<div align="center">Original Article</div>

Morphine-induced Nitric Oxide Production in PC12 Cells

Mohammad Reza Zarrindast PhD[1,2,3,4], Mehrak Javadi-Paydar PhD[5], Ladan Delphi MSc[6], Nasim Vousooghi PhD•[2,7]

Abstract

Background: The role of nitric oxide (NO) in many well-known effects of morphine is well defined. NO is involved in the signaling pathway of the *N*-methyl-D-aspartate (NMDA) receptor, which is proposed to mediate some of morphine's effects. This research studies the effect of morphine and NMDA on lipopolysaccharide (LPS)-stimulated NO production by clonal rat pheochromocytoma (PC12) cells.

Methods: We used the Griess reaction to measure NO concentrations in cell culture medium.

Results: PC12 cells that were incubated for 24 h with varying concentrations of morphine (0.1, 1, 10, 100, and 1000µM) plus LPS (1 µg/ml) did not significantly alter the concentration of NO in the medium. However, NO production increased when cells were treated for both 48 h with 100 and 1000 µM morphine and for 72 h with 10,100, and 1000 µM of morphine. After 72 h, 1 µM naloxone significantly decreased NO concentration. Naloxone, at doses of 0.1, 1, and 10µM prevented NO production by 1000 µM of morphine. NMDA (0.1, 1, and 10 µM) did not alter NO concentrations after 24, 48 or 72 h. Morphine (1 µM)-induced NO production was inhibited by 10 µM NMDA after 48 h. Inhibition of NO was also noted with1 and 10 µM concentrations of morphine after 72 h.

Conclusion: Chronic treatment of PC12 cells with morphine significantly increases LPS-stimulated NO production via naloxone-sensitive receptors.The cells seem to release endogenous morphine in medium. NMDA does not affect NO production, which may be due to the lack of functional NMDA receptor expression in PC12 cells.

Keywords: Morphine, Nitric oxide, NMDA, PC12 cells

Introduction

Nitric oxide (NO) is a small, gaseous molecule which functions as a neuronal messenger that diffuses easily across neuronal membranes.[1] Nitric oxide synthase (NOS) acts on L-arginine to produce NO and L-citrulline.[2] There are two forms of NOS, constitutive (cNOS) and inducible (iNOS). iNOS is expressed when it is induced by bacterial lipopolysaccharide (LPS) or some proinflammatory cytokines.[3]

Opioid drugs like morphine exert many of their well-known effects via the NO pathway. It has been shown that morphine up-regulates NOS biosynthesis and affects its function by activating opioid receptors.[4] NO can activate the enzyme guanylyl cyclase and generate cyclic GMP (cGMP) from guanosine triphosphate (GTP).[5] In vitro studies have shown that morphine is able to increase cGMP concentration.[6] The NO-cGMP pathway may be involved in morphine-induced analgesia[7] since administration of NOS inhibitors like N^G-nitro-L-arginine (L-NNA) prevent mor-
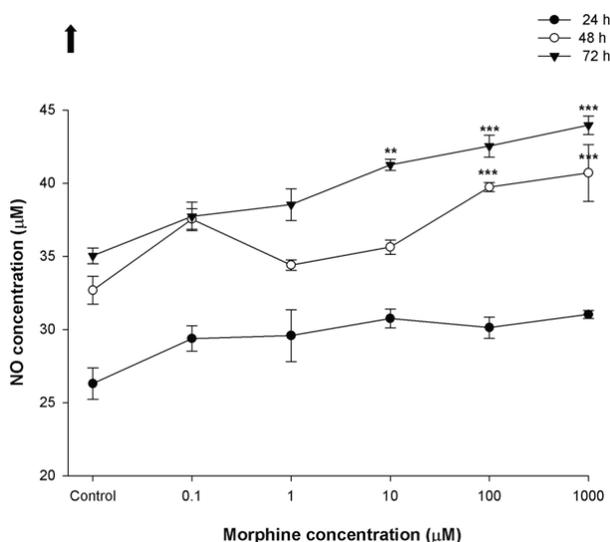
phine analgesia.[8] It has been shown that NOS inhibitors can prevent the development of morphine tolerance [9] or block morphine-induced CPP.[10]

