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MORPHINE ANALGESIA AND CEREBRAL OPIATE RECEPTORS: A DEVELOPMENTAL STUDY

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1 Development of the analgesic response to morphine and ontogenesis of central opiate receptors were analyzed in rats 5 to 120 days old.

2 The analgesic effect of morphine increased until day 15, after which it decreased to reach a plateau at about day 30. With phenoperidine, on the other hand, the analgesic effect increased until day 15, remained constant between day 15 and day 30 after which it decreased slowly.

3 The ratio of the amounts of morphine in blood over those in brain increased about 3 fold between day 15 and day 30.

4 Opiate receptors were detected in the brain of newborn rats: stereospecific binding of [^{3}H]-naloxone at 10 and 50 nM indicated the presence of low and high affinity binding sites.

5 The number of [^{3}H]-naloxone binding sites increased rapidly during the second and third week after birth. Their affinity for several opiates remained constant throughout development.

6 These results indicate that the analgesic activity of opiates varies with age: until day 15, the analgesic effect of opiates increases in parallel with the number of opiate brain receptors. Then, the formation of the blood brain barrier introduces an additional step in the regulation of opiate activity.

Introduction

The increased sensitivity of the newborn to morphine is well established. Toxicity of morphine decreases with age (Kupferberg & Way, 1963). Nicàk & Masnyk (1966) and Johannesson & Becker (1973) have shown that the analgesic activity of morphine decreased after day 14 in rats. These changes have been attributed to the development of the blood-brain barrier (Kupferberg & Way, 1963; Way, Costley & Way, 1965).

On the other hand, Pert, Pasternak & Snyder (1973), Simon, Hiller & Edelman (1973) and Terenius (1973) have demonstrated the existence of stereospecific opiate binding sites in rat brain. Recently, Clendeninn, Petraitis & Simon (1976) and Coyle & Pert (1976) have studied the ontogenesis of these opiate receptors in the rat and guinea-pig.

In the present study, we have analyzed in the rat the development with age of morphine analgesia, of permeation through the blood-brain barrier and of central opiate receptor sites. Our purpose was to determine the developmental stage at which the opiate receptors become functional.

Methods

Animals

Wistar male and female rats weighing 250 to 300 g were used. They were maintained under constant environmental conditions. Males were mixed with females in a ratio of 1/5. Gravid females were kept from the last few days of gestation in individual cages where they remained with their litters.

Analgesia testing

The method used was derived from that of Janssen, Niemegeers & Dony (1963) adapted for young rats. It consists of immersing the terminal half of the tail in water at 57°C which induces a tail-withdrawal response after a measurable latency time.

Analgesia manifested itself by an increase in reaction time. Drugs were injected in a final volume of:

$$V = \frac{wt}{100} \times n$$

where wt = animal weight (g); n = 1 (for rats 5, 10, 15, 20, 25, 30 days old), n = 2 for 45 days old rats and n = 4 for 60 and 120 days old). The response times were measured once before administration of the drug, and 5, 10 and 15 min after phenoperidine or 15 min after morphine, then every 15 min until the disappearance of analgesia. A series of control rats, treated with 0.9% w/v NaCl solution (saline) was investigated under the same conditions. The quantitative analysis of activity was established from the percentage analgesia obtained for each animal at the maximum effect of the drug; 100% analgesia corresponds to a cut-off time selected as ten times the control response latency. T_0 is the reaction time before treatment:

$$T = 10\,T_0 = 100\% \text{ analgesia}$$

$$\text{i.e. } 1 \leqslant \frac{T}{T_0} \leqslant 10$$

and

$$T = T_0 = 0\% \text{ analgesia}$$

Since the variation of the ratio T/T_0 is linear, the percentage of analgesia is:

$$p = \left(\frac{T}{T_0} - 1\right)\left(\frac{100}{9}\right).$$

Transfer of morphine from blood to brain

In order to study the distribution of morphine in brain and blood, a number of animals in each age group were injected subcutaneously with morphine 1 mg/kg (containing 1.5 μCi of [^{3}H]-morphine). Thirty minutes later the rats were killed by decapitation, an aliquot of blood was collected under heparin and the brain was quickly removed. The collected samples were placed on a combusto-cone, weighed and combusted in an oxidizer (Model 306 Packard); 10 ml of liquid scintillator was added and radioactivity measured in a Beckman 3150 P liquid scintillation counter.

