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Supraspinal administration of opioids with selectivity for μ-, δ- and κ-opioid receptors produces analgesia in amphibians

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Abstract

Previous results using an amphibian model showed that systemic and spinal administration of opioids selective for μ, δ and κ-opioid receptors produce analgesia. It is not known whether non-mammalian vertebrates also contain supraspinal sites mediating opioid analgesia. Thus, opioid agonists selective for μ (morphine; fentanyl), δ (DADLE, [D-Ala^2, D-Leu^5]-enkephalin; DPDPE, [D-Pen^2, D-Pen^5]-enkephalin) and κ (U50488, trans-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl] benzeneacetamide methanesulfonate; CI977, (5R)-(544α,744α,845β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4,5]dec-8yl]-4-benzofuranacetamide monohydrochloride) opioid receptors were tested for analgesia following i.c.v. administration in the Northern grass frog, *Rana pipiens*. Morphine, administered at 0.3, 1, 3 and 10 nmol/frog, produced a dose-dependent and long-lasting analgesic effect. Concurrent naltrexone (10 nmol) significantly blocked analgesia produced by i.c.v. morphine (10 nmol). ED_{50} values for the six opioids ranged from 2.0 for morphine to 63.9 nmol for U50488. The rank order of analgesic potency was morphine > DADLE > DPDPE > CI977 > fentanyl > U50488. These results show that supraspinal sites mediate opioid analgesia in amphibians and suggest that mechanisms of supraspinal opioid analgesia may be common to all vertebrates. © 1997 Elsevier Science B.V.

Keywords: Opioid analgesia; Amphibian; (*Rana pipiens*); Acetic acid test; Intracerebroventricular (i.c.v.) administration

1. Introduction

There are a number of sites within the central nervous system of vertebrates that produce analgesia after opioid administration. In mammals, direct injections of morphine and other opioids into the lateral cerebral ventricle (i.c.v.) produces a potent, dose-dependent analgesia reversed by opioid antagonists (Vaught et al., 1982; Porreca et al., 1984; Fang et al., 1986; Leander et al., 1986; Porreca et al., 1987a; Leighton et al., 1988). Studies to determine the type of supraspinal opioid receptor using selective antagonists for μ (Ward and Takemori, 1982), δ (Porreca et al., 1987b; Calcagnetti and Holtzman, 1991; Sofuoglu et al., 1991) or κ (Horan et al., 1992; Jones and Holtzman, 1992) opioid receptors have shown that each type of opioid receptor can mediate supraspinal analgesia. Further research using subtype-selective opioid antagonists and agonists in rodents demonstrated that sites defined as μ_1 and μ_2 (Paul et al., 1989), δ_1 or δ_2 (Jiang et al., 1991; Mattia et al., 1991; Haaseth et al., 1994) and κ_1, κ_2, or κ_3 opioid receptors (Horan et al., 1991; Paul et al., 1991; Horan et al., 1993) each contribute to the observed analgesia after i.c.v. administration.

Opioid analgesia after i.c.v. administration is thought to occur indirectly, by opioid disinhibition of brainstem aminergic nuclei which release endogenous analgesic substances (serotonin or catecholamines) at descending terminations in the spinal cord. Thus i.c.v. morphine in rats produces a behavioral analgesia blocked by intrathecal (i.t.) administration of 5-HT receptor antagonists and α-adrenoceptor antagonists (Wigdor and Wilcox, 1987). Besides modulation of descending influences, direct analgesic effects of i.c.v. opioid receptor agonists occur if these agents inhibit the flow of noxious information through the midbrain. For example, opioid receptor agonists may act directly on thalamic neurons to decrease the ascending pain signal which ultimately reaches the limbic and cerebral cortex (Yaksh and Rudy, 1978). Finally, opioid receptor agonists may be producing analgesia in part by a direct action on neurons of the limbic cortex or cerebral neocortex which contain high densities of opioid receptors

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(Mansour et al., 1988). While it is known that opioid receptor agonists given i.c.v. act in a synergistic fashion with opioid receptor agonists given i.t. (Yeung and Rudy, 1980; Roerig and Fujimoto, 1989), the role of a direct action of opioid agonists in specific brain regions to contribute to the overall analgesic effect is not known.