The *N*-methyl-D-aspartate (NMDA) receptor is an important excitatory amino acid receptor involved in many neuronal functions like synaptic plasticity and neuronal development.[11] However, over-activation of NMDA receptors can result in neuronal excito-toxicity and cell death.[12] These ionotropic receptors are members of the glutamate receptor family and are highly permeable to Ca^{2+}. Influx of calcium through the activated NMDA receptor channel leads to various actions, of which some occur through the initiation of NO synthesis followed by continuous NO emission.[13] In fact, in some areas of the central nervous system, NMDA receptors are co-localized in neurons that contain NOS.[14] Studies have indicated that NMDA receptor antagonists, similar to NOS inhibitors, can prevent some effects of morphine. For example, morphine-induced tolerance, analgesia,[15,16] and withdrawal[17] can be inhibited by administration of NMDA receptor antagonists. Acquisition of morphine-induced CPP has been shown to be blocked by the NMDA receptor antagonist memantine.[18]

As mentioned above, the relation between NO production and morphine effects and also between NMDA receptors and morphine have been indirectly evaluated by researchers. However, studies investigating the direct interaction between morphine, NMDA and NO production in cell culture systems are limited. Clonal rat pheochromocytoma (PC12) cells derived from chromaffin cells of the adrenal medulla are extensively used as a model system to study neurons as they show a number of neuronal characteristics.[19] Therefore, we have selected the PC12 cell line to study the effects of morphine and NMDA on LPS-stimulated NO production by these cells. This study has also evaluated the direct interaction between morphine and NMDA in PC12 cells with regards to the NO pathway.

Authors' Affiliations: [1]Department of Neuroscience, School of Advanced Medical Technologies and Department of Pharmacology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran, [2]Iranian National Center for Addiction Studies, Tehran University of Medical Sciences, Tehran, Iran,[3]Institute for Studies in Theoretical Physics and Mathematics, School of Cognitive Sciences, Tehran, Iran, [4]Institute for Cognitive Science Studies, Tehran, Iran, [5]Brain and Spinal Injury Repair Research Center, Imam Khomeini Hospital, Tehran University of Medical Sciences, Tehran, Iran, [6]School of Biology, University College of Science, University of Tehran, Tehran, Iran, [7]Department of Neuroscience, School of Advanced Medical Technologies, Tehran University of Medical Sciences, Tehran, Iran.
•**Corresponding author and reprints:** Nasim Vousooghi PhD, Department of Neuroscience, School of Advanced Medical Technologies, Tehran University of Medical Sciences, P.O. BOX: 1417755469, Tehran, Iran. Tel: (+9821)-8899-1118, Fax: (+9821)-8899-1117, E-mail: n-vousooghi@tums.ac.ir.
Accepted for publication: 7 September 2011

Figure 1. The effects of 24,48, and 72 h incubation of PC12 cells treated with different concentrations of morphine (0.1, 1, 10, 100, and 1000 µM) plus LPS (1 µg/ml) for 24,48, and 72 h on the amount of NO in cell culture medium. NO concentration (µM) was measured by the Griess reaction. Data are presented as mean±SEM of 3 samples. **P<0.01, ***P<0.001 different from the respective control group.

Figure 2. The effects of 24,48 and 72 h incubation of PC12 cells treated with different concentrations of naloxone(0.1, 1, and 10 µM) plus LPS (1 µg/ml) for 24,48, and 72 h on the amount of NO in cell culture medium. NO concentration (µM) was measured by the Griess reaction. Data are presented as mean±SEM of 3 samples. *P<0.05 different from the respective control group.

Materials and Methods

Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Invitrogen. LPS, NMDA, naloxone hydrochloride, sulfanilamide and N-1-naphtylethylenediamine dihydrochloride were purchased from Sigma-Aldrich Co. (UK). Morphine sulfate was purchased from Temad Co. (Tehran, Iran).
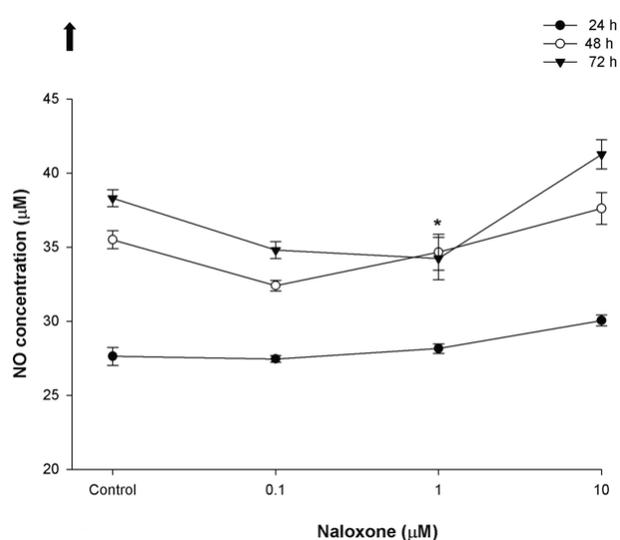
Cell culture and sample treatment