Opiate binding assay

Male and female Wistar rats of various ages were used. After decapitation the brain minus the cerebellum was homogenized in a Potter Elvehjem tissue grinder with a teflon pestle in 10 volumes of 0.05 M Tris HCl buffer (pH 7.4; 4°C). The homogenate was then diluted tenfold with the same cold buffer.

In some experiments binding of [^{3}H]-naloxone was measured on the crude mitochondrial fraction (P_2 fraction) prepared according to Whittaker, Michaelson & Kirkland (1964): the brain was homogenized in 6 volumes of sucrose 0.32 M, Tris 1 mM, pH 7.4 at 4°C. The homogenate was centrifuged (1000 *g* for 5 min), the pellet (P_1) washed once and the combined supernatants centrifuged at 20000 *g* for 15 minutes. The resulting pellet (P_2) was homogenized in Tris buffer 0.05 M pH 7.4 and then diluted with the same buffer to give a final concentration of protein of 1 mg/ml; 0.9 ml of homogenate or P_2 fraction was first incubated for 5 min at 35°C with either levorphanol or dextrorphan (10^{-5} M). Subsequently [^{3}H]-naloxone was added at a final concentration of 10 nM in the reaction mixture (final volume 1 ml) and incubated for an additional 15 min at 35°C. Each experiment was performed in quadruplicate. At the end of the incubation, samples were immediately filtered under reduced pressure through Whatman glass fibre disks (GFB) and washed twice with 10 ml of cold Tris buffer. The filters were dried under an infrared lamp and counted in 10 ml of toluene scintillation cocktail.

Protein concentrations were estimated by the method of Lowry, Rosebrough, Farr & Randall (1951). Specific [^{3}H]-naloxone binding was defined as the difference between binding of [^{3}H]-naloxone that occurs in the presence of dextrorphan and the binding of [^{3}H]-naloxone that occurs in presence of levorphanol.

Analysis of data

The ED_{50} values with 95% fiducial limits were calculated by the method of Litchfield & Wilcoxon (1949). An analysis of variance was used to compare the dose-response curves of analgesia among the different ages. All other comparisons were made using Student's *t* test where the variants are the same or Cochran's test in the other cases (Snedecor, 1956). Mean values are expressed ± s.e. mean.

Although the analgesic data were subjected to a statistical test that applies to a normal distribution, it should be noted that in some instances, due to the imposition of a cut-off time, the data were of a non-Gaussian nature.

Drugs

The following drugs were used in this study: morphine hydrochloride (Francopia, Paris), phenoperidine hydrochloride (R 1406, Lebrun, Paris), levorphanol and dextrorphan tartrate (Hoffman Laroche, Basel), [^{3}H]-morphine (sp. act. 28 Ci/mmol) radiochemical purity 98% (The Radiochemical Centre, Amersham) and [^{3}H]-naloxone (sp. act. 19.985 Ci/mmol) radiochemical purity 98% (New England Nuclear).