There have been no studies of opioid analgesia at supraspinal sites in amphibians or any other non-mammalian species. We used an amphibian model in this study for scientific, economic, and ethical reasons (Stevens, 1988; Stevens, 1992; Stevens, 1997). As the amphibian brain does not contain telencephalic specialization beyond rudimentary olfactory nuclei, there is no limbic nor cerebral cortex (Stevens, 1995). Previous work using an amphibian model demonstrated that systemic and spinal administration of opioids produced a potent and dose-dependent analgesia as measured on the acetic acid test (Stevens and Pezalla, 1983; Stevens and Pezalla, 1984; Pezalla and Stevens, 1984; Stevens et al., 1987; Stevens and Kirkendall, 1993). The rank order of analgesic potency of opioids selective for μ, δ and κ opioid receptors in amphibians was highly-correlated with that seen in mammals after systemic and spinal administration (Stevens et al., 1994; Stevens, 1996). After spinal administration in amphibians, doses of opioids selective for μ and δ opioid receptors were directly comparable with those obtained in rodents, as expressed in terms of nmol/animal (Stevens, 1996). The present studies were designed to provide the first data on opioid analgesia after i.c.v. administration of opioids selective for μ, δ and κ opioid receptors in amphibians.

2. Methods

2.1. Animals

Male or female common grass frogs, *Rana pipiens*, were obtained from commercial suppliers (Sullivan, Nashville, TN, USA). Animals with a snout-vent length of 5–7 cm (mean weight 35 g) were used, as this size is generally available from commercial distributors on a year-round basis. Upon arrival, frogs were kept in stainless-steel group holding aquaria, provided with flowing water and fed crickets three to four times weekly. Two days before the start of an experiment, frogs were randomly assigned to individual plastic cages with soft mesh lids, for acclimatization to laboratory conditions.

2.2. Drugs and administration

The drugs used were fentanyl hydrochloride, morphine sulfate, DADLE ([D-Ala^2, D-Leu^5]-enkephalin), DPDPE ([D-Pen^2, D-Pen^5]-enkephalin) and naltrexone hydrochloride from the NIDA Drug Supply Program (obtained through the kind assistance of Mr. Robert Walsh of the Research Technology Branch). CI-977 (a.k.a. enadoline, (5R)-(5$4\alpha$,7$4\alpha$,8$4\beta$)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4,5]dec-8yl]-4-benzofuranacetamide monohydrochloride) was obtained from Ms. Carol Germain of Parke-Davis (Ann Arbor, MI, USA). U50488 (trans-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl] benzeneacetamide methanesulfonate) was a generous gift from Dr. Philip VonVoigtlander of Upjohn (Kalamazoo, MI, USA). Drugs were mixed in sterile saline to yield nmol/μl solutions of the free base. Serial dilutions were made from stock solutions to obtain 3–6 dosages per tested opioid agent. The volume of i.c.v. injection was 3 μl/frog.

Supraspinal administration of drugs was done using a modification of the original paper describing i.c.v administration in rodents (Haley and McCormick, 1957), with targeted delivery into the third ventricle in amphibians. Briefly, animals were administered a local anesthetic (0.5 ml, 2% lidocaine) suffused just under the skin of the skull region. 15 min later, a midline incision was made with a scalpel and the top of the skull exposed by retraction. A hand-held microsyringe, fitted with 8 cm length of PE-10 catheter tubing and a 4 cm, 29 gauge needle was used to deliver the drugs. Placement of the needle was made through the skull at a depth of 2.5 mm (set by an adjustable stopper) midline and just anterior to the optic tectum. This site was chosen by referring to the frog brain atlas (Kemali and Braitenberg, 1969) and shown in pilot studies to fill the brain ventricles after dye injection. Visualization of the brain structures through the thin cartilaginous skull of amphibians aided in the consistency of injection sites. Control animals showed no significant changes in baseline pain thresholds or any untoward effects. For opioid receptor antagonist experiments, naltrexone was injected concurrent with the i.c.v. agonist, or given as a pretreatment by the intraspinal (i.s.) route in amphibians (Stevens and Pezalla, 1983).

