A molecular basis for opiate action

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Introduction

Alkaloids, from the Arabian word al-qali meaning saltwort, consist of a large family of compounds synthesized almost exclusively in plants. In spite of their elusive biological role, a lot of the molecules that accumulate in the plant cell vacuoles may exert strong effects on animals and humans. Opiates are alkaloids which have been known and used for some 4000 years, since the Sumerians cultivated poppies (Papaver somniferum) and isolated the liquid appearing on notched unripe seed capsules. The so-called opium (from the Greek word opos, meaning juice) may first have been employed in religious rituals because it evokes euphoria. It was also long used to fight coughing or diarrhoea, or to relieve pain, although variability in both the quality of preparations and the rate of absorbance made its use rather uncertain. Opium seems to have been introduced by the Arabs to India and Asia during the 8th century and to have reached Europe between the 10th and 13th centuries. Addiction resulting from its repeated use was reported as early as the 16th century, but the situation was nowhere as acute as in China, where opium replaced the banned tobacco smoking in the mid-17th century.

In 1806 Sertürner isolated the first active substance from opium, which he named morphine (Figure 1) after the god of dreams, Morpheus. A few years later codeine, an intermediate of the morphine biosynthetic pathway, was isolated, and several other constituents were subsequently identified (e.g. papaverine, noscapine, thebaine). After the invention of the hypodermic syringe and hollow

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needle in the 1850s, morphine was slowly introduced into medicine for the treatment of severe or chronic pain, as well as in surgical practice to reduce postoperative pain or as an adjunct to general anaesthetics. During the next 100 years many attempts were made to find molecules which could mimic morphine analgesia but which were devoid of its secondary effects (nausea, constipation, respiratory depression) and would not induce addiction. In 1898 heroin (Figure 1)
was synthesized with the hope that it would fulfill these requirements. Unfortunately, heroin was later found to be even more addictive than morphine and became a major drug of abuse. Indeed, heroin is more lipid-soluble than morphine and can therefore readily enter the brain, where it is converted into morphine and produces the euphoria or ‘high’ anticipated by drug addicts.

The opioid system: discovery of a complex neurotransmitter system

Several observations suggested that opiates interact with specific binding sites, which were likely to be receptors. This could be shown when radioligands with high specific activities were developed. In 1973, three groups [1–3] showed simultaneously the existence of high-affinity, saturable, stereospecific binding sites for [3H]naloxone or [3H]etorphine on brain membranes. In 1976, Martin et al. [4] reported the first evidence for multiple opioid receptors. Pharmacological studies led to the classification of opioid-binding sites into three receptor types, referred to as µ, δ and κ receptors. Later, the availability of numerous synthetic opiates and their use in biological assays indicated a possible heterogeneity within each receptor class, and the existence of δ₁, δ₂, µ₁, and µ₂, and κ₁, κ₁b, κ₂, and κ₃ opioid receptors was postulated [5].

The demonstration of the existence of opioid receptors suggested that the receptor sites might be the target for endogenous opiate-like (named opioid) molecules. In 1975, Hughes et al. [6] isolated two pentapeptides, Leu-enkephalin (YGGFL) and Met-enkephalin (YGGFM), from pig brain. Shortly afterwards, other endogenous peptides were identified (Table 1). Opioid peptides are derived from proteolysis of larger precursor proteins which are encoded by three distinct genes (Figure 2) [7].

Endogenous opioid peptides exhibit weak selectivity towards the three opioid receptor types. β-Endorphin binds to µ and δ receptors with comparable affinity. Met- and Leu-enkephalins are considered to be endogenous ligands for δ receptors, and similarly dynorphins for κ receptors. No endogenous peptide with high affinity and specificity for the µ receptor type was described until Zadina et al. [8] reported the characterization of two novel tetrapeptides isolated from bovine cortex. This discovery resulted from an unusual interplay between pharmacology and combinatorial chemistry. The isolated peptides, endomorphin 1 (YPWF-NH₂) and endomorphin 2 (YPFF-NH₂), were found to bind to the µ receptor with affinities up to 24 times higher than other known endogenous opioid peptides, and with 4000- and 15000-fold preference over binding to the δ and κ receptors respectively. Definite demonstration of their physiological existence now requires the identification of the encoding genes. Besides the four categories of endogenous opioid peptides described above, a few other natural peptides have been isolated which show opioid activity [9].

