ELSEVIER

European Journal of Pharmacology 328 (1997) 31–36

ejp

Activation of c-*fos* mRNA in the brain by the κ-opioid receptor agonist enadoline and the NMDA receptor antagonist dizocilpine

Peter K. Panegyres *, John Hughes

Parke-Davis Neuroscience Research Centre, Cambridge University Forvie Site, Robinson Way, Cambridge CB2 2QB, UK

Received 9 December 1996; revised 25 February 1997; accepted 25 March 1997

Abstract

Using in situ hybridization, we have shown that the κ-opioid receptor agonist enadoline (CI-977) and the non-competitive NMDA receptor antagonist dizocilpine (MK-901) induced the immediate early gene c-*fos* in dorsal medial thalamic nuclei. Dizocilpine, and not enadoline, also induced c-*fos* in the posterior cingulate cortex and retrosplenial cortex. Enadoline's stimulation of c-*fos* mRNA was dose and time dependent and completely inhibited by pretreatment with naltrexone. Morphine did not stimulate c-*fos* in the thalamus. It is suggested that the stimulation of c-*fos* with enadoline represents a specific κ-opioid receptor effect.

Keywords: κ-Opioid receptor agonist; NMDA receptor antagonist; Immediate early gene

1. Introduction

Neurones have the potential to modify their responses to changes in input. This capacity is indicative of a molecular machinery that can relate changes in stimuli to changes in phenotype. Proto-oncogenes, e.g. c-*fos* and c-*jun*, are members of a set of genes known as cellular immediate early genes, and are believed to play an important role in stimulus-transcription coupling (Morgan and Curran, 1991). Many stimuli induce c-*fos* mRNA and protein including long-term potentiation, seizures, ischaemia and electrical stimulation (Sheng and Greenberg, 1990; Morgan and Curran, 1991). These stimuli require the influx of Ca^{2+} through the *N*-methyl-D-aspartate (NMDA) ionophore and L-type Ca^{2+} channels (Bading et al., 1993). The induction of c-*fos* by ischaemia, seizures and other stimuli can be blocked by NMDA receptor antagonists such as the non-competitive antagonist dizocilpine maleate (MK-801) (Dragunow et al., 1989; Herrera and Robertson, 1989; Szekely et al., 1987, 1989).

Contrasting with these findings on the induction of immediate early genes expression, it has been shown that dizocilpine, a drug which blocks the NMDA Ca^{2+} ionophore, can itself stimulate Fos protein and other immediate early genes (Dragunow and Faull, 1990; Gass et al., 1993). Both of these studies demonstrated immediate early genes activation in dorsal medial thalamic nuclei, in the cingulate and splenial cortex, and the deeper layers of the cerebral cortex. The findings in the cingulum and splenium have also been associated with the induction of heat shock gene and protein (Olney et al., 1991; Sharp et al., 1991), morphological evidence of neuronal injury with vacuolation and death (Olney et al., 1989; Allen and Iversen, 1990), and related by some to the psychotic potential of non-competitive NMDA antagonists (Olney et al., 1991).

The highly selective κ-opioid receptor agonist of the arylacetamide series enadoline (CI-977) has analgesic properties (Hunter et al., 1990). It has also been shown to be anticonvulsant (Singh et al., 1990) and to be neuroprotective in global and focal models of cerebral ischaemia (Hayward et al., 1992, 1993). In vitro it has been shown to inhibit glutamate release (Lambert et al., 1991). Electrophysiologically it reduces excitatory post-synaptic potentials in the rat locus coeruleus and dorsal raphe by a presynaptic mechanism (Pinnock, 1992a,b).

Since enadoline is believed to operate through a presynaptic effect on glutamate release, we were interested to determine what effect the drug had on c-*fos* expression in the central nervous system, and to compare it with the known effects of dizocilpine.

