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# Perinatal exposure to morphine: reactive changes in the brain after 6-hydroxydopamine

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**Abstract**

The effects of neonatal 6-hydroxydopamine treatment on the brain of control rats and of rats perinatally exposed to morphine were examined. Noradrenaline levels were increased in the pons-medulla, mesencephalon and caudate of 8-week-old control rats lesioned with neonatal 6-hydroxydopamine; perinatal morphine treatment prevented such an increase. In the caudate, there was a loss of dopamine and an increase of serotonin following the neurotoxic lesion; exposure to perinatal morphine prevented the serotonin increase. Brain expression of synapsin I mRNA was particularly abundant in cerebral cortex, hippocampus, dentate gyrus and olfactory bulb. In perinatal morphine-treated rats, the expression of synapsin I mRNA was significantly reduced; interestingly, the neonatal treatment with 6-hydroxydopamine normalized its expression. Therefore, brain-reactive neurochemical changes triggered by 6-hydroxydopamine were suppressed by perinatal morphine exposure whereas the association of morphine exposure and 6-hydroxydopamine lesion promoted the normal mRNA expression of the synaptic marker synapsin I.

*Keywords:* Opiate receptor; Neuronal plasticity; Synapsin I; Pruning effect

## 1. Introduction

Several reports have implicated the endogenous opioid system in the modulation of brain development and growth. Opioid receptors and peptides are both present in the brain of mammals before birth and their distribution and level vary according to the particular developmental stage (Kent et al., 1982; Loughlin et al., 1985; Mcdowell and Kitchen, 1986). The activation of fetal or neonatal receptors by morphine or heroin affects body weight and the development of the nervous system, with concomitant reduced body weight at birth, behavioral deficit and increased rate of mortality (Hammer et al., 1989; Vathy et al., 1985; Wilson et al., 1973, 1979). In addition, the formation of cortical neuron dendrites is impaired and cell maturation and proliferation are restricted (Hui et al., 1978; Ricalde and Hammer, 1990; Seatrix and Hammer, 1993). Neuronal and glial cell thymidine incorporation and DNA synthesis are also affected by opioid exposure (Isayama et al., 1991; Kornblum et al., 1987; Stien-Martin and Hauser, 1990). However, prenatal treatment of rats with opioid antagonists promotes larger brains with a higher number of neurons and glial cells (Zagon and McLaughlin, 1983a, b, 1984).

Previous studies from our laboratories have shown that perinatal exposure to morphine affects [Met$^5$]enkephalinergic and substance P-ergic innervation of the striatum (Tenconi et al., 1992; Di Giulio et al., 1995). In addition, the expression of synapsin I mRNA is reduced throughout the brain, suggesting that perinatal morphine may affect synaptic maturation in the brain (Di Giulio et al., 1995). The effects of perinatal administration of morphine were further examined by monitoring the reactive changes of the serotoninergic system after neonatal 5,7-dihydroxytryptamine lesion (Gorio et al., 1993). It was observed that morphine inhibits the typical rapid increase of 5-hydroxytrypatmine level in the pons-medulla ('pruning effect') and markedly impairs the extent of restoration of 5-HT levels and fibers in the spinal cord (Gorio et al., 1993). Perinatal morphine exposure has also been observed to impair reactive cortical plasticity triggered by selective noradrenergic denervation of the frontal cortex caused by

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neonatal 6-hydroxydopamine (Germani et al., 1995). The distal cortical noradrenergic degeneration is accompanied by reactive hypergrowth of the short noradrenaline-containing axons (Aoki and Nakamura, 1991; Sachs and Jonsson, 1975; Oke et al., 1978; Gustafson and Moore, 1987). In the present study, we monitored whether 6-hydroxydopamine-induced noradrenaline depletion in the frontal cortex is correlated with increased noradrenaline levels in areas innervated by shorter noradrenaline-containing axons and whether perinatal morphine affects such a process. In addition, the expression of mRNA levels of synapsin I was monitored.

## 2. Materials and methods

### 2.1. General procedures

Female Sprague-Dawley rats of 250–275 g (Charles River, Como, Italy) were paired with males of 300–350 g. Morphine treatment was initiated after spermatozoa were found in the vaginal fluids. The opioid was added to the drinking water at an initial dose of 0.1 mg/ml and the dose was gradually increased to 0.3 mg/ml. The latter dosage was maintained until the pups were separated from their mothers (21 days), when the dose supplied to pups was reduced to 0.1 mg/ml. The detailed amounts of morphine supplied and taken up by the rats is shown in Table 1. The animals were killed at the appropriate time following anesthesia with i.p. injection of pentobarbital (30 mg/kg). Specific brain areas were dissected out, frozen in liquid nitrogen and kept at −80°C.