PC12 cells were purchased from Pasteur Institute (Tehran, Iran). Cell culture medium consisted of DMEM (4.5 g/l) that contained 10% (v/v) heat-inactivated FBS, penicillin (100 units/ml), and streptomycin sulfate (100 µg/ml). Cells were maintained at 37ºC in a humidified atmosphere (90%) with 5% CO_2. After overnight incubation (which lead to 40% – 50% confluency),[20] cells were stimulated with LPS (1 µg/ml) and simultaneously treated with different doses of morphine, naloxone and NMDA alone (experiments 1, 2, and 3) or in combination (experiments 4, 5, and 6) for 24, 48, and 72 h. For each experiment, there was a control sample with equal volumes of medium that underwent no treatment.

All drugs were dissolved in phosphate buffered saline and then diluted with the medium accordingly to reach the intended drug concentration.

Measurement of NO production

Using the Griess reaction, we measured the accumulated nitrite in the culture medium as an indicator of NO production.[21] Briefly, 50 µl of cell culture medium was mixed with an equal volume of 1% (w/v) sulfanilamide [in 5% (v/v) phosphoric acid] and 0.1% (w/v) N-1-naphtylethylenediamine dihydrochloride, and incubated at room temperature for 10 min. The absorbance at 548 nm was measured in a microplate reader. Fresh culture medium was used as the blank in all experiments. Sodium nitrite serial dilution standard curve was used to calculate concentrations in the samples. All samples were analyzed in triplicate.
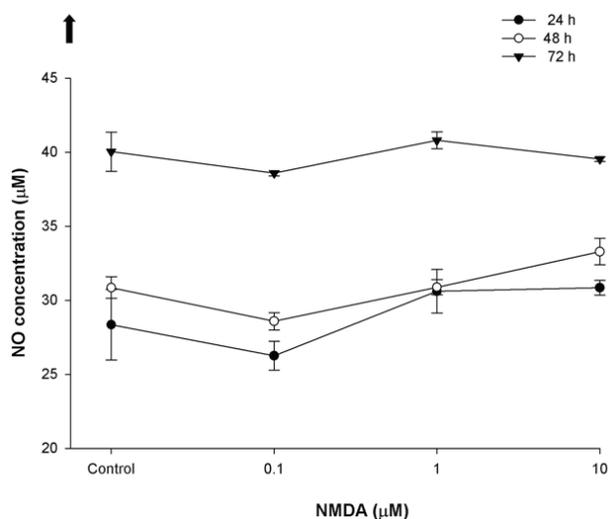
Data analysis

Data are reported as mean ± SEM. Differences among treatment groups were tested using one-way ANOVA followed by Tukey's test. $P < 0.05$ was considered statistically significant.
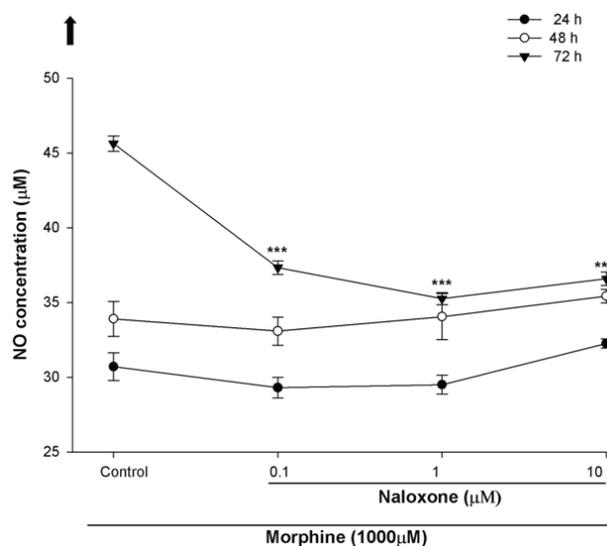
Results

Figure 1 shows NO concentration amounts (µM) in the culture medium of PC12 cells that contained LPS (1 µg/ml) plus different morphine concentrations (0.1, 1, 10, 100, and 1000 µM) for 24, 48, and 72 h incubation times. After 24h incubation with different morphine doses, the NO concentration was not significantly altered in the medium when compared with the control sample. However, morphine concentrations of 100 and 1000 µM significantly increased NO concentration after 48 and 72 h incubation with PC12 cells ($P < 0.001$). The 10 µM morphine concentration after a 72 h incubation period also significantly increased NO production ($P < 0.01$).