* Corresponding author. Current address: Department of Neuropathology, Royal Perth Hospital, Perth 6000, WA, Australia. Tel.: (61-9) 224-2433; Fax: (61-9) 224-2556;
e-mail: petepane@dunamis.rph.uwa.edu.au

2. Materials and methods

2.1. Animals

Male Wistar rats (270–290 g) were supplied by B&K Universal. They were housed in cages with 5–6 animals per cage. Food and water were freely available. They were kept in rooms with a 12 h light-dark cycle. There were 6 animals in each experimental and control group.

2.2. Compounds

Enadoline [CI-977 = 5*R*-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4,5]-dec-8-yl]-4-benzofuranacetamide monohydrochloride] was synthesized in the Department of Medical Chemistry, Parke-Davis Neuroscience Research Centre (Cambridge, UK). It was dissolved in sterile water.

Dizocilpine maleate [(+)-MK-801 hydrogen maleate = (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]-

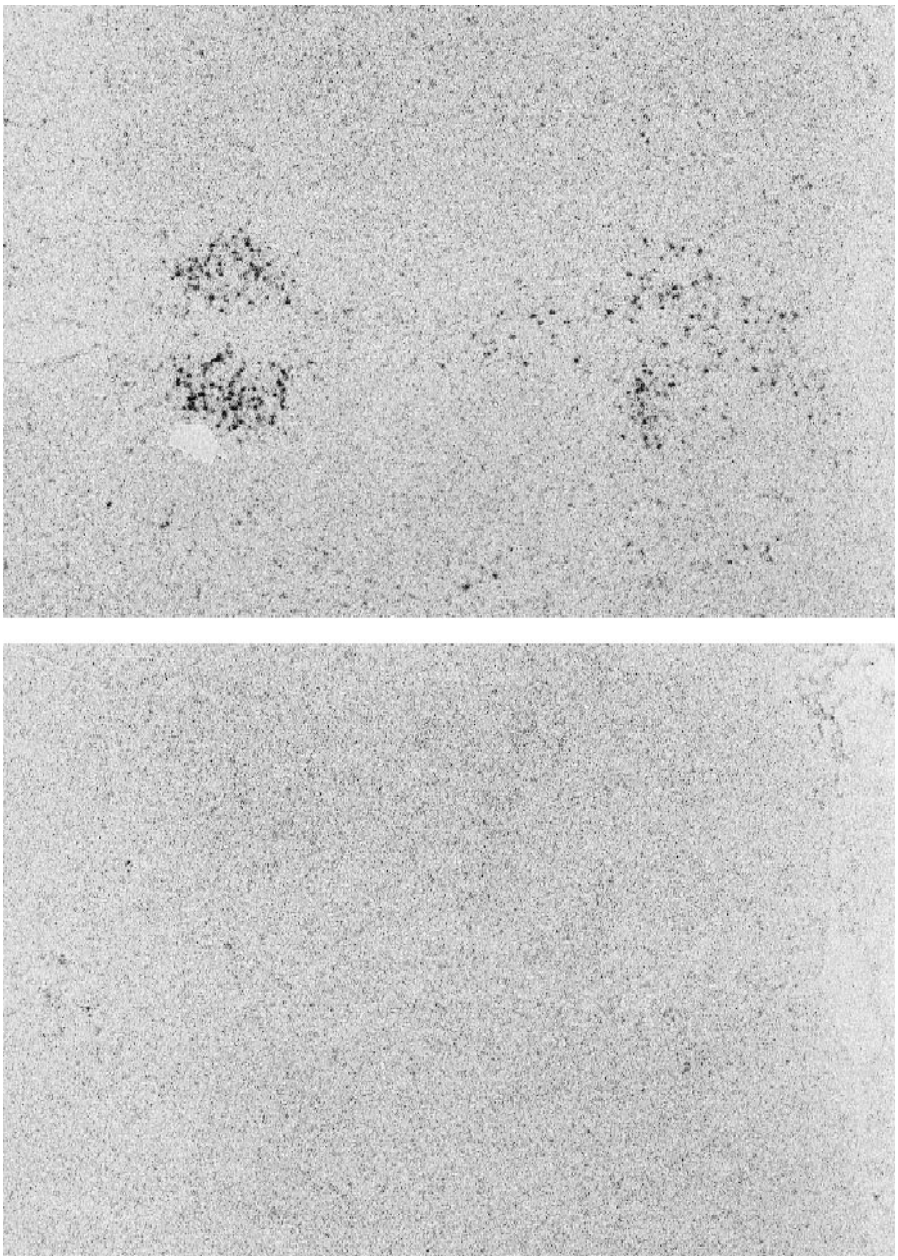

Fig. 1. Photomicrographs of emulsions of transverse rat sections shaving c-*fos* mRNA containing cells in an animal given enadoline (upper) and vehicle control (lower). The arrow shows the paraventricular thalamic nucleus where there are a number of c-*fos* containing cells. Rostrally, to the right of the photograph, shows c-*fos* mRNA containing cells in centromedian thalamic nuclei (H&E × 32).

cyclohepten-5,10-imine hydrogen maleate] was obtained from Research Biochemicals International (Natick, MA, USA).

Naltrexone hydrochloride was obtained from Sigma (Poole, UK) and morphine sulphate from Martindale, UK. These drugs were also dissolved in sterile water.

2.3. Experimental design

Animals were given all drugs by intraperitoneal injection (i.p.). Enadoline was used at a neuroprotective dose of 1 mg/kg, as was dizocilpine at 3 mg/kg (Hayward et al., 1992, 1993). The naltrexone dose was 10 mg/kg, and morphine dose 1 mg/kg. Animals were killed 1 h after administration of each drug unless described otherwise.

2.4. In situ hybridization

The brains were quickly dissected and frozen on aluminium foil on dry ice. The brains were stored at −70°C until required. Sections of 10 μm thickness were obtained using a microtome. Coronal sections from the same levels were taken in all animals (using the coordinates from the interaural line of Paxinos and Watson, 1982): claustrum and nucleus accumbens from the interaural line (2.7 mm, coronal), thalamic nuclei and posterior cingulate cortex (−2.8 mm, coronal from bregma), central gray and retrospenial cortex – including the interpeduncular nuclei – (−6.3 mm, coronal); transverse encompassing neocortex, thalamus, striatum and hippocampus (5.6 mm).

