ABSTRACT Morphine is the most widely used compound among narcotic analgesics and remains the gold standard when the effects of other analgetic drugs are compared. The most characteristic effect of morphine is the modulation of pain perception resulting in an increase in the threshold of noxious stimuli. Antinociception induced by morphine is mediated via opioid receptors, namely the \( \mu \)-type opioid receptor. Apart from the \( \mu \)-opioid receptor, two other classical opioid receptors, \( \kappa \)- and \( \delta \)- and one non-classical opioid receptor, the nociceptin receptor was discovered and cloned so far. At the same time endogenous opioids were also discovered, such as enkephalins, endorphins, and dynorphins. The opioid receptors together with the endogenous opioids form the so called endogenous opioid system, which is highly distributed throughout the body and apart from analgesia it has several other important physiological functions. In this article we will review the historical milestones of opioid research – in detail with morphine. The review will also cover the upmost knowledge in the molecular structure and physiological effects of opioid receptors and endogenous opioids and we will discuss opioid receptor modelling – a rapidly evolving field in opioid receptor research.

KEY WORDS heterogeneity molecular modelling molecular structure morphine oligomerization opioid receptor

INTRODUCTION

The narcotic analgesics, of which morphine is the prototype, produce a large variety of pharmacological responses by interacting with the opioid receptors in the nervous system. Morphine is one of the agonist ligands for multiple opioid receptors (\( \mu \), \( \delta \) and \( \kappa \)) and therefore the majority of its effects is attributed to specific drug-receptor interactions. This idea is based on morphine actions that can be competitively blocked by opiate antagonists, such as naloxone, naltrexone or diprenorphine. Morphine is a natural compound, the principal alkaloid in opium and becoming the prototype opiate analgesic and narcotic.

Analgesic and other important effects of morphine-type drugs, including natural, semisynthetic and synthetic derivatives have been well established (Martin 1983; Yaksh and Noueihed 1985; Wood and Iyengar 1988; Jaffe and Martin 1990; Pasternak 1993; Spetea 2013). The question of morphine tolerance and dependence is not included in this paper, thus the reader can refer to several reviews (Cox 1991; Trujillo and Akil 1991; Di Chiara and North 1992; Meunier 1992).

Historical background

Many of pharmacological actions of morphine, including analgesia, euphoria and constipation had clearly been known long before morphine itself was isolated. In several early civilizations, such as Sumeria, Egypt, ancient Greece and the Roman empire, extracts of the poppy plant (Papaver somniferum) were thoroughly used as medicine. Theriaca, opium, laudanum pulvis Doveri and paregoric, each contains morphine in substantial amounts, and they have been used for centuries in western medicine. Several similar extracts are still in use, e.g., to treat some kind of diarrhoeas. Morphine, as the major active ingredient of opium, was isolated in 1805 by the German pharmacist Wilhelm Sertürner (1805) and subsequently named after Morpheus the god of dreams in Greek-Roman mythology. Morphine was the first alkaloid to be discovered, and its isolation therefore was a breakthrough in organic chemistry. Soon thereafter morphine became one of the most popular drugs for therapy. Besides their medicinal use, morphine-containing drugs and later pure morphine, served as recreational agents and this contributed to the illicit applications. Thus opium was accepted in classical Islamic countries such as Arabia, Turkey and Iran, where opium eating and smoking replaced the consumption of alcoholic drinks. Opium was also consumed as a favourite substance of pleasure in India and China. Unfortunately, morphine and
heroin are still among the most dangerous drugs all over the world, and the abuse of opiates represents serious problems in societies. A schematic timeline illustrating the milestones in morphine and opioid receptor research is shown in Figure 1.

**Chemistry**

Morphine is a member of the morphinan-framed alkaloids (other name of the ring system is: perhydrophenantrene), including codeine, thebaine, neopinone and morphine itself. They are predominantly found in the plant genus of *Papaver*. The structure of morphine (Gulland and Robinson 1925) consists of five condensed rings: phenolic A, cyclohexane B, cyclohexenol C, N-methyl-piperidine D and a partially saturated furan ring E (Fig. 2).

The stereochemical structure has been confirmed by X-ray diffraction analysis (Mackay and Crowfoot-Hodgkin 1955; Fridrichsons et al. 1968). This frame is rather structurally rigid, but some functional groups of morphine, such as...
as the C3 phenolic and C6 secondary alcoholic, allow the compound to be chemically reactive. Due to the piperidine ring, morphine is a weak base: the collective name ‘alkaloid’, that is plant-derived natural compounds having an N-atom in their structure reflects this property. Natural morphine is optically active being levorotatory molecule, but its synthetic counterpart (+)-morphine has been shown to display entirely different biological effects (Jacquet et al. 1977). The morphine molecule has five chiral centers on the carbon atoms of 5, 6, 9, 13, 14. The absolute configuration of asymmetric carbon atoms in the natural morphine is: 5\(R\), 6\(S\), 9\(R\), 13\(S\), 14\(R\).