Figure 2 represents the effects of different naloxone concentrations (0.1, 1, and 10 µM) on NO production by PC12 cells in the presence of LPS (1 µg/ml) for incubation periods of 24, 48, and 72 h. There were no significant changes in NO concentration after the 24 and 48 h incubation periods of PC12 cells with different naloxone concentrations. However, NO concentration significantly decreased when cells were treated with a naloxone concentration of 1 µM for 72 h ($P < 0.05$).
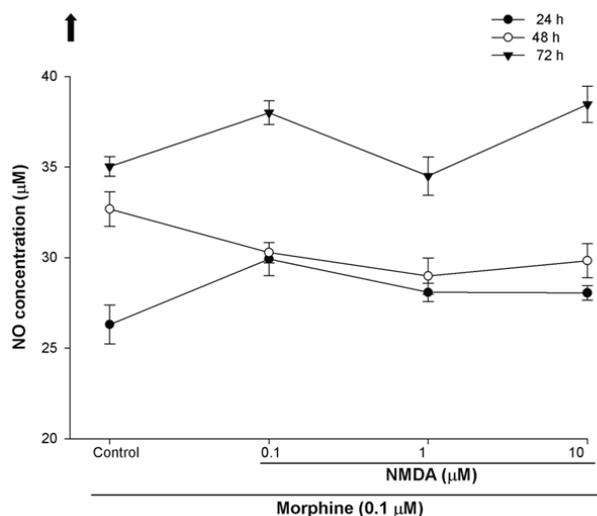
Figure 3 shows the NO concentrations in culture medium when PC12 cells were treated with different NMDA concentrations (0.1,

Figure 3. The effects of 24,48, and 72 h incubation of PC12 cells treated with different concentrations of NMDA(0.1, 1, and 10 μM) plus LPS (1 μg/ml) for 24,48, and 72 h on the amount of NO in cell culture medium. NO concentration (μM) was measured by the Griess reaction. Data are presented as mean±SEM of 3 samples.

Figure 4. The effects of 24,48, and 72 h incubation of PC12 cells treated with a fixed concentration of morphine (1000 μM) alone or in combination with different naloxone concentrations (0.1, 1, and 10 μM) in medium that contained LPS (1 μg/ml) for 24, 48, and 72 h on the amount of NO in cell culture medium.. NO concentration (μM) was measured by the Griess reaction. Data are presented as mean±SEM of 3 samples. ***$P<0.001$ different from the respective control group.

Figure 5. The effects of 24,48, and 72 h incubation of PC12 cells treated with a fixed concentration of morphine (0.1 μM) alone or in combination with different NMDA concentrations (0.1, 1, and 10 μM) in the medium that contained LPS (1 μg/ml) for 24, 48, and 72 h on the amount of NO in cell culture medium. NO concentration (μM) was measured by the Griess reaction. Data are presented as mean±SEM of 3 samples.
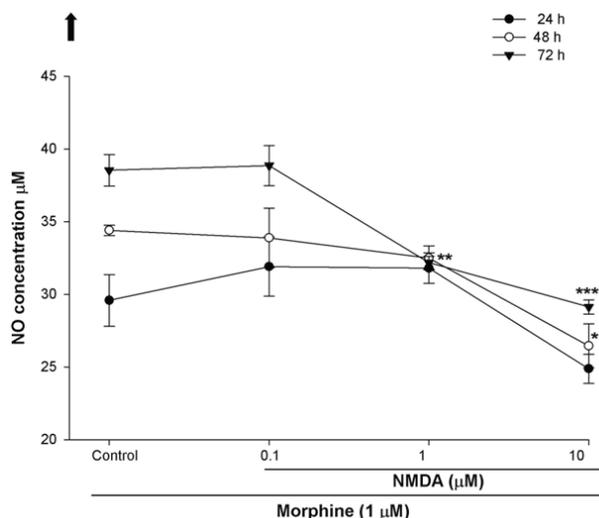
Figure 6. The effects of 24,48, and 72 h incubation of PC12 cells treated with a fixed concentration of morphine (1 μM) alone or in combination with different NMDA concentrations (0.1, 1, and 10 μM) in the medium that contained LPS (1 μg/ml) for 24, 48, and 72 h on the amount of NO in cell culture medium. NO concentration (μM) was measured by the Griess reaction. Data are presented as mean±SEM of 3 samples. *$P<0.05$, **$P<0.01$, ***$P<0.001$ different from the respective control group.