Pups were injected i.p. with 6-hydroxydopamine (100 mg/kg) within 6–8 h after birth, i.e. day 0 after delivery.

### 2.2. Monoamine assay

Tissue specimens were processed as described previously (Di Giulio et al., 1989; Gorio et al., 1993). After homogenization in ice-cooled 0.4 N perchloric acid and addition of a known amount of 3,4-dihydroxybenzylamine hydrobromide (Sigma) as an internal standard, part of the sample was processed for protein determination (Lowry et al., 1951) and the remnant was used for assay of monoamines using a high-pressure liquid chromatography (HPLC) system coupled to an electrochemical detector (Spectra Physics, SP8750). The aliquot for cathecolamine detection was initially subjected to cathecol absorption with alumina and was then re-extracted by shaking with 0.4 N perchloric acid for 8 min. After centrifugation, samples were injected into the HPLC system. The method employed is a slight modification of that of De Saint Blanquat et al. (1987). The aliquot for serotonin assay was centrifuged at $40000 \times g$ for 15 min and the resulting supernatant was directly injected into the HPLC apparatus (Di Giulio et al., 1987).

Data are the means ± S.E. from at least six specimens.

Table 1
Daily mean morphine intake/single rat

| Age (days) | Dose (mg/ml) | Morphine intake (mg/day) |
|---|---|---|
| Pregnancy | | |
| 1 | 0.1 | 2.1 ± 0.1 |
| 7 | 0.2 | 4.5 ± 0.31 |
| 14 | 0.3 | 6.4 ± 0.68 |
| 19 | 0.3 | 10.2 ± 0.81 |
| After delivery | | |
| 1 | 0.3 | 8.1 ± 0.86 |
| 10 | 0.3 | 17.3 ± 0.98 |
| 20 | 0.3 | 20.6 ± 1.71 |
| Pups | | |
| 21 | 0.1 | 1.2 ± 0.1 |
| 30 | 0.1 | 2.0 ± 0.09 |
| 40 | 0.1 | 3.4 ± 0.22 |
| 55 | 0.1 | 4.4 ± 0.61 |

### 2.3. In situ hybridization

The oligonucleotide complementary to synapsin I (NEP 555) was labeled at the 3′ end with terminal deoxynucleotidyl transferase in the presence of SdATP (NEN, 1200 Ci/mmol) at a specific activity between $4.4 \times 10^9$ and $8.9 \times 10^9$ cpm/$\mu$g of oligonucleotide. The in situ hybridization was carried out as previously described (Wisden et al., 1991) with ~200000 cpm of labeled probe/section. Sections of 12-$\mu$m thickness were cut on a Zeiss cryostat and the specimens were then exposed to Kodak X-Omat AR filo for up to 2 weeks. For control experiments, the antisense oligonucleotide was hybridized in the

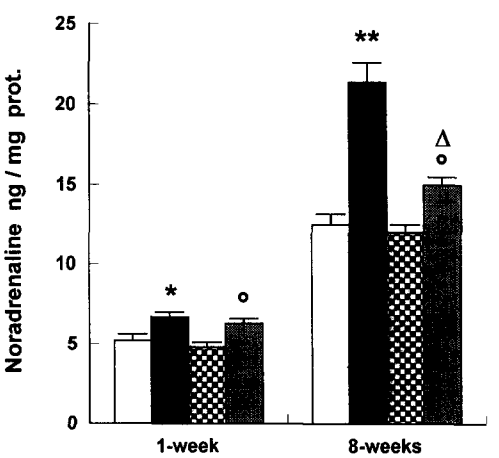


Fig. 1. Noradrenaline (NE) level (ng/mg protein) in mesencephalon of control (open columns), perinatal morphine-exposed (checked columns), contro + neonatal 6-hydroxydopamine (black columns) and morphine + neonatal 6-hydroxydopamine (stippled columns)-treated rats. The neonatal treatment with 6-hydroxydopamine promoted a significant increase of noradrenaline content in both groups; the effect was similar at 1 week after birth, but significantly smaller in perinatal morphine treated at 8 weeks. * $P < 0.05$ vs. control; ** $P < 0.01$ vs. control; $^{\circ}$ $P < 0.05$ vs. morphine; $^{\Delta}$ $P < 0.05$ vs. control + 6-hydroxydopamine.

presence of a large excess of unlabeled oligonucleotide (50-fold); no labeling above background was observed.