2.3. Algesiometric testing

The acetic acid test was used to determine the nociceptive threshold in frogs (Pezalla, 1983; Stevens, 1997). Briefly, the acetic acid test consists of eleven concentrations of acetic acid serially diluted from glacial acetic acid. The concentrations were given a code number from 0 to 10 with the lowest code number equal to the lowest concentration of acetic acid. Nociceptive testing was done by placing, with a Pasteur pipette, a single drop of acid on the dorsal surface of the frog's thigh. Testing began with the lowest concentration and proceeded with increasing concentrations on alternate hindlimbs until the nociceptive threshold was reached. The nociceptive threshold was defined as the code number of the lowest concentration of acid which caused the frog to vigorously wipe the treated leg with either hindlimb. To prevent tissue damage, the acetic acid was immediately wiped off with a gentle stream of distilled water once the animal responded or

after 4 s if the animal fails to respond. If the animal failed to respond, testing continues on the opposite hindlimb with the next higher concentration. An animal which fails to respond to the highest concentration (#10) is assigned the cut-off value of 11.

2.4. Data collection and analysis

For agonist effects, the nociceptive threshold was determined before the administration of the agonist dose (baseline nociceptive threshold) and at 60 and 240 min after administration. Each individual animal's nociceptive threshold (NT) was transformed into maximum percent effect (MPE) at each time point by the formula below:

$$\text{MPE} = \frac{(\text{posttreatment NT} - \text{baseline NT})}{(\text{cutoff NT}\,(11) - \text{baseline NT})} \times 100$$

M.P.E. data was plotted for dose groups as a time-course after administration and the maximum M.P.E. values over that time-course were pooled from individual animals with the same treatment for construction of dose–response curves. Computer software (Pharm/PCS, Microcomputer Specialists, Philadelphia, PA, USA) was used to calculate the median effective dose (ED_{50}) and 95% confidence interval and for statistical testing of the significant differences between treatments. Significant effects were considered at $P < 0.05$.

3. Results

In pilot studies, the i.c.v. administration of saline or distilled water was without significant effect on nociceptive threshold (data not shown). Animals tolerated the i.c.v. administration procedure well with no signs of neurological dysfunction and appeared normal after return to home cages for analgesic testing. However, a small number of animals (< 5%) across treatment groups including controls showed unexplained lethality after the 4 h time-course and data from these animals were not used. All reported agonist doses in this study did not produce lethality or any other untoward effects.

3.1. Morphine time-course curves

Administration of morphine (0.1 to 10 nmol/frog) into the third ventricle of amphibians produced a potent and dose-dependent analgesic effect, which lasted at least 4 h (see Fig. 1). Area under the curve analysis for each dose showed that there was a significant dose-dependent effect of morphine analgesia (one-way ANOVA, $P < 0.05$). Time-course curves for the other opioid selective for μ-opioid receptors, fentanyl; for opioids selective for δ-opioid receptors, DADLE and DPDPE and the for opioids selective for κ-opioid receptors, CI-977 and U50488, also showed dose-dependent analgesia within the doses tested (curves not shown, doses plotted in Fig. 3).

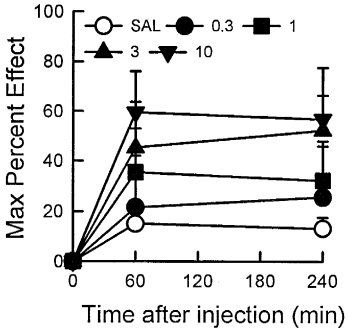

Fig. 1. Time-course of morphine analgesia after i.c.v. administration in amphibians. Data were plotted as mean + SEM for four log-spaced doses of morphine. $n = 6$–8 animals per morphine dose. Treatment groups and doses are given.