Results

Analgesic effects

Morphine induced analgesia at all stages of postnatal development in rats. Figure 1 shows the developmen-

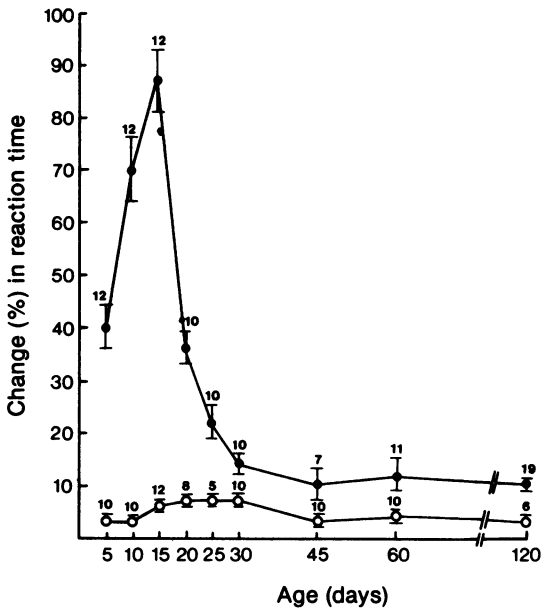

Figure 1 Analgesic effects of morphine in rats treated subcutaneously with 1 mg/kg morphine (●) or saline (○) during ontogenesis. Changes (%) in reaction time were determined 45 min after drug injection (time of peak effect). Data represent mean results from the number of animals indicated above each point, vertical lines show s.e. means. All the morphine results are significantly different from saline controls ($P < 0.05$ or better).

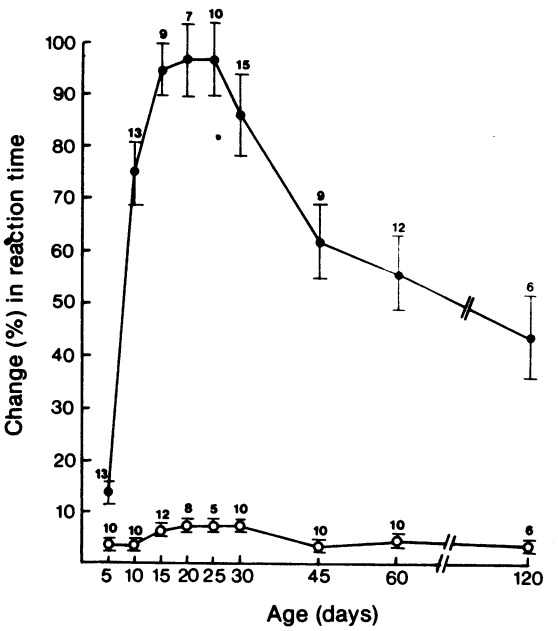

Figure 2 Analgesic effects of phenoperidine in rats treated with 0.25 mg/kg phenoperidine (●) or saline (○) during ontogenesis. Changes (%) in reaction time were determined 15 min after drug injection (time of peak effect). Data represent mean results from the number of animals indicated above each point, vertical lines show s.e. means. All the results are significantly different from saline controls ($P < 0.05$ or better).

tal profile of morphine analgesia 45 min after a subcutaneous injection of the drug (1 mg/kg). The intensity of the analgesic activity increased significantly from 5 to 15 days and decreased rapidly until day 30 and remained constant until day 120. The assays made with several doses gave similar patterns. Dose-effect relationships were established from which ED_{50} values were calculated (Table 1).

Table 1 Variation of the ED_{50} for morphine with age

Age (days)	n	*ED_{50} value (mg/kg) with fiducial limits*
5	34	1.7 (1.2–2.3)
10	31	1.0 (0.7–1.5)
15	32	0.4 (0.2–0.6)
20	18	1.6 (1.0–2.4)
30	36	5.0 (3.3–7.6)
120	38	9.0 (6.5–12.4)

ED_{50} is the dose of morphine that produces 50% analgesia (the quantitative determination of analgesia is described in Methods); *n* = total number of animals for all the tested doses. Each ED_{50} value differs significantly from the previous value except between day 30 and day 120.

Phenoperidine at 0.25 mg/kg also produced significant analgesia at each age. The variations in analgesic activity of this dose of phenoperidine in relation to age are shown in Figure 2 at the peak effect (15 minutes). A significant increase in activity between day 5 and day 15 was also found. This activity was constant until day 30, then it decreased gradually.