The autoradiograms were analyzed by the NIH IMAGE 1.44 program written by Wayne Rasband, performed on a Macintosh model computer (available from Internet by anonymous ftp from zipping, nih, gov). Data were expressed as optical density and are the means ± S.E. from at least six specimens.

### 2.4. Data analysis

The data were evaluated statistically by Student's *t*-test and the differences between the mean values (calculated as *P* values) were considered significant when the *P* values were $< 0.5$.

## 3. Results

### 3.1. Morphine intake

The amount of morphine taken up by dams and its relative concentration in drinking water increased steadily during pregnancy (Table 1). Pups were separated from their mothers 21 days after birth and the dosage of morphine supplied to pups was decreased to 0.1 mg/ml; no signs of withdrawal were associated with the reduction in morphine intake. As previously reported (Tenconi et al., 1992), a reduction of body weight of the order of 20% was observed between 14 and 60 days of life of young rats perinatally exposed to morphine.

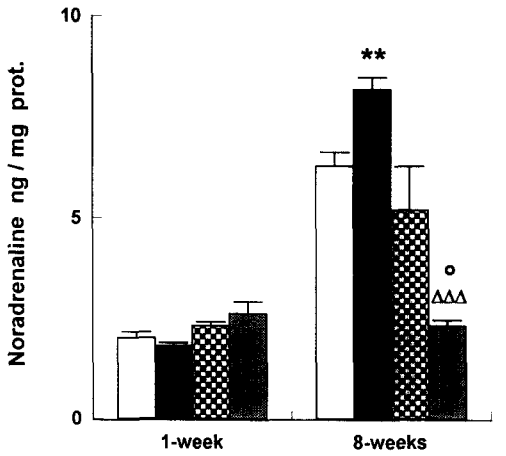


Fig. 3. Noradrenaline (NE) level (ng/mg. protein) in caudate of control (open columns), perinatal morphine-exposed (checked columns), control + 6-hydroxydopamine (black columns)- and morphine + 6-hydroxydopamine (stippled columns)-treated rats. At 8 weeks after birth, noradrenaline content was higher in lesioned control rats while in lesioned morphine-treated rats it was significantly reduced. $^{**}$ $P < 0.01$ vs. control; $^{\circ}$ $P < 0.05$ vs. morphine; $^{\Delta\Delta\Delta}$ $P < 0.001$ vs. control + 6-hydroxydopamine.

### 3.2. Monoaminergic changes

Morphine exposure did not affect baseline noradrenaline levels in the brain areas under study (Figs. 1–3). After neonatal injection of 6-hydroxydopamine, noradrenaline was selectively depleted in the frontal cortex and lumbar spinal cord (Germani et al., 1995). In the mesencephalon of 7-day-old rats neonatally lesioned with 6-hydroxydopamine, however, the level of noradrenaline was signifi-

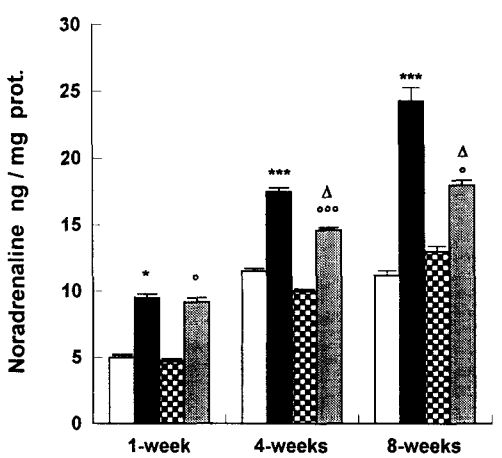


Fig. 2. Noadrenaline (NE) level (ng/mg protein) in pons medulla of control (open columns), perinatal morphine-exposed (checked columns), control + 6-hydroxydopamine (black columns)- and perinatal morphine + 6-hydroxydopamine (stippled columns)-treated rats. The neonatal neurotoxin treatment promoted a gradual increase in noradrenaline content that was markedly higher in lesioned control vs. lesioned morphine-treated rats. $^{*}$ $P < 0.05$ vs. control; $^{**}$ $P < 0.01$ vs. control; $^{***}$ $P < 0.001$ vs. control $^{\circ}$ $P < 0.05$ vs. morphine; $^{\circ\circ\circ}$ $P < 0.001$ vs. morphine; $^{\Delta}$ $P < 0.05$ vs. control + 6-hydroxydopamine.