In situ hybridization was performed using synthetic oligonucleotides 3′-labelled with [^{35}S]dATP using terminal deoxynucleotide transferase. The sequences used were: c-*fos*, a 45-mer probe corresponding to amino acids 1–15 of the peptide (Curran et al., 1987). The labelled probe had a specific activity of 2×10^6 Ci/mmol and was made up to a concentration of 3×10^3 dpm/μl with hybridization buffer (50% formamide, 4 × saline sodium phosphate/sodium pyrophosphate/EDTA, 10% dextran sulfate, 5 × Denhardt's solution, denatured salmon sperm DNA 200 μg/ml, dithiothreitol (40 mM) and polyadenylic acid (100 μg/ml)). 100 μl of the buffer containing labelled probe was added to each slide and incubated overnight at 42°C. The following day the sections were washed at room temperature for 0.5 h, and then at 60°C for 0.5 h in 1 × standard saline citrate. The sections were dehydrated in ethanol solutions and exposed to autoradiography film (Hyperfilm-B max, Amersham, Amersham, UK). In all experiments treatment and control groups were examined simultaneously, exposed to the same film, for the same duration, and developed and photographed under identical conditions.

2.5. Emulsion microautoradiography

Slides were dipped in molten emulsion (LM-1, Amersham) for 5 s and then allowed to dry. They were placed in light-protected boxes for up to 6–8 weeks and experimental and control sections were developed under standard conditions. The emulsions were stained with haematoxylin and eosin (H&E).

2.6. Quantification

Films were quantified using the Microcomputer Imaging Device (MCID, Imaging Research, Canada). The system was set such that changes in optical density were in the linear range. Relative optical density units were normalized to an anatomical region in which gene expression remained constant. The same areas of interest were outlined using the computer tools and identical regions were used in experimental and control. The films were read blind without knowledge of treatments.

2.7. Statistics

The Kruskal-Wallis nonparametric analysis of variance (ANOVA) was used to analyze the data.