The identification of opioid receptors together with their specific endogenous ligands led to the suggestion that they could organize into a complex neurotransmitter system (Figure 3). Indeed, besides a major role in
endogenous pain-controlling pathways (see [10]), the opioid system is involved in a large variety of biological events (reviewed annually [11]). Numerous pharmacological studies have demonstrated that the opioid system plays a role in stress-induced analgesia [7] and contributes to some pathophysiological conditions associated with stress. Regulation of affective behaviour, including motivation and reward, is another crucial function of the opioid system. Indeed the dysregulation of rewarding pathways by exogenous opiates is thought to underlie opiate addiction by neurobiological [12] and molecular [13] mechanisms which are being intensively investigated. Opioids might also play a role in cognitive functions, such as learning and memory, and alterations in opioiergic systems have been hypothesized to be associated with some psychiatric or neurological disorders. The opioid system modulates neuroendocrine physiology and controls autonomic functions, such as respiration, blood pressure, thermoregulation and gastrointestinal motility, and is involved in immune function.

Anatomical studies have shown that the components of the opioid system are widely distributed throughout the central nervous system (CNS) [14], in agreement with the broad diversity of opiate biological actions. Noteworthy is the
observation that μ, δ and κ binding sites display distinct distribution patterns, indicating that each receptor class contributes differently to opioid function. On the peptide side, prepro-opiomelanocortin (POMC) synthesis is highly restricted, while prepro-enkephalin and prepro-dynorphin transcripts both display a similar...
widespread expression pattern in the CNS. Some reports indicate that both receptor sites and peptides are also present in non-neuronal tissues.

In summary, opioids act generally along neural pathways related to behaviour that is essential for self and species survival, and it is widely accepted that the endogenous opioid network is recruited in response to threatening stimuli.

**Opioid receptors: first steps towards molecular mechanisms of opioid action**

In 1992, some 20 years after opioid receptors had been postulated, a cDNA encoding the mouse $\delta$-opioid receptor ($mDOR$) was isolated simultaneously by two independent laboratories using expression cloning in mammalian cells [15,16]. Both groups constructed a random-primed expression library from the rat/mouse glioma × neuroblastoma hybrid cell line (NG108-15) known to express the $\delta$-opioid receptor at high levels. The library was transfected into a mammalian cell line (COS cells) lacking endogenous opioid receptors. Cells expressing the opioid receptor were detected using a pharmacological assay relying on high-affinity binding of a $\delta$-receptor-specific radiolabelled ligand, and both groups isolated the same cDNA using enkephalin-derived peptides as probes. Cloning by homology was then initiated using $mDOR$ as a probe in PCR or low-stringency screening procedures, leading to the identification of cDNAs encoding $\delta$ ($DOR$), $\mu$ ($MOR$) and $\kappa$ ($KOR$) opioid receptors in

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**Figure 3. The complex opioid neurotransmitter system**

The endogenous opioid system (blue oval) consists of three classes of opioid receptors and a family of opioid peptides which interact to modulate a wide variety of physiological functions (blue circle). Opioid receptors are also the target for natural exogenous substances (opiates) and synthetic analogues which induce analgesia or may lead to drug addiction.
rodents and humans [17,18]. Genes encoding opioid receptors have now been identified throughout vertebrate evolution.