Distribution of radioactivity in brain and blood after administration of [^3H]-morphine. Figure 3 shows that the ratio between the concentration of morphine in blood over that in brain was constant during the first two weeks *post partum* and, then increased with age. In 30 day old animals it was approximately a third of the value obtained with 15 day old animals. No attempt was made to identify the chemical nature of the radioactivity but the results have been arbitrarily expressed in morphine concentration, taking into

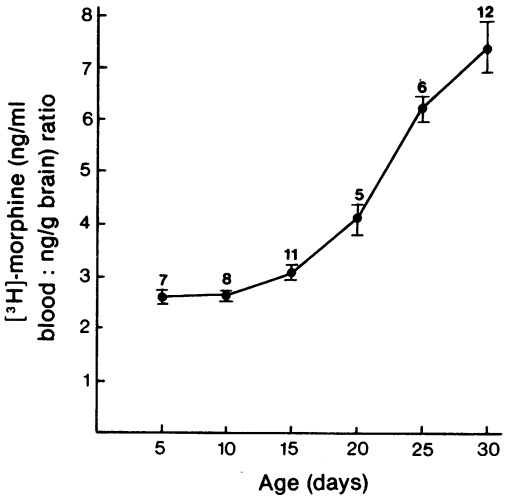

Figure 3 Evolution of the morphine (blood/brain) ratio in relation to age. Amount of morphine in brain (ng/g) and blood (ng/ml) was measured 20 min after the subcutaneous injection of morphine (1 mg/kg containing 1.5 μCi of [3H]-morphine, sp. act. 28 Ci/mmole). Data represent means of the number of experiments indicated above each point, vertical lines show s.e. means.

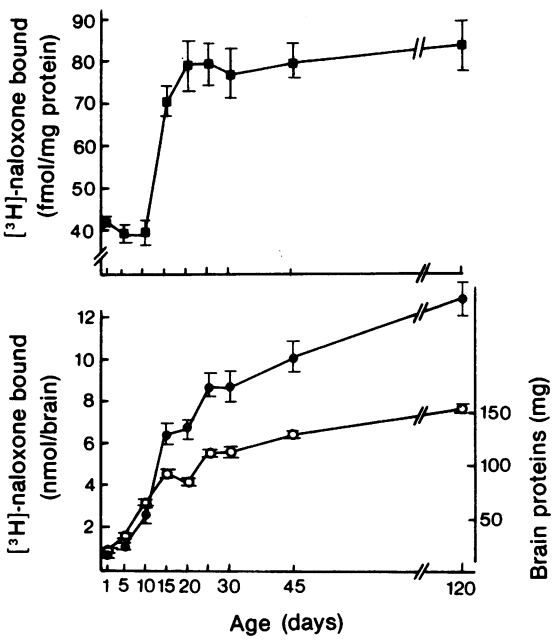

Figure 4 Stereospecific binding of [3H]-naloxone and proteins in brain during development. [3H]-naloxone binding is expressed as femtomol/mg of protein (■) in (a) and nanomol/brain (●) in (b). Brain proteins are expressed as mg/brain (○). Each point represent mean of determinations from 5 to 15 animals; vertical lines show s.e. means.

account the high specific activity of the injected material.

Development of opiate binding sites in rat brain

Figure 4 shows the developmental pattern of [3H]-naloxone stereospecific binding sites to brain homogenate. The level of binding per mg of brain protein was constant during the first ten days *post partum*, then increased rapidly between 10 and 15 days of age and slowly between 2 weeks to adult age. The apparent total number of binding sites in the whole brain increased until the adult age.

A kinetic study of [3H]-naloxone binding on the P_2 fraction prepared from rat brain of various ages (5, 10, 15, 20, 30 and 60 days old) indicated that the increase in binding during development reflects an increase in the maximal number of binding sites (*B max*) whereas the affinity value (K_D) does not change. In Figure 5 the results for rats 10, 30 and 60 days old are shown. The apparent affinity of the receptor site for [3H]-naloxone was 4×10^{-9} M. These data agree well with those of Coyle & Pert (1976).