3. Results

Enadoline caused sedation but no motor effects. Dizocilpine, however, gave rise to increased head movements, increased locomotion, ataxia and rolling movements.

Enadoline stimulated c-*fos* mRNA in the paraventricular and the centromedian thalamic nucleus, but not the

Table 1
The stimulation of c-*fos* mRNA in limbic structures relative to the hippocampus after enadoline and dizocilpine

	Dizocilpine [a]	Vehicle	Enadoline [b]	Vehicle
Posterior cingulate cortex	4.76 ± 0.24	1.37 ± 0.13	0.81 ± 0.08	1.5 ± 0.19
Retrosplenial cortex	4.61 ± 0.26	1.03 ± 0.08	0.86 ± 0.17	1.0 ± 0.07
PVTN	5.51 ± 0.80	0.75 ± 0.12	7.80 ± 0.41	1.21 ± 0.25
CMTN	7.48 ± 0.07	0.91 ± 0.14	5.11 ± 0.29	0.99 ± 0.14
Hippocampus	1.0	1.0	1.0	1.0

PVTN = paraventricular thalamic nucleus; CMTN = centromedian thalamic nucleus. Kruskal-Wallis ANOVA: (a) KW = 21.083, $P = 0.0036$; (b) KW = 17.6, $P = 0.0244$. Six animals in each treatment group, results expressed as means ± S.E.

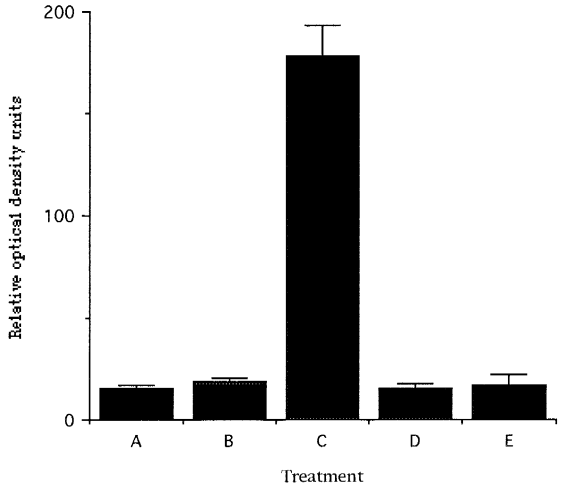

Fig. 2. The effects of dose and time on the expression of c-*fos* mRNA in the paraventricular thalamic nucleus. A = dose; B = time. RNA units are relative optical density units expressed as mean ± S.E., $n = 6$ in each group. Kruskal-Wallis nonparametric anova $P < 0.1$ for 0.1 mg/kg; $P < 0.05$ for 1 mg/kg; $P < 0.001$ for 10 mg/kg dose of enadoline. For time after 1 mg/kg enadoline $P < 0.001$ for 1h, $P < 0.05$ for 6 h.