Binding studies using recombinant mouse, rat and human receptors expressed in heterologous host cells unambiguously assigned each of the three cloned receptors to the three opioid receptor classes described previously in nervous tissues. Interestingly, only one gene has been isolated for each opioid receptor type. Genes for the multiple \(\mu_1, \mu_2\), \(\delta_1, \delta_2\) and \(\kappa_1, \kappa_2, \kappa_3\) receptor subtypes have not been isolated by molecular approaches, nor have alternative splicing mechanisms been identified that could be responsible for the postulated receptor heterogeneity. Perhaps other opioid receptor subtypes are encoded by genes structurally unrelated to the known MOR, DOR or KOR genes. Alternatively, receptor variants could arise from distinct conformations of the three known receptor proteins depending on the cellular context or intrinsic properties of the interacting ligand.

Analysis of the cloned opioid receptor sequences indicated that they belong to the G-protein-coupled receptor family (Figure 4). The three opioid receptor types show high sequence similarity with somatostatin, angiotensin and chemoattractant receptors. Seven putative transmembrane \(\alpha\)-helical segments were identified, as well as potential glycosylation sites in the N-terminal domain and several phosphorylation sites in the third intracellular loop and the

![Figure 4. Schematic representation of the \(\delta\)-opioid receptor](image)

The \(\mu\), \(\delta\), and \(\kappa\) receptor subtypes are highly similar. Each circle indicates an amino acid residue, and the seven putative \(\alpha\)-helical segments are represented inserted in the membrane. Putative glycosylation (Y), phosphorylation (P) and palmitoylation (blue zig-zag line) sites and a postulated disulphide bridge are represented. The N-terminal part of the protein is located on the extracellular side of the membrane and the C-terminal part on the cytoplasmic face. The deduced protein sequences of all the cloned receptors (\(\mu\), \(\delta\), \(\kappa\) of mouse, rat and human origin) are given in [17].
C-terminus. These consensus sites for post-translational modifications are located at different positions in the MOR, DOR and KOR sequences. By analogy with other receptors, the presence of an important disulphide bridge between the first and second extracellular loops was also postulated.

The cloning of the opioid receptor genes opened up a new era in the molecular understanding of the structure–activity relationships in opioid receptors. It is now possible to express the receptors as recombinant proteins in various cell lines, to modify their sequences by site-directed mutagenesis or to create chimaeras by fusing parts of different receptor types. Ligand recognition, signal transduction and receptor desensitization can now be analysed extensively using unlimited sources of proteins. Furthermore, structure–function studies may now be performed on human recombinant receptors, an important factor in drug screening programmes.

Ligand recognition is presently being explored by many groups, and several three-dimensional models have been proposed (reviewed in [19]). Mutagenesis data show that extracellular loops, which differ greatly between opioid receptors, are critical for μ, δ and κ selectivity. On the other hand, the three opioid receptor types share high sequence homology in the transmembrane regions, and an opioid-binding pocket has been located within the helical bundle. Accordingly, studies of point mutants showed that a number of transmembrane residues found in helices 2–7, and conserved across opioid receptor types, are important to maintain ligand binding. Interestingly, descriptions of ligand-binding mechanisms led to the conclusion that there is no unique opioid-binding pocket, but rather a specific network of multiple interactions within each ligand–receptor complex [20]. More extensive modelling studies and experimental identification of critical structural determinants of the receptors will be available once the three-dimensional structure is resolved.

Biochemical and electrophysiological studies on various primary neuron cultures and neuroblastoma cell lines have shown that the μ, δ and κ receptors inhibit the cAMP pathway, decrease the conductance of various voltage-gated Ca^{2+} channels or activate inwardly rectifying K^+ channels, depending on the cell under study (Figure 5). Pertussis-sensitive G\(_{\alpha_i}\) or G\(_{\alpha_o}\) types seem to be the preferred coupling partners of opioid receptors over other G\(_{\alpha}\) protein types. Functional coupling to cellular effectors has now been demonstrated for the recombinant receptors produced in a number of heterologous expression systems [17]. Such studies have also shown that not only the intrinsic properties of the receptor, but also the availability of G-proteins and downstream effectors in the cell, drive the signalling process. As an example, co-expression of a G\(_{\alpha}\) protein subunit modifies receptor–G-protein interactions and switches the receptor coupling from the endogenous to the heterologous \(\alpha\) subunit. Functional studies have also demonstrated that each receptor type may simultaneously activate multiple G-protein \(\alpha\) subunits, as well as multiple effector pathways in the same cell. Peptide competition studies showed that the second
and third intracellular loops, as well as the membrane-proximal part of the C-terminal tail, interact with G-proteins.