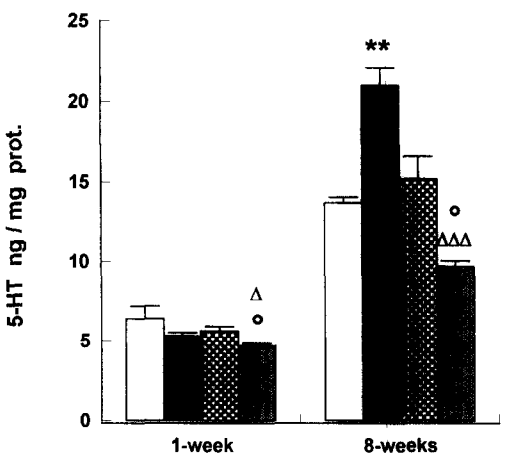


Fig. 4. Serotonin (5-HT) level (ng/mg protein) in caudate of control (open columns), perinatal morphine (checked columns), control + neonatal 6-hydroxydopamine (black columns)- and morphine + neonatal 6-hydroxydopamine (stippled columns)-treated rats. At 8 weeks after birth, there was a marked increase of 5-HT content in lesioned control rats whereas in lesioned morphine-exposed rats there was a significant loss. $^{**}$ $P < 0.01$ vs. control; $^{\circ}$ $P < 0.05$ vs. morphine; $^{\Delta}$ $P < 0.05$ vs. control + 6-hydroxydopamine; $^{\Delta\Delta\Delta}$ $P < 0.001$ vs. control + 6-hydroxydopamine.

cantly increased and this increase was even greater at 8 weeks (Fig. 1). In morphine-exposed rats, the effects of 6-hydroxydopamine were less marked, with the difference between control and lesioned rats being particularly evident at 8 weeks (Fig. 1). In the pons-medulla of 6-hydroxydopamine-treated rats, the noradrenaline content gradually increased over the control value and this was evident even after 7 days. Perinatal morphine exposure partially counteracted this increase (Fig. 2). In the caudate, changes of noradrenaline content after 6-hydroxydopamine were apparent only at 8 weeks (Fig. 3). In lesioned controls, the level was significantly increased whereas in morphine-exposed rats 6-hydroxydopamine treatment caused a marked loss of noradrenaline (Fig. 2).