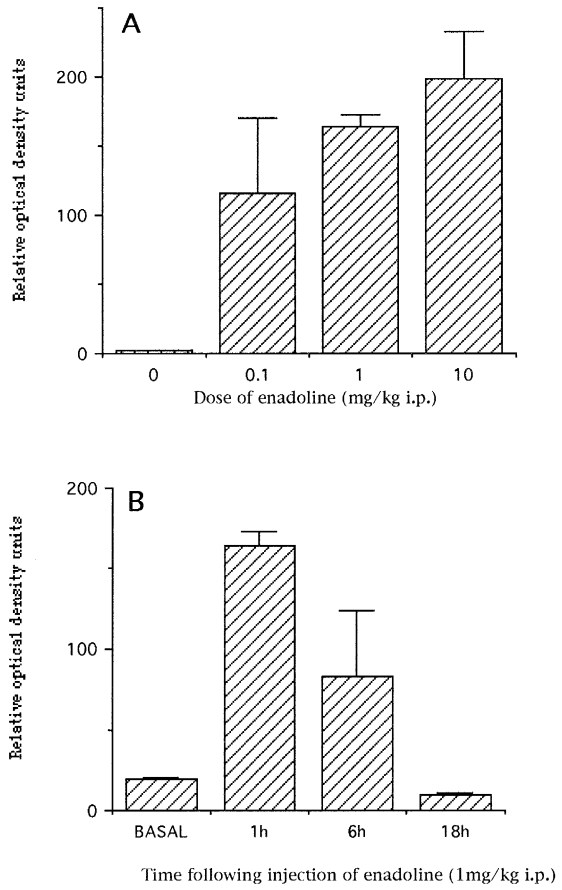

Fig. 3. The effects of naltrexone pretreatment on the expression of c-*fos* mRNA in the paraventricular thalamic nucleus after enadoline. Pretreatments were given 0.5 h before enadoline (1 mg/kg i.p.). A = naltrexone (10 mg/kg i.p.) + vehicle; B = naltrexone + enadoline; C = vehicle + enadoline; D = vehicle + vehicle; E = vehicle + morphine (1 mg/kg i.p.). Results are expressed as relative optical density units, means ± S.E., $n = 6$ in each group.

posterior cingulate and retrosplenial cortex (Fig. 1). Enadoline stimulated c-*fos* by a magnitude of 5–7 times in thalamic nuclei (Table 1). No rise was seen within the cingulate and retrosplenial cortex. In contrast, dizocilpine stimulated c-*fos* mRNA 4.6–7.5 times in all these brain regions.

The stimulation of c-*fos* was dose and time dependent. Stimulation was found at 0.1 mg/kg and increased further by doses of 1 and 10 mg/kg (Fig. 2A). Following a dose of 1 mg/kg expression of c-*fos* was maximal at 1 h and had returned to basal levels by 18 h (Fig. 2B). Time points at 0.5 h and between 1 and 6 h were not studied.

Naltrexone pretreatment completely inhibited enadoline's stimulation of c-*fos* (Fig. 3). Naltrexone and morphine had no affect on c-*fos* by themselves (Fig. 3).

4. Discussion

We have confirmed that the non-competitive NMDA receptor antagonist and neuroprotective agent dizocilpine can, by itself, induce c-*fos* in limbic regions including dorsal medial thalamic nuclei and the retrosplenial and posterior cingulate cortex (Dragunow and Faull, 1990; Gass et al., 1993). In these same structures neuronal vacuolation and death have been observed and related to the psychotic side effects of non-competitive NMDA antagonists (Olney et al., 1989).

This study demonstrates that the neuroprotective κ-opioid receptor agonist enadoline can stimulate c-*fos* by itself. However, the effect has similarities and differences when compared to dizocilpine. Dorsal medial thalamic nuclei were stimulated by both agents. Enadoline did not induce c-*fos* in the cingulum and splenium. Possibly this effect on c-*fos* in dorsal medial thalamic nuclei represents an affect on glutamatergic fibres. Enadoline has been shown to inhibit glutamate release in vitro (Lambert et al., 1991), and dizocilpine binds to a site within the ionophore of the NMDA receptor (Monaghan et al., 1989). However, there is no in vivo evidence that enadoline inhibits glutamate release in the thalamus.

The stimulation of c-*fos* mRNA in thalamic nuclei was completely inhibited by naltrexone, indicating opioid stimulation. Morphine, a predominantly μ-agonist had no effect on c-*fos* expression in the thalamus, confirming the probable κ-opioid nature of the response. κ-Opioid receptors are found in dorsal medial thalamic nuclei which is consistent with their stimulation by enadoline (Mansour et al., 1988). Morphine has been shown to stimulate c-*fos* in the striatum but not in other regions of the brain (Chang et al., 1988).

Dizocilpine stimulates 2-deoxyglucose metabolism in limbic regions, including thalamic nuclei and the cingulum (Nehis et al., 1988). These regions conform closely to the anatomical regions of c-*fos* stimulation. Enadoline, however, decreases glucose utilization in all areas except the

lateral habenula (Mackay et al., 1993) and has no effect upon cerebral blood flow (Mackay et al., 1992). Therefore the changes in c-*fos* expression recorded here do not represent non-specific metabolic activation and toxicity but probably specific neuronal stimulation via the κ-opioid receptor.