3.2. Naltrexone antagonism of i.c.v. morphine analgesia

Concurrent administration of naltrexone at an equimolar dose to morphine (both at 10 nmol) was used to assess the opioid receptor antagonist sensitivity of morphine analgesia. As shown in Fig. 2, saline (3 μl) or naltrexone (10 nmol) did not significantly alter the nociceptive threshold. Morphine at 10 nmol/frog produced an MPE (SEM) of 69.3 (10.3) which was significantly different from saline-injected control (*t*-test, $P < 0.05$). Morphine plus naltrexone in the same injection volume (each at 10 nmol) gave an MPE value of 14.8 (2.8) which was significantly lower than the MPE of morphine alone (*t*-test, $P < 0.05$). Naltrexone (10 nmol) given by the intraspinal route 15 min before the i.c.v. administration of morphine had no effect on morphine analgesia (data not shown).

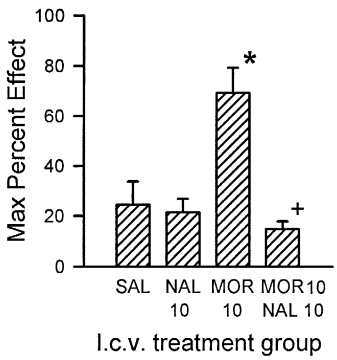

Fig. 2. Naltrexone antagonism of i.c.v. morphine analgesia. Data were plotted as mean + SEM for i.c.v. saline (SAL, 3 μl), naltrexone (NAL, 10 nmol), morphine (MOR 10 nmol) or concurrent i.c.v. morphine and naltrexone (both at 10 nmol). $n = 6$ animals per group. * Indicates significant difference from SAL group. + Indicates significant difference from MOR alone group (Student's *t*-test, $P < 0.05$).

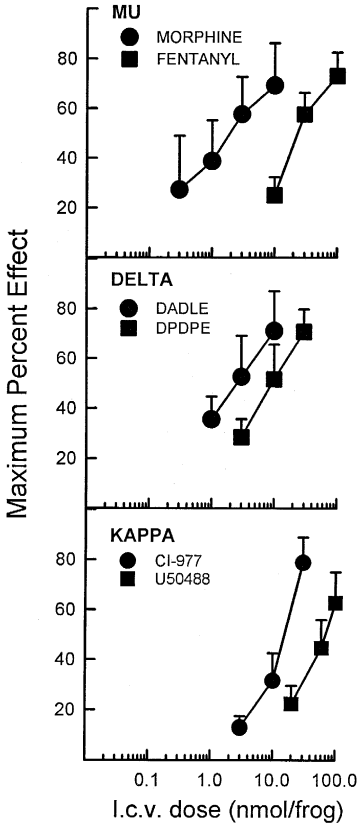

Fig. 3. Dose–response curves of the analgesic effect of opioid receptor agonists selective for μ, δ and κ-opioid receptors after i.c.v. administration in amphibians. Data are plotted as mean + SEM of maximum MPE value for individual animals over the time-course of testing for each drug and dose. $n = 6–10$ animals per dose. Doses can be obtained by referring to the log axis; ED_{50} and slope values are given in Table 1.

3.3. Dose–response curves of opioids selective for μ, δ and κ-opioid receptors

The dose–response curves of the analgesic effects following the i.c.v. administration of opioids selective for μ, δ and κ-opioid receptors are shown in Fig. 3. The dose–response curve for morphine was linear on a semilog plot (see top panel, Fig. 3) with an analgesic ED_{50} value (95% confidence interval) of 2.0 (0.9–4.6) nmol/animal and a slope of 29.0 (9.5–48.4). The slopes of the six opioid agonist dose–response curves did not differ from one another and the ED_{50} values for the six opioids ranged from 2.0 for morphine to 68.9 nmol/animal for U50488 (see Table 1). The rank order of the relative analgesic potency of i.c.v. opioid agonists was morphine > DADLE > DPDPE > CI-977 > fentanyl > U50488.