1, and 10 μM) plus LPS (1 μg/ml) for 24, 48, and 72 h. None of the applied concentrations were able to significantly alter NO production at any of the incubation times (24, 48, and 72 h).

Figure 4 shows the effect of a fixed concentration of morphine (1000 μM) alone or in combination with different naloxone concentrations (0.1, 1, and 10 μM) on NO concentration in the me-

dium that contained LPS (1 μg/ml) after 24, 48, and 72 h. The data indicated that all naloxone concentrations significantly prevented NO production by this concentration of morphine after 72 h ($P < 0.001$). However, the same effect was not observed with any naloxone concentration after 24 or 48 h.

In Figure 5, the effect of a fixed morphine concentration (0.1 μM)

alone or in combination with different NMDA concentrations (0.1, 1, and 10 μM) on NO concentration in the medium that contained LPS (1 μg/ml) after 24, 48, and 72 h of incubation is seen. None of the NMDA concentrations significantly altered NO production by this concentration of morphine at any of the incubation times.

Figure 6 shows the effect of a fixed morphine concentration (1 μM) alone or in combination with different NMDA concentrations (0.1, 1, and 10 μM) on NO production in the medium that contained LPS (1 μg/ml) after 24, 48, and 72 h incubation. The data indicated that NMDA concentrations did not change NO production by 1 μM morphine after 24 h incubation. However, after 48 h, NMDA (10 μM) significantly prevented NO production by morphine ($P < 0.05$). NO concentration was also significantly reduced by the NMDA concentrations of 1 μM ($P < 0.01$) and 10 μM ($P < 0.001$) after 72 h incubation.

Discussion

In the present study we investigated the effects of morphine and NMDA on LPS-stimulated NO production by PC12 cells. It has been shown that LPS and cytokines are able to induce NOS at the transcriptional level and lead to NO production in the medium,[22,23] a process that takes several hours.[24] In vitro studies have indicated that LPS-induced NO production can be modulated by opioids.[25,26] There are reports which claim that morphine may increase[27] or decrease[25] NO production in response to LPS. The conflicting results may be due to several factors, such as the type of studied cell, dose of morphine, or duration of exposure.[28]

Our data indicated that acute treatment with different doses of morphine was not able to increase NO production in LPS-stimulated PC12 cells. However, after 48 and 72 h of exposure to morphine, NO production significantly increased in these cells. Our results have confirmed studies which claim that phenomenon such as tolerance and physical or psychological dependence observed after chronic or sustained exposure to opioids are critically dependent on NO production.[29,30]

Molecular mechanisms that have been proposed for morphine-induced NO production may be the modulation of intracellular calcium currents by morphine which may activate Ca^{2+}/calmodulin-dependent NOS.[31] It has been shown that chronic morphine exposure can lead to an increase in NOS expression[32,33] or activity.[34] NO production by morphine has been observed to occur both under basal and LPS-activated states.[26] Our data have also indicated that naloxone is able to significantly prevent NO production after 72 h incubation of PC12 cells with morphine. This is also in agreement with previous findings that morphine-induced NO release is mediated through naloxone-sensitive receptors.[35,36] It has been shown that PC12 cells express a functional mu opioid receptor protein;[37] however, expression of kappa or delta opioid receptors is controversial and varies with the PC12 cell lines used.[38,39]

In addition, our results showed that 72 h incubation with only naloxone (1μM) has significantly inhibited NO production in LPS-stimulated PC12 cells, which may propose the existence of an opioid-like compound in the culture medium. This may support previous findings which have indicated that PC12 cells have the ability to produce morphine chemically identical to the synthetic molecule.[40] It seems that the inhibitory effect of naloxone on NO production is related to antagonizing the effect of morphine released by PC12 cells. Further studies are needed to prove this hypothesis.