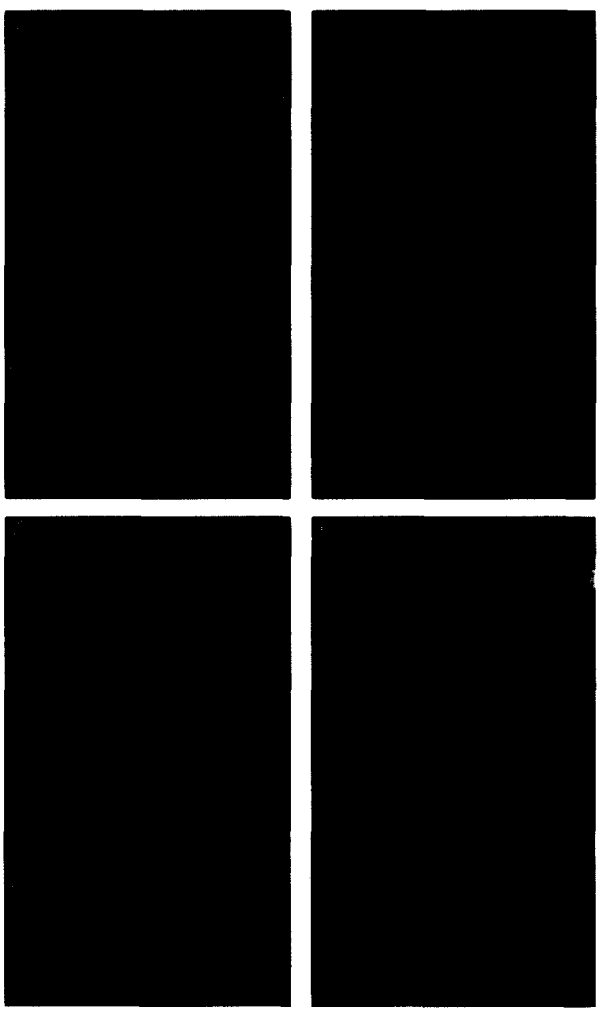

Fig. 5. In situ hybridization of synapsin I mRNA expression in the brain of control (A), perinatal morphine exposure (B), control + neonatal 6-hydroxydopamine (C) and perinatal morphine exposure + neonatal 6-hydroxydopamine (D). Morphine exposure reduced markedly brain mRNA expression (B). This effect was counteracted by neonatal 6-hydroxydopamine treatment. The picture in B has a high contrast to show the light autoradiography. CB, cerebellum; CPu: caudate putamen; Cx, cerebral cortex; DG, dentate girus; Hi, hippocampus; OB, olfactory bulb; Su, subiculum; Th, thalamus.

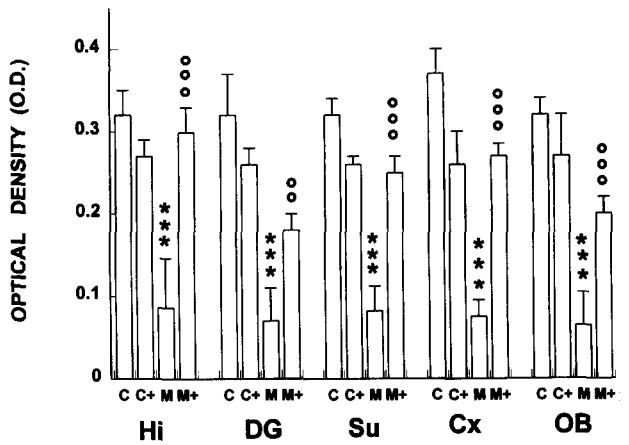


Fig. 6. Quantification of brain synapsin I shown in Fig. 9 from control (C), control + neonatal 6-hydroxydopamine (C+), perinatal morphine exposure (M) and perinatal morphine exposure + neonatal 6-hydroxydopamine (M+) 7-day-old rats. The data show that in every brain area examined the expression of synapsin I mRNA was reduced very markedly by morphine (M) and that 6-hydroxydopamine treatment restored full mRNA expression (M+), counteracting the effects of morphine. Cx, cerebral cortex; DG, dentate girus; Hi, hippocampus; OB, olfactory bulb; Su, subiculum. *** $P < 0.001$ vs. control; ooo $P < 0.001$ vs. morphine.

The 5-HT content was unaffected by morphine and 6-hydroxydopamine in the pons-medulla and mesencephalon (data not shown). In the caudate of controls, the neurotoxic treatment caused a marked increase in 5-HT content; however, in lesioned morphine-exposed rats there was a loss of 5-HT (Fig. 4). In the mesencephalon and pons-medulla, no changes in dopamine content were observed whereas in the caudate 6-hydroxydopamine caused a loss of dopamine in both control and perinatal morphine-exposed rats. In the caudate of 8-week-old rats, the control level was $11.46 \pm 0.81$ (ng/mg tissue), which dropped to $6.89 \pm 0.42$ after neonatal 6-hydroxydopamine. In morphine-exposed rats, the content was $10.01 \pm 1.12$ and $4.85 \pm 0.55$ after 6-hydroxydopamine. The dopamine loss was significant at the 0.01 level.

### 3.3. Quantitative mRNA expression studies

In situ hybridization studies were performed in brains of 7-day-old rats with oligonucleotides complementary to synapsin I (Fig. 5). The expression of brain synapsin I mRNA was higher in hippocampus, dentate gyrus, subiculum, cerebral cortex and olfactory bulb of control rats. Morphine exposure greatly reduced this expression (Figs. 5 and 6). Also, 6-hydroxydopamine treatment lowered synapsin I mRNA expression; however, quantification of six specimens showed that the reduction was seen throughout the brain but was not significant (Figs. 5 and 6). The association of the two negative effects (morphine Fig. 5B and 6-hydroxydopamine Fig. 5C) led to almost complete restoration of brain synapsin I expression (Fig. 5D, Fig. 6).