Table 1
Analgesic ED_{50}, slope and relative potency of opioid agonists selective for μ, δ and κ-opioid receptors after i.c.v. administration in amphibians

Opioid	Selectivity	ED_{50} [a]	95% CI [b]	Slope	95% CI	R.P. [c]
Morphine	μ	2.0	0.9–4.6	29.0	9.5–48.4	1.0
DADLE	δ	2.5	0.8–7.8	35.4	6.0–76.9	0.8
DPDPE	δ	9.5	4.9–18.6	42.2	12.1–72.3	0.21
CI-977	κ	12.8	8.5–19.1	67.3	39.0–95.5	0.16
Fentanyl	μ	23.0	15.1–35.0	50.3	28.6–72.0	0.09
U50488	κ	63.9	36.4–112	57.3	9.8–104	0.03

[a] In nmol/animal, i.c.v.
[b] 95% confidence interval.
[c] Relative potency compared to morphine, R.P. = ED_{50} morphine/ ED_{50} of opioid.

4. Discussion

Although this study was not designed to assess the toxicity of supraspinal opioids, there were no signs of untoward effects following administration of opioids selective for μ, δ and κ-opioid receptors within the dosage range employed. The lethality seen in less than 5% of animals were likely due to misguided injections, as it was observed in both control and treatment groups. Additionally, as this was the first time attempting this route of administration in amphibians, it is not surprising that lethality abated as the studies progressed.

4.1. Relative potency of opioid receptor agonists in amphibians and mammals

These data are the first to report analgesic potency studies of opioid receptor agonists administered by the supraspinal route in amphibians and confirm and extend previous findings after systemic and intraspinal administration of opioid receptor agonists (see Section 1). The rank order of relative analgesic potency was closely correlated between i.c.v. and i.s. opioid receptor agonists in amphibians, with the exception of the opioid selective for μ-opioid receptors, fentanyl. Although the other opioid selective for μ-opioid receptors, morphine, was one of the most potent agents after i.c.v. administration (Table 1), morphine is known to be less-selective for μ-opioid receptors than fentanyl. Thus morphine was shown to produce analgesia at δ-opioid receptors in rodent models (Takemori and Portoghese, 1987; Vanderah et al., 1993) and in the present studies it is possible that morphine produced analgesia at a non-μ-opioid receptor sites. Together with the low potency of i.c.v. fentanyl, this suggest that μ-opioid receptors play less a role mediating supraspinal analgesia compared to spinal analgesia in amphibians. Comparing i.s. (Stevens, 1996) and i.c.v. routes (present study) in amphibians, analgesic ED_{50} values were generally 2 to 10-fold lower for all six opioid receptor agonists after intraspinal administration in frogs. This is in agreement with studies of supraspinal and spinal administration of opioid receptor agonists in rodents where a greater sensitivity of spinal sites to the analgesic effects of opioid receptor agonists has been noted (Porreca et al., 1987b). This finding suggests that there are fewer or less important supraspinal sites mediating opioid analgesia relative to the spinal sites in

amphibians and perhaps in all vertebrates. However, it is also possible that opioids are more potent after spinal administration than by the i.c.v. route due to greater access or higher concentrations of drug at opioid receptors following spinal administration.

With regard to type and subtype of opioid receptor mediating supraspinal analgesia, studies of i.c.v. administration of opioid receptor agonists in rodents demonstrated that agents selective at μ, δ and κ-opioid receptors produce a behaviorally-defined analgesia and that subtypes of these opioid receptors also can be shown to mediate supraspinal analgesia (see Section 1). The pharmacological specificity of opioid analgesia has not been as thoroughly investigated in amphibians. Studies with type- and subtype-selective opioid receptor antagonists and agonists have begun and are needed to further characterize the putative opioid receptor types present in amphibians.