The NMDA receptors are believed to be critically involved in excitatory synaptic transmission in the central nervous system; they have a major role in brain development and memory formation.[41] However, a variety of neurological disorders are manifested when NMDA receptors are over-stimulated. In order to have a functional receptor, the NR1 subunit of the NMDA receptor must co-assemble with one or more NR2A-D and/or NR3A-B subunits.[42] Our data have shown that PC12 cells treated with NMDA did not significantly induce NO production after 24, 48, or 72 h. These results do not confirm studies with other cell lines such as neuroblastoma SH-SY5Y cells, in which the activation of NMDA receptors leads to NOS activation and NO production.[43] The reason for this discrepancy may be found in some reports indicating that PC12 cells express no or only negligible amounts of NMDA receptor protein when cultivated in an undifferentiated state (without nerve growth factor) despite the presence of mRNA-transcripts that correspond to some NMDA receptor subunits.[44,45] Although different variants of PC12 cells have different profiles of NMDA receptor subunit expressions,[44] it has been claimed that NR2 subunits are not found in significant levels in PC12 cells.[46] This matter may lead to rapid degradation of unassembled NR1 subunits and lack of functional NMDA receptor production on the cell surface[47] which may be an important factor that limits functional responses to NMDA in PC12 cells. Current flow recording in response to applied NMDA has been unsuccessful in PC12 cells.[44]

Surprisingly, according to our data the same ineffective doses of NMDA are able to significantly decrease NO production by a chronic sub-effective dose of morphine (1 μM). This is difficult to explain as previous findings claim that NMDA receptors are not functionally expressed in PC12 cells (see above). Considering that the viability of cells were unaffected by the doses of drugs used (data not shown), other reasons come to mind. One possible explanation may be based upon previous data which has revealed that chronic morphine administration may affect NMDA receptor subunit expression.[48] Whether this interaction happens in PC12 cells or not, needs to be further investigated.

In conclusion, chronic treatment of PC12 cells with morphine significantly increases LPS-stimulated NO production via naloxone-sensitive receptors. It seems that PC12 cells are able to synthesize and release endogenous morphine in the medium. Treatment of cells by NMDA itself did not affect NO production which may be related to the absence of functional NMDA receptor expression in PC12 cells. However, NMDA significantly decreased NO production when co-administered with morphine. The reason for the latter observation needs to be clarified in the future.

References

1. Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev.* 1991; **43:** 109 – 142.
2. Bredt DS, Hwang PM, Snyder SH. Localization of nitric oxide synthase indicating a neural role for nitric oxide. *Nature.* 1990; **347:** 768 – 770.
3. Yun HY, Dawson VL, Dawson TM. Neurobiology of nitric oxide. *Crit Rev Neurobiol.* 1996; **10:** 291 – 316.
4. Kumar S, Bhargava HN. Time course of the changes in central nitric oxide synthase activity following chronic treatment with morphine in the mouse: reversal by naltrexone. *Gen Pharmacol.* 1997; **29:** 223 – 227.
5. Toda N, Kishioka S, Hatano Y, Toda H. Interactions between morphine and nitric oxide in various organs. *J Anesth.* 2009; **23:** 554 – 568.
6. Minneman KP, Iversen IL. Enkephalin and opiate narcotics increase cyclic GMP accumulation in slices of rat neostriatum. *Nature.* 1976;

262: 313 – 314.

7. Duarte ID, Ferreira SH. The molecular mechanism of central analgesia induced by morphine or carbachol and the L-arginine-nitric oxide-cGMP pathway. *Eur J Pharmacol*. 1992; **221:** 171 – 174.

8. Pataki I, Telegdy G. Further evidence that nitric oxide modifies acute and chronic morphine actions in mice. *Eur J Pharmacol*. 1998; **357:** 157 – 162.

9. Kolesnikov YA, Pick CG, Pasternak GW. NG-nitro-L-arginine prevents morphine tolerance. *Eur J Pharmacol*. 1992; **221:** 399 – 400.

10. Kivastik T, Rutkauskaite J, Zharkovsky A. Nitric oxide synthesis inhibition attenuates morphine-induced place preference. *Pharmacol Biochem Behav*. 1996; **53:** 1013 – 1015.