Signal transduction is tightly associated with receptor desensitization, defined as a rapid loss of receptor function upon sustained exposure to an agonist. This process tends to limit the biological action of the stimulating drug or neurotransmitter at the cellular level. Opioid receptor desensitization was first shown in neuroblastoma cell lines expressing endogenous receptors or in isolated organ preparations, and has now been described in several heterologous host cell lines expressing the recombinant receptors. Such model cell lines provide convenient tools with which to dissect the molecular events underlying decreased cellular response under chronic opiate stimulation, including phosphorylation of the receptor by endogenous specific (G-coupled-receptor kinases) or non-specific (protein kinase A or C) kinases, receptor internalization and down-regulation [19]. These studies represent a first step towards the elucidation of opiate tolerance and dependence at the cellular level [21].

The situation in vivo is much more complex, and the chronic activation of opioid receptors triggers long-term adaptative changes at the level of neuronal networks [12,13] that ultimately lead to opiate addiction. Although these phenomena are highly complex and poorly understood, future studies should clarify whether opioid receptor desensitization plays a role in the development of opiate tolerance and dependence in vivo.

**Figure 5. Functional coupling of opioid receptors**

Upon activation by an agonist (dark blue arrow), all three opioid receptor subtypes can inhibit adenylate cyclase activity and voltage-gated Ca\(^{2+}\) channels or activate inwardly rectifying K\(^+\) channels. These effects are mediated by heterotrimeric G-proteins whose G\(\alpha\) subunit is of the G\(_i\) or G\(_o\) type. Coupling to these effector systems has been demonstrated in nervous tissue and using heterologous expression in various host cells.
Recent approaches: from gene to function

Gene targeting techniques allow us to inactivate any gene of interest in mice. This is an extremely powerful approach to investigating the action of a defined gene product in development, as well as in any physiological function or behaviour in the adult. Genes encoding all the components of the opioid system (opioid peptides and receptors) have now been disrupted in mice by homologous recombination [22]. All these mutant mice reached the adult stage without apparent anatomical abnormalities, indicating that the absence of a single component of the opioid system is not detrimental to development. Although only a few behavioural studies have been performed to date, the first results have indicated increased pain thresholds in mice lacking prepro-enkephalin, μ or κ receptors, increased anxiety in the prepro-enkephalin knock-out mice and an altered response to stress in β-endorphin-deficient mutant mice. Comparative analysis of the various mutant mice in identical behavioural paradigms will highlight the specific contribution of each component of the endogenous opioid system in the response to stressful situations. The inactivation of multiple opioid peptide and/or receptor genes in the same animal has not yet been described.

Of great interest is the study of opiate action in opioid receptor-deficient mice. These mutant mouse strains represent exquisite tools with which to define the mode of action in vivo of prototypic, as well as novel, opioid compounds at the molecular level. Morphine was long believed to act at multiple receptors in vivo, because morphine affinity towards μ receptor binding sites exceeds that towards δ and κ receptors by only 100-fold. Although this selectivity factor is reasonably high for in vitro experiments, where the amount of drug available to the receptors is well controlled, it is rather low when considering in vivo protocols using repeated systemic administration of high doses of drug. The pharmacological action of morphine was extensively investigated by Matthes et al. [23] in mice lacking the μ-opioid receptor. These experiments showed clearly that all responses to morphine were abolished in mutant mice, whatever the dose or mode of administration. Indeed, morphine was unable to induce analgesia, reward or physical dependence in mice lacking the μ receptors [23]. Furthermore, both respiratory depression and immunosuppression, well-established morphine side-effects, were absent in these mice. The genetic approach, therefore, nicely demonstrated that the receptor protein encoded by the MOR gene represents the essential molecular target of morphine in vivo. As a consequence, it seems that both the desired effects and the adverse side-effects of morphine, one of the most clinically useful opiates, result from the activation of the same receptor protein. Therefore it appears unlikely that an ideal analgesic could be developed by drug design strategies that adopt the MOR-encoded receptor as their target.
**Perspectives: an arduous march to therapeutics**