4.2. Nature of opioid receptors in amphibian central nervous system

Early opioid binding studies using amphibian central nervous system (CNS) tissue demonstrated μ, δ and κ-opioid binding sites, using the most selective agents available at that time (for references, see Stevens, 1988). More recent studies have noted a predominance of κ-opioid receptors, with little or no μ or δ-opioid binding sites (Ruegg et al., 1981; Simon et al., 1982; Wollemann, 1987). Further subclassification of μ and δ opioid receptors in frogs has not been made due to their scarcity in amphibian tissue. However, the subtype of κ opioid receptors has been examined in amphibians with controversial results. Some authors report that the predominant amphibian opioid receptor type is like the mammalian κ_2 opioid receptor (Benyhe et al., 1990; Wollemann et al., 1993) whereas others classified it as κ_3 opioid receptor (Brooks et al., 1993; Stevens and Paul, 1996). In contrast, other investigators find sufficient difference in the binding characteristics of the amphibian opioid receptor compared to mammals to declare a new type of opioid binding site in amphibians that is non-μ, non-δ and non-κ opioid receptor in nature (Mollereau et al., 1988). At present, the tentative conclusion may be that the predominant opioid receptor site in amphibians is κ-opioid-like receptor. The main difference is a greater affinity of opioid selective for μ and δ-opioid receptors for the amphibian κ-opioid receptors compared to the mammalian κ-opioid receptors and a lesser affinity for the opioids selective for κ-opioid receptors, U50488 and U69593 (Mollereau et al., 1988; Benyhe et al., 1990; Stevens and Paul, 1996). The binding data suggests the possibility that opioids selective for μ and δ-opioid receptors, as well as opioids selective for κ-opioid receptors, could be mediating at least part of their analgesic effects following i.c.v. administration by binding to a predominant and promiscuous κ-opioid-like receptor in amphibian brain.

There has been only a preliminary comparison of μ, δ and κ opioid receptor clones across different classes of vertebrates, including an unspecified bullfrog species (Li et al., 1995). The full sequence of frog opioid receptor clones has not been determined, but amphibian μ, δ and κ-opioid receptor fragments corresponding to the region from the second to third transmembrane domains, show about 96% homology to rat MOR-1 and DOR-1, but only 90% homology compared to rat KOR-1 (Dr. K. Evans, personal communication). As this sequence region examined is highly conserved across each of the mammalian opioid receptor types, the divergence seen in the cloned frog KOR fragment may indicate even greater divergence in other regions of the frog KOR gene that are known to be less conserved between mammalian opioid receptor types. This divergence may account for the unique κ-opioid-like receptor binding profile seen in amphibian CNS homogenates (see above). Definitive evidence identifying the opioid receptors present in amphibians will only come after the completion of full opioid receptor cloning, which for behavioral correlation should be done using *Rana pipiens* tissue.

4.3. Mechanism of supraspinal analgesia in amphibians

It is unlikely that the analgesic effect of i.c.v. opioid receptor agonists was due to caudal diffusion to sites of action in the frog spinal cord as spinal administration of naltrexone did not block the analgesic effects of i.c.v. morphine. Concurrent i.c.v. administration of equimolar naltrexone and morphine blocked analgesia suggesting that a classical opioid receptor located in supraspinal brain regions was mediating the analgesic effects of i.c.v. opioid receptor agonists. Although there is no opioid receptor localization studies in amphibians to help ascertain the precise supraspinal sites of action, binding studies in amphibian CNS homogenates demonstrate high density of opioid receptor binding sites (see above). The role of descending endogenous analgesic systems, such as serotonin from the dorsal raphe or epinephrine from the locus coeruleus, is unknown in mediating the observed analgesia in amphibians. Descending pathways homologous to those implicated in supraspinal analgesia in mammals have been identified histochemically in amphibians, but functional studies have not been reported (Parent, 1973; Soller and Erulkar, 1979; Tan and Miletic, 1990). The present data suggests, by the process of elimination, that later-evolved neural structures missing in amphibians (such as the limbic cortex or neocortex) are not essential for the expression of supraspinal opioid analgesia in vertebrates.

4.4. Conclusions

These results are the first to show that supraspinal administration of opioid receptor agonists in amphibians produces analgesia. Opioid receptor agonists selective for

μ, δ and κ-opioid receptors were all effective in producing analgesia as measured on the acetic acid test. The analgesic effects of i.c.v. opioid receptor agonists is generally similar in amphibians and mammals, suggesting that higher brain structures missing in earlier-evolved vertebrates are not by themselves necessary for supraspinal analgesia. Studies with type- and subtype-selective opioid receptor antagonists and agonists are needed to further characterize the putative receptor types present in the frog. As it is likely that molecular biologists will clone the amphibian opioid receptors to better understand the evolution of opioid receptor genes, research utilizing an amphibian model for opioid analgesia will provide the only opportunity for assessing the in vivo pharmacology of newly characterized amphibian opioid receptors.

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