11. Sucher NJ, Awobuluyi M, Choi YB, Lipton SA. NMDA receptors: from genes to channels. *Trends Pharmacol Sci*. 1996; **17:** 348 – 355.

12. Choi DW. Glutamate neurotoxicity and diseases of the nervous system. *Neuron*. 1988; **1:** 623 – 634.

13. Devulder JE. Could nitric oxide be an important mediator in opioid tolerance and morphine side effects? *J ClinAnesth*. 2002; **14:** 81 – 82.

14. Gracy KN, Pickel VM. Ultrastructural localization and comparative distribution of nitric oxide synthase and N-methyl-D-aspartate receptors in the shell of the rat nucleus accumbens. *Brain Res*. 1997; **747:** 259 – 272.

15. Gonzalez P, Cabello P, Germany A, Norris B, Contreras E. Decrease of tolerance to, and physical dependence on morphine by, glutamate receptor antagonists. *Eur J Pharmacol*. 1997; **332:** 257 – 262.

16. Lutfy K, Hurlbut DE, Weber E. Blockade of morphine-induced analgesia and tolerance in mice by MK-801. *Brain Res*. 1993; **616:** 83 – 88.

17. Bristow LJ, Hogg JE, Hutson PH. Competitive and glycine/NMDA receptor antagonists attenuate withdrawal-induced behaviours and increased hippocampal acetylcholine efflux in morphine-dependent rats. *Neuropharmacology*. 1997; **36:** 241 – 250.

18. Ribeiro D, Couto B, Aguilar MA, Manzanedo C, Rodriguez-Arias M, Minarro J. Effects of NMDA receptor antagonists (MK-801 and memantine) on the acquisition of morphine-induced conditioned place preference in mice. *Prog Neuropsychopharmacol Biol Psychiatry*. 2004; **28:** 1035 – 1043.

19. Nakagawa T, Minami M, Satoh M. Up-regulation of RGS4 mRNA by opioid receptor agonists in PC12 cells expressing cloned mu- or kappa-opioid receptors. *Eur J Pharmacol*. 2001; **433:** 29 – 36.

20. Taupenot L. Analysis of regulated secretion using PC12cells. *Curr Protoc Cell Biol*.2007; Chapter 15:Unit 15.12

21. Ding AH, Nathan CF, Stuehr DJ. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. *J Immunol*. 1988; **141:** 2407 – 2412.

22. Xie QW, Cho HJ, Calaycay J, Mumford RA, Swiderek KM, Lee TD, et al. Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science*.1992**; 256:** 225–228.

23. Seyffarth G, Nelson PN, Dunmore SJ, Rodrigo N, Murphy DJ, Carson RJ. Lipopolysaccharide induces nitric oxide synthase expression and platelet-activating factor increases nitric oxide production in human fetal membranes in culture. *Reprod Biol Endocrinol*. 2004; **2:** 29.

24. Mustafa SB, Olson MS. Expression of nitric-oxide synthase in rat Kupffer cells is regulated by cAMP. *J Biol Chem*. 1998; **273:** 5073 – 5080.

25. Iuvone T, Capasso A, D'Acquisto F, Carnuccio R. Opioids inhibit the induction of nitric oxide synthase in J774 macrophages. *Biochem Biophys Res Commun*. 1995; **212:** 975 – 980.

26. Singhal PC, Sharma P, Kapasi AA, Reddy K, Franki N, Gibbons N. Morphine enhances macrophage apoptosis. *J Immunol*. 1998; **160:** 1886 – 1893.

27. Martucci C, Franchi S, Lattuada D, Panerai AE, Sacerdote P. Differential involvement of Rel B in morphine-induced modulation of chemotaxis, NO, and cytokine production in murine macrophages and lymphocytes. *J Leukoc Biol*. 2007; **81:** 344 – 354.

28. Saurer TB, Ijames SG, Lysle DT. Evidence for the nucleus accumbens as a neural substrate of heroin-induced immune alterations. *J Pharmacol Exp Ther*. 2009; **329:** 1040 – 1047.

29. Bhargava HN. Attenuation of tolerance to, and physical dependence on, morphine in the rat by inhibition of nitric oxide synthase. *Gen Pharmacol*. 1995; **26:** 1049 – 1053.

