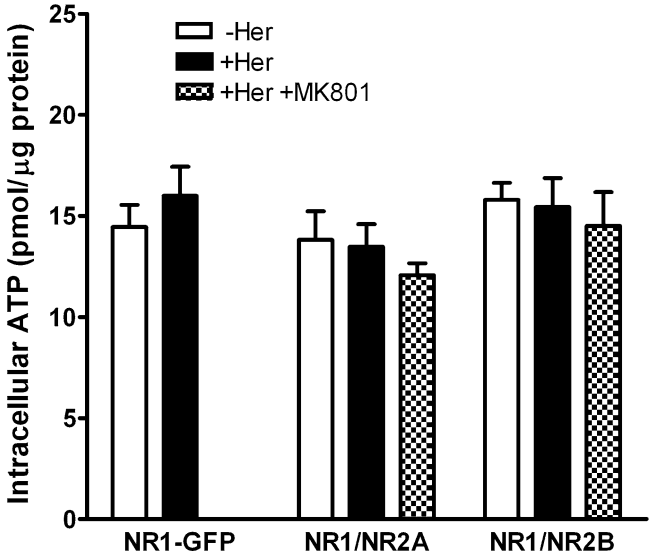

FIGURE 3. Measurement of intracellular ATP levels in HEK293 cells transfected with different subunits of the NMDAR upon acute exposure to street heroin. Cells transfected with NR1-GFP (transfection control) or with the different subunits of the NMDAR (NR1/NR2A or NR1/NR2B) were treated as described in FIGURE 2. Intracellular ATP levels were analyzed by HPLC with UV detection. Data were expressed as pmol/μg protein and presented as mean ± SEM of at least three experiments, performed in triplicate.

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REFERENCES

1. TZSCHENTKE, T.M. & W.J. SCHMIDT. 2003. Glutamatergic mechanisms in addiction. Mol. Psychiatry **8**: 373–382.
2. OHMORI, T., T. ABEKAWA & T. KOYAMA. 1996. The role of glutamate in behavioral and neurotoxic effects of methamphetamine. Neurochem. Int. **29**: 301–307.
3. OLIVEIRA, M.T., A.C. REGO, M.T. MORGADINHO, *et al.* 2002. Toxic effects of opioid and stimulant drugs on undifferentiated PC12 cells. Ann. N. Y. Acad. Sci. **965**: 487–496.
4. OLIVEIRA, M.T., A.C. REGO, T.R. MACEDO & C.R. OLIVEIRA. 2003. Drugs of abuse induce apoptotic features in PC12 cells. Ann. N. Y. Acad. Sci. **1010**: 667–670.
5. MAO, J., B. SUNG, R.R. JI & G. LIM. 2002. Neuronal apoptosis associated with morphine tolerance: evidence for an opioid-induced neurotoxic mechanism. J. Neurosci. **22**: 7650–7661.

6. HU, S., W.S. SHENG, J.R. LOKENSGARD & P.K. PETERSON. 2002. Morphine induces apoptosis of human microglia and neurons. Neuropharmacology **42**: 829–836.
7. REGO, A.C. & C.R. OLIVEIRA. 2003. Mitochondrial dysfunction and reactive oxygen species in excitotoxicity and apoptosis: implications for the pathogenesis of neurodegenerative diseases. Neurochem. Res. **28**: 1563–1574.
8. HARDINGHAM, G.E., Y. FUKUNAGA & H. BADING. 2002. Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. Nat. Neurosci. **5**: 405–414.
9. BISAGA, A., S.D. COMER, A.S. WARD, *et al.* 2001. The NMDA antagonist memantine attenuates the expression of opioid physical dependence in humans. Psychopharmacology (Berl.) **157**: 1–10.
10. ZERON, M.M., O. HANSSON, N. CHEN, *et al.* 2002. Increased sensitivity to N-methyl-D-aspartate receptor-mediated excitotoxicity in a mouse model of Huntington's disease. Neuron **33**: 849–860.
11. NICOTERA, P. & G. MELINO. 2004. Regulation of the apoptosis-necrosis switch. Oncogene **23**: 2757–2765.
12. SCHWAB, B.L., D. GUERINI, C. DIDSZUN, et al. 2002. Cleavage of plasma membrane calcium pumps by caspases: a link between apoptosis and necrosis. Cell Death Differ. **9**: 818–831.
13. HUTCHINSON, M.R. & A.A. SOMOGYI. 2002. Diacetylmorphine degradation to 6-monoacetylmorphine and morphine in cell culture: implications for in vitro studies Eur. J. Pharmacol. **453**: 27–32.
14. MARTIN, G., A. GUADANO-FERRAZ, B. MORTE, *et al.* 2004. Chronic morphine treatment alters N-methyl-D-aspartate receptors in freshly isolated neurons from nucleus accumbens. J Pharmacol. Exp. Ther. **311**: 265–273.