## 4. Discussion

Using electrophysiological, morphological and biochemical methods, it has been shown previously that neonatal treatment with 6-hydroxydopamine causes the permanent loss of afferent noradrenergic axons and noradrenaline in the cerebral cortex and the hypergrowth of short noradrenaline-containing axons in the brainstem and thalamus (Sachs and Jonsson, 1975; Oke et al., 1978; Gustafson and Moore, 1987; Aoki and Nakamura, 1991). Such an irreversible plastic phenomenon is termed the 'pruning effect' (Schneider, 1973). In the present study, perinatal exposure to morphine impaired the reactive increase in noradrenaline content in the brainstem and caudate following neonatal treatment with 6-hydroxydopamine. Morphine treatment, therefore, prevented the 'pruning effect'. Interestingly, however, 6-hydroxydopamine treatment promoted the restoration of mRNA expression of synapsin I throughout the brain. Thus, in agreement with previous reports (Luthman et al., 1987; Germani et al., 1995), neonatal 6-hydroxydopamine caused a loss of dopamine, and an increase of noradrenaline and serotonin levels, in the caudate; in perinatal morphine-exposed rats, however, the serotonin and noradrenaline content decreased in a similar way to dopamine.

We have previously shown that perinatal morphine treatment prevents the dopaminergic, serotoninergic anf [Met$^5$]enkephalinergic compensatory increase in noradrenaline-depleted cerebral cortex of neonatal 6-hydroxydopamine-lesioned rats (Germani et al., 1995) as well as the restoration of 5-HT content in the spinal cord following selective 5,7-dihydroxydopamine neonatal lesion (Gorio et al., 1993). The inhibitory effects of perinatal morphine on neuronal plasticity, therefore, were observed on lesioned neurons, capable of regrowth, as well as on unlesioned neurons, capable of taking over territories previously denervated. These effects were blocked by the opioid antagonist naltrexone (Gorio et al., 1993; Germani et al., 1995). It had also been shown that perinatal morphine exposure affects striatal development, increasing [Met$^5$]enkephalin and substance P levels and decreasing dopamine content with a reduction in the amount of tyrosine hydroxylase mRNA in the substantia nigra. These effects are antagonized by naltrexone (Tenconi et al., 1992; Di Giulio et al., 1995; Gorio et al., 1996). It is also well-known that the striatal endogenous opioid system is regulated by the dopaminergic innervation as demonstrated with 6-hydroxydopamine lesions (Sivam et al., 1986; Angulo et al., 1986). Therefore, the activation of opioid receptors during perinatal brain development mainly affects the striatum and inhibits reactive neuronal plasticity triggered by selective lesions in the brain and spinal cord. In this study, morphine exposure was observed to also inhibit the reactive increase in noradrenaline in the caudate, mesencephalon and pons-medulla. Conversely, this study also showed that synapsin I mRNA expression in the brain was reduced by morphine and then restored to normal levels by treatment with 6-hydroxydopamine. It seems, therefore, that some developmental alterations caused by morphine can be effectively reversed by the challenging 6-hydroxydopamine lesion, while other cellular mechanisms, related to neuronal plasticity, are not.

Recent results have shown that the expression of mRNA for neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) and neurotrophin-3, is unaffected by perinatal morphine exposure (Gorio et al., 1996). Conversely, G protein may be involved as it is known that chronic morphine treatment may alter the coupling between G proteins and opioid receptors (Tao et al., 1993). Furthermore, we have reported that inhibition of PC12 cell neuritogenesis by morphine is prevented by GTP-$\gamma$-S, a stable activator of G proteins (Tenconi et al., 1996). Therefore, these data would clearly suggest a role for G proteins in the inhibition of neuronal plasticity caused by morphine. There is evidence that an opiate-binding cell adhesion molecule (OBCAM) may be involved in the coupling of opioid receptors with G proteins. OBCAM may be part of an opioid receptor complex, since antibodies to this protein inhibit opioid binding (Roy et al., 1988a; Roy et al., 1988b) and chronic exposure to $\delta$ opioid agonists downregulates OBCAM (Lane et al., 1992). Antisense OBCAM cDNA transfected NG108-15 cells show altered coupling between opioid receptors and G proteins (Govritapong et al., 1993). Chronic morphine treatment specifically downregulates OBCAM in specific areas (Kalyuzhny et al., 1995), thus, it is possible that perinatal morphine exposure might alter neuronal plasticity by affecting both G protein function and OBCAM via a $\delta$-opioid receptor as shown by the in vitro inhibition of PC12 neurite formation by morphine (Tenconi et al., 1996). In addition to binding opioids, OBCAM has a potential role in promoting cell contact (Schofield et al., 1989).

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