30. Cuellar B, Fernandez AP, Lizasoain I, Moro MA, Lorenzo P, Bentura ML, et al. Up-regulation of neuronal NO synthase immunoreactivity in opiate dependence and withdrawal. *Psychopharmacology (Berl)*. 2000; **148:** 66 – 73.

31. Nieto-Fernandez FE, Mattocks D, Cavani F, Salzet M, Stefano GB. Morphine coupling to invertebrate immunocyte nitric oxide release is dependent on intracellular calcium transients. *Comp BiochemPhysiol B Biochem Mol Biol*. 1999; **123:** 295 – 299.

32. Lanier RK, Ijames SG, Carrigan KA, Carelli RM, Lysle DT. Self-administration of heroin produces alterations in the expression of inducible nitric oxide synthase. *Drug Alcohol Depend*. 2002; **66:** 225 – 233.

33. Lysle DT, Carrigan KA. Morphine-6beta-glucuronide modulates the expression of inducible nitric oxide synthase. *Inflammation*. 2001; **25:** 267 – 275.

34. Barjavel MJ, Bhargava HN. Effect of opioid receptor agonists on nitric oxide synthase activity in rat cerebral cortex homogenate. *Neurosci-Lett*. 1994; **181:** 27 – 30.

35. Leza JC, Lizasoain I, San-Martin-Clark O, Lorenzo P. Morphine-induced changes in cerebral and cerebellar nitric oxide synthase activity. *Eur J Pharmacol*. 1995; **285:** 95 – 98.

36. Mastronicola D, Arcuri E, Arese M, Bacchi A, Mercadante S, Cardelli P, et al. Morphine but not fentanyl and methadone affects mitochondrial membrane potential by inducing nitric oxide release in glioma cells. *Cell Mol Life Sci*. 2004; **61:** 2991 – 2997.

37. Yoshikawa M, Nakayama H, Ueno S, Hirano M, Hatanaka H, Furuya H. Chronic fentanyl treatments induce the up-regulation of mu opioid receptor mRNA in rat pheochromocytoma cells. *Brain Res*. 2000; **859:** 217 – 223.

38. Abood ME, Tao Q. Characterization of a delta opioid receptor in rat pheochromocytoma cells. *J Pharmacol Exp Ther*. 1995; **274:** 1566 – 1573.

39. Margioris AN, Venihaki M, Stournaras C, Gravanis A. PC12 cells as a model to study the effects of opioids on normal and tumoral adrenal chromaffin cells. *Ann N Y Acad Sci*. 1995; **771:** 166 – 172.

40. Goumon Y, Zhu W, Weeks BS, Casares F, Cadet P, Bougaeva M, et al. Identification of morphine in the adrenal medullary chromaffin PC-12 cell line. *Brain Res Mol Brain Res*. 2000; **81:** 177 – 180.

41. Albensi BC. The NMDA receptor/ion channel complex: a drug target for modulating synaptic plasticity and excitotoxicity. *Curr Pharm Des*. 2007; **13:** 3185 – 3194.

42. Le DA, Lipton SA. Potential and current use of N-methyl-D-aspartate (NMDA) receptor antagonists in diseases of aging. *Drugs Aging*. 2001; **18:** 717 – 724.

43. Bredt DS, Snyder SH. Nitric oxide: a physiologic messenger molecule. *Annu Rev Biochem*. 1994; **63:** 175 – 195.

44. Edwards MA, Loxley RA, Williams AJ, Connor M, Phillips JK. Lack of functional expression of NMDA receptors in PC12 cells. *Neurotoxicology*. 2007; **28:** 876 – 885.

45. Schubert D, Kimura H, Maher P. Growth factors and vitamin E modify neuronal glutamate toxicity. *Proc Natl Acad Sci U S A*. 1992; **89:** 8264 – 8267.

46. Vazhappilly R, Sucher NJ. Translational regulation of the N-methyl-D-aspartate receptor subunit NR1. *Neurosignals*. 2004; **13:** 190 – 193.

47. Vazhappilly R, Sucher NJ. Turnover analysis of N-methyl-D-aspartate receptor subunit NR1 protein in PC12 cells. *Neuro sci Lett*. 2002; **318:** 153 – 157.

48. Bajo M, Crawford EF, Roberto M, Madamba SG, Siggins GR. Chronic morphine treatment alters expression of N-methyl-D-aspartate receptor subunits in the extended amygdala. *J Neurosci Res*. 2006; **83:** 532 – 537.