The competitive inhibition of [3H]-naloxone binding to the P_2 fraction by morphine and phenoperidine was studied at various ages. The ED_{50} values (concentrations that inhibit stereospecific binding by 50%) were 3×10^{-8} M for morphine and 2×10^{-7} M for phenoperidine: these values remained constant during the ontogenic period.

Pasternak & Snyder (1975) have reported the presence of high affinity and low affinity binding sites. Under the experimental conditions described here, we measured the high affinity binding site. Table 2 shows the developmental pattern of the stereospecific [3H]-naloxone binding at 10 nM and 50 nM. The two binding sites are present at all stages of ontogenesis in rat brain.

Discussion

Our results show that in rats there is a biphasic change in the animals' sensitivity to morphine. The analgesic effect increases between day 5 and day 15 after which it decreases to reach a steady value at day 30. The decreased sensitivity to morphine after day 15 was reported by Nicàk & Masnyk (1966), Way (1967) and Johannesson & Becker (1973). It is best explained (Way, 1967) by the formation of the blood-brain barrier which regulates penetration of drugs into brain according to their lipophilicity; our results

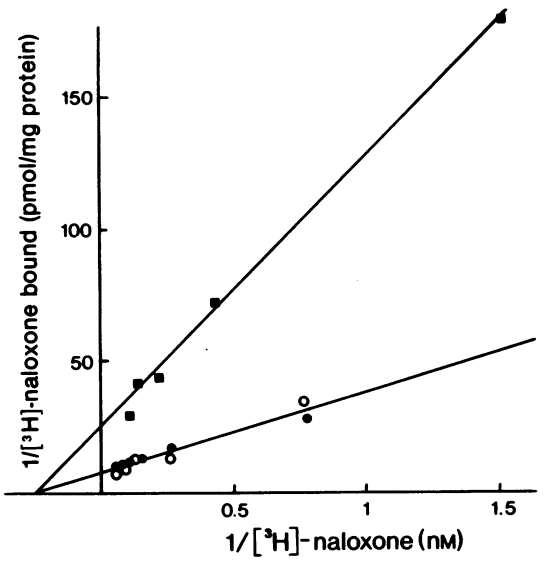

Figure 5 Lineweaver-Burk plot of opiate binding at different ages: (■) 10 days old; (○) 30 days old; (●) 60 days old. Rat brain P_2 fraction (1 mg protein/ml) was incubated with various concentrations of [³H]-naloxone as described in Methods. The reciprocal of [³H]-naloxone concentration (abscissa scale) was plotted against the reciprocal of stereospecific [³H]-naloxone binding (ordinate scale).

agree with this conclusion. The decreased analgesic power of morphine alters in parallel with the increased ratio between morphine concentration in blood and brain. Indeed, the present study demonstrates that phenoperidine which, in contrast to morphine passes freely from blood to brain, has the same analgesic activity between day 15 and day 30. The specific number of binding sites (per mg of brain protein) remains constant during this period. These results suggest that, after day 15, the analgesic activity of opiates is not only regulated by the properties of the receptor site but also by the lipophilic properties of these drugs.

Table 2 [³H]-naloxone stereospecific binding (fmol/mg protein) at 10 and 50 nM of [³H]-naloxone at various ages

Age (days)	n	*Stereospecific binding* 10 nM	50 nM
5–10	7	40 ± 4.6	168 ± 19
15–20	5	70 ± 2.4	188 ± 19
30	8	84 ± 6.1	206 ± 10

Brain homogenate (1 mg protein/ml) was preincubated either with dextrorphan (10^{-5} M) or levorphanol (10^{-5} M), then incubated with [³H]-naloxone 10 or 50 nM. Each value is the mean ± s.e. mean.