Fibres from the centromedian and intermediodorsal thalamic nuclei provide a diffuse cortical projection system that is believed to control the rhythmical electrical activity of the cerebral cortex (Dempsey and Morrison, 1942, 1943; Jasper, 1949), influence central pain mechanisms (Emmers, 1976) and to regulate striatal motor activity (Jones, 1985). It is proposed that the activation of these thalamic nuclei by enadoline is a potential mechanism by which these agents affect the cerebral cortex and other structures such as the hippocampus. The modulation of these diffuse projections by κ-opioid receptors agonists may be related to neuroprotective, analgesic and anticonvulsant effects.

5. Conclusions

We have demonstrated that both enadoline and dizocilpine induce c-*fos* mRNA in dorsal medial thalamic nuclei. The stimulation of c-*fos* by enadoline is thought to be a κ-opioid receptor effect.

Acknowledgements

The authors are thankful to Dr Sandy McKnight for his helpful comments.

References

Allen, H.L., Iversen, L.L., 1990. Phencyclidine, dizocilpine and cerebrocortical neurons. Science 247, 221.

Bading, H., Ginty, D.D., Greenberg, M.E., 1993. Regulation of gene expression in hippocampal neurones by distinct signalling pathways. Science 260, 181–186.

Chang, S.L., Squinto, S.P., Harlen, R.E., 1988. Morphine activation of c-*fos* expression in rat brain. Biochem. Biophys. Res. Commun. 157, 698–704.

Curran, T., Gordan, M.B., Rubino, K.L., Sambucetti, L.C., 1987. Isolation and characterization of the c-*fos* (rat) cDNA and analysis of post-translational modification in vitro. Oncogene 2, 79–84.

Dempsey, E.W., Morrison, R.S., 1942. The production of rhythmically recurrent cortical potentials after localized thalamic stimulation. Am. J. Physiol. 135, 293–300.

Dempsey, E.W., Morrison, R.S., 1943. The electrical activity of a thalamocortical relay system. Am. J. Physiol. 138, 283–293.

Dragunow, M., Faull, R.L.M., 1990. MK-901 induces c-*fos* protein in thalamic and neocortical neurons of rat brain. Neurosci. Lett. 113, 144–150.

Dragunow, M., Abraham, W.C., Goulding, M., Mason, S.E., Robertson, H.A., Faull, R.L.M., 1989. Long-term potentiation and the induction of c-*fos* mRNA proteins in the dentate gyrus of unanaesthetized rats. Neurosci. Lett. 101, 274–280.

Emmers, R., 1976. Thalamic mechanisms that process a temporal pulse code for pain. Brain Res. 103, 425–441.

Gass, P., Herdegen, T., Bravo, R., Kiessling, M., 1993. Induction and suppression of immediate early genes in specific rat brain regions by the non-competitive *N*-methyl-D-asparate receptor antagonist MK-801. Neuroscience 53, 749–758.

Hayward, N.J., McKnight, A.T., Woodruff, G.N., 1992. The neuroprotective action of κ-opioid agonists in the gerbil. Mol. Neuropharmacol. 2, 221–227.

Hayward, N.J., McKnight, A.T., Woodruff, G.N., 1993. Neuroprotective effect of the κ-agonist enadoline (CI-977) in rat models of focal cerebral ischaemia. Eur. J. Neurosci. 5, 961–967.

Herrera, D.E., Robertson, H.A., 1989. Unilateral induction of c-*fos* protein in cortex following cortical devascularization. Brain Res. 503, 205–213.

Hunter, J.C., Leighton, G.E., Meecham, K.G., Boyle, S.J., Horwell, D.C., Rees, D.C., Hughes, J., 1990. CI-977, a novel and selective agonist for the κ-opioid receptor. Br. J. Pharmacol. 101, 183–189.

Jasper, H.H., 1949. Diffuse projection systems: the integrative action of the thalamic reticular system. Electroencephalogr. Clin. Neurophysiol. 1, 405–420.