Numerous morphine derivatives have been synthesized with the hope that they would have analgesic effects comparable with those of morphine but without its secondary effects of respiratory depression, constipation, abuse liability, euphoria, nausea and vomiting. The hope for new efficient analgesics, free of the usual morphine side-effects, has motivated the synthesis of many new molecules. Numerous prototypic ligands, peptidic analogues and non-peptide opioid alkaloid derivatives have been developed for each of the opioid receptors, both as pharmacological tools and as potential therapeutic agents [24]. These ligands are characterized by high-affinity binding and significant \( \mu \), \( \delta \) and \( \kappa \) selectivity, as well as by greater stability in the case of the peptide derivatives. Most of these compounds contain the classical opioid pharmacophore, i.e. a phenolic ring located at a fixed distance from a positively charged nitrogen atom, a structure which is common to opioid peptides and morphine analogues.

A tremendous diversity of compounds has been studied in receptor binding experiments, isolated organ bioassays and physiological studies in vivo. None of the synthetic molecules has yet proven entirely satisfactory; however, some of these compounds are now used as therapeutic agents. The development of piperidine derivatives, such as Fentanyl (Figure 1), was pioneered by Janssen and led to the synthesis of several potent analgesics with different clinical applications. The thebaine-derived etorphine is about 1000 times more potent than morphine, but its side-effects restrict its veterinary use as a sedative for large animals. Morphinans, e.g. levorphanol (Figure 1), were created by removal of one or more of the morphine rings. Compounds of the methadone type represent the ultimate simplification of the morphine structure. One of these is a widely used analgesic (\( d \)-propoxyphene, or Darvon), while methadone itself (Figure 1), a \( \mu \)-receptor partial agonist, is used to withdraw human addicts because it can be taken orally, has long-lasting action and does not induce detectable psychotropic effects.

All opiate drugs used clinically act mainly at the \( \mu \)-receptor. \( \kappa \)-Agonists have been developed with the hope that their opposing action in mood control compared with that of \( \mu \)- and \( \delta \)-agonists would make them non-addictive. Unfortunately, their strong aversive properties in humans hamper their use for therapeutic purposes. Great efforts are now being made to design highly selective and potent \( \delta \)-agonist compounds, to develop peripherally acting opiates that would be devoid of central effects, and to combine low doses of opiates with drugs acting at different pharmacological targets.

In conclusion, the cloning of opioid receptor genes has recently opened up an entirely new field of investigation in opioid research. The amount of information obtained from these newly developed molecular tools should increase exponentially in the very near future. Understanding the molecular basis of opiate action both at cellular level and in vivo will allow us to establish novel therapeutic strategies for the treatment of pain and addiction.
Summary

- Opioid receptors mediate the strong analgesic and addictive actions of exogenous opiates, the prototype of which is morphine.
- The opioid system consists of a family of endogenous opioid peptides and three receptor types, m, d and k. It is widely distributed throughout the CNS and regulates a large diversity of physiological functions, including pain perception and mood control.
- The recent cloning of opioid receptors has opened up a new era in opioid research. The molecular basis of opioid action may now be addressed by in vitro structure–function studies of recombinant receptors and by in vivo gene targeting.
- Novel drug design strategies based on data obtained from molecular approaches will be developed in order to generate the long-sought non-addictive analgesic.

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References