The morphine ED_{50} value for analgesia decreases significantly from day 5 to day 15. This increased analgesic sensitivity was also seen with phenoperidine. There was also a parallel increase in the number of binding sites per brain from day 5 to day 15. Thus the increase of the opiate analgesic activity seems to be correlated principally with the augmentation of the opiate binding capacity. The properties of the opiate receptor appear to remain constant during the development; thus there is a high and a low affinity binding site and the high affinity site for [³H]-naloxone retains the same value of 4×10^{-9} M during development.

This study suggests that narcotic activity during the development of newborn rats is dependent on two factors: before day 15, analgesia increases in parallel with the opiate brain receptor after which penetration across the blood-brain barrier becomes a significant factor.

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References

CLENDENINN, N.J., PETRAITIS, M. & SIMON, E.J. (1976). Ontological development of opiate receptor in rodent brain. *Brain Research*, **118**, 157–160.

COYLE, J.T. & PERT, C.B. (1976). Ontogenetic development of [³H]-naloxone binding in rat brain. *Neuropharmacology*, **15**, 555–560.

JANSSEN, P.A., NIEMEGEERS, C.J. & DONY, J.G. (1963). The inhibitory effect of fentanyl and other morphine-like analgesics on the warm-water induced tail withdrawal reflex in rats. *Arzneim. Forsch.*, **13**, 502–507.

JOHANNESSON, T. & BECKER, B.A. (1973). Morphine analgesia in rats at various ages. *Acta pharmac. tox.*, **33**, 429–441.

KUPFERBERG, H.J. & WAY, E.L. (1963). Pharmacologic basis for the increased sensitivity of the newborn rat to morphine. *J. Pharmac. exp. Ther.*, **141**, 105–112.

LITCHFIELD, J.T. & WILCOXON, F. (1949). A simplified method for evaluating dose effect experiments. *J. Pharmac. exp. Ther.* **96**, 99–113.

LOWRY, O.M., ROSEBROUGH, N.J., FARR, A.L. & RAN-

DALL, R.J. (1951). Protein measurement with the film phenol reagent. *J. biol. Chem.*, **193**, 265–275.

NICÀK, A. & MASNYK, S. (1966). Modification of the analgetic effect in relation to the development stage in rats. *Med. Pharmac. exp.*, **14**, 273–275.

PASTERNAK, G.K. & SNYDER, S.H. (1975). Identification of novel high affinity opiate receptor binding in rat brain. *Nature*, **253**, 563–565.

PERT, C.B., PASTERNAK, G.W. & SNYDER, S.H. (1973). Opiate receptor: demonstration in nervous tissue. *Science, N.Y.*, **179**, 1011–1014.

SIMON, E.J., HILLER, J.M. & EDELMAN, I. (1973). Stereospecific binding of a potent narcotic analgesic [^{3}H] etorphine to rat brain homogenate. *Proc. natn. Acad. Sci. U.S.A.* **70**, 1947–1949.

SNEDECOR, G.W. (1956). *Statistical Methods.* Ames. 5th ed., 534 p. The Iowa State University Press.

TERENIUS, L. (1973). Stereospecific interaction between narcotic analgesics and a synaptic membrane fraction of rat cerebral cortex. *Acta Pharmac. tox.*, **32**, 311–320.

WAY, E.L. (1967). Brain uptake of morphine pharmacologic implications. *Fedn Proc.*, **26**, 4, 1115–1119.

WAY, W.L., COSTLEY, E.C. & WAY, E.L. (1965). Respiratory sensitivity of the newborn infant to meperidine and morphine. *Clin. Pharmac. Ther.*, **6**, 454–461.

WHITTAKER, V.P., MICHAELSON, I.A. & KIRKLAND, R.J.A. (1964). The separation of synaptic vesicles from nerve-ending particles (synaptosomes). *Biochem. J.*, **90**, 293–303.

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