Jones, E.G., 1985. The Thalamus. Plenum Press, New York, NY.

Lambert, P.D., Woodruff, G.N., Hughes, J., Hunter, J.C., 1991. Inhibition of L-glutamate release: a possible mechanism of action for the neuroprotective effects of the κ-selective agonist CI-977. Mol. Neuropharmacol. 1, 77–82.

Mackay, K.B., Kusumoto, K., Graham, D.I., McCulloch, J., 1992. Effect of CI-977 on ischaemic brain damage and cerebral blood flow after middle cerebral artery occlusion in the rat. Soc. Neurosci. Abstr. 18, 1254.

Mackay, K.B., Browne, S.E., McCulloch, J., 1993. Modulation of glutamate mechanisms with enadoline and NBQX: Local cerebral glucose utilization in the rat. J. Cereb. Blood Flow Met. 13 (Suppl.), S786.

Mansour, A., Khachaturian, H., Lewis, M.E., Akil, H., Watson, J.J., 1988. Anatomy of CNS opioid receptors. Trends Neurosci. 11, 308–314.

Monoghan, D.T., Bridges, R.J., Cotman, C.W., 1989. The excitatory amino acid receptors: their classes, pharmacology and distinct properties in the function of the central nervous system. Annu. Rev. Pharmacol. Toxicol. 29, 365–402.

Morgan, J.I., Curran, T., 1991. Stimulus-transcription coupling in the nervous system: Involvement of the inducible proto-oncogenes *fos* and *jun*. Ann. Rev. Neurosci. 14, 421–451.

Nehis, D.G., Kurumaji, A., Park, C.K., McCulloch, J., 1988. Differential effects of competitive and non-competitive *N*-methyl-D-aspartate antagonists on glucose use in the limbic system. Neurosci. Lett. 91, 204–210.

Olney, J.W., Labruyere, J., Prict, M.T., 1989. Pathological changes induced in cerebrocortical neurons by phencyclidine and related drugs. Science 244, 1360–1362.

Olney, J.W., Labruyere, J., Wang, G., Wozniak, D.F., Price, M.T., Sesman, M.A., 1991. NMDA antagonist neurotoxicity: mechanism and prevention. Science 264, 1515–1518.

Paxinos, G., Watson, C., 1982. The Rat Brain in Sterotaxic Coordinates. Academic Press, Australia.

Pinnock, R.D., 1992a. A highly selective κ-opioid receptor agonist, CI-977, reduces excitatory postsynaptic potentials in the rat locus coeruleus in vitro. Neuroscience 47, 87–94.

Pinnock, R.D., 1992b. Activation of κ-opioid receptors depresses electrically evoked excitatory postsynaptic potentials on 5-HT-sensitive neurones in the rat dorsal raphe nucleus in vitro. Brain Res. 583, 237–246.

Sharp, F.R., Jasper, P., Hall, J., Noble, L., Sagar, S.M., 1991. MK-801 and ketamine induce heat shock protein HSP72 in injured neurones in posterior cingulate and retrosplenial cortex. Ann. Neurol. 30, 801–809.

Sheng, M., Greenberg, M.E., 1990. The regulation and function of c-*fos* and other immediate early genes in the nervous system. Neuron 4, 477–485.

Singh, L., Vass, C.A., Hunter, J.C., Woodruff, G.N., Hughes, J., 1990. The anticonvulsant action of CI-977, a selective kappa-opioid agonist: a possible involvement of the glycine/NMDA receptor complex. Eur. J. Pharmacol. 4, 477–480.

Szekely, A.M., Barbaccia, M.L., Costa, E., 1987. Activation of specific glutamate receptor subtypes increases c-*fos* proto-oncogene expression in primary cultures of neonatal rat cerebellar granule cells. Neuropharmacology 26, 1779–1782.

Szekely, A.M., Barbaccia, M.L., Alho, H., Costa, E., 1989. In primary cultures of cerebellar granule cells the activation of *N*-methyl-D-aspartate-sensitive glutamate receptors induces c-*fos* mRNA expression. Mol. Pharmacol. 35, 401–408.