Multiple pharmacological effects of morphine are ascribed to this stereochemical structure and only limited action of (+) morphine has been recorded (Adams et al. 1991).

**Receptors for opioids**

**Receptor theory**

Bioactive compounds (alkaloids, drugs, toxins, etc.) produce a wide range of biological responses in the body. At the beginning of the 20. century Paul Ehrlich postulated that „Corpora non agunt nisi fixata” (compounds only act when they are bound). Central nervous system (CNS) mechanisms of various drugs have not been fully understood. It was proposed by Ehrlich, Dale and others that therapeutic and toxic effects of drugs result from their interactions with specific molecules in the body. These molecules are called ‘receptors’: receptors are usually proteinaceous macromolecules consisting of single chain or multiple glycoproteins. Interaction of a small (first messenger) molecule with the receptor is followed by receptor activation that initiates further, mainly intracellular, events in the signal transduction cascade. Receptor activation often modulates synaptic transmission in nervous tissues. In fact, neurotransmitters are typical compounds that interact with pre- or postsynaptic receptor molecules. Response to a messenger molecule depends on the binding capacity of the receptors. Localisation of the receptors may be in the cell surface, in the cytosol or in the nucleus. We can refer to the signaling ‘messenger’ molecule (e.g. hormone or neurotransmitter) as the ligand, which interact with specific recognition sites, named ‘binding pocket’ on the receptor. Ligand binding to the receptors is followed by conformational changes and a sequence of intracellular reactions (transmembrane signaling). They generate, amplify, coordinate, and terminate post receptor signaling by chemical secondary messengers located in the cytoplasm, leading to a change in cellular function. Being aware of these facts, receptors have become the central focus investigating drug effects.

In a pharmacological point of view ligands may be agonists or antagonists. An agonist can bind to the receptor and activates it producing cellular and other responses. Antagonists are capable of blocking the agonist binding, thus the pharmacological effect of the primary drug is inhibited. Pure antagonists alone do not mediate pharmacological effects unless they are able to inhibit the receptor stimulating effects of endogenous agonist ligands. The typical mechanism by which antagonists work is competitive binding and inhibition. Partial agonists can produce less biological responses than full agonists do. Inverse agonists are compounds that interact with the same receptor as an agonist but induce biological responses opposite to that of antagonists.

The interaction between ligand and receptors can be quantified in radioligand binding assays or radioreceptor binding assays. In such studies a radiolabelled chemical named ‘radioligand’ (L\(^{\ast}\)), usually a small molecule will interact with the receptor protein macromolecule:

\[ L^{\ast} + [R] \leftrightarrow [RL^{\ast}] \]

where [R] is the receptor concentration, [RL\(^{\ast}\)] is the concentration of the receptor ligand complex, or the ‘bound ligand’.

In practice, the quantity of the bound ligand is measured by filtration methods. Ideally, candidate ligands for radiolabeling should have the following desirable properties: (i) high affinity to favour specific over non-specific binding, (ii) low non-specific binding, (iii) high specific (molar) radioactivity to detect low receptor densities, (iv) receptor specificity. Further properties of specific binding are reversibility, saturability, stereospecificity and competitiveness. Radioligand binding assays evolved rapidly during the characterization of opioid receptor.
Opioid receptors: discovery and heterogeneity

Among others the opioid system is involved in regulating pain, some vegetative functions, reward mechanisms and drug addiction. Natural and synthetic opioids exert their pharmacological actions through three opioid receptors. As the opioid receptor concentration is very low in different tissues, its discovery had been challenged until highly specific \([3H]\) labelled radioligands with high molar radioactivity became available. By the use of brain membrane preparations and \([3H]\)dihydromorphine (agonist) or \([3H]\)naloxone (antagonist) radioligands with high molar activity led to the discovery of the brain’s opioid receptors (Terenius 1973; Pert and Snyder 1973; Simon et al. 1973). The key step in identifying opioid receptors was to establish an in vitro method to determine non-specific binding. Non-specific binding can be measured in the simultaneous presence of the labelled ligand (in nanomolar concentration) and an excess of unlabelled ligand (in micromolar concentration). Latter may be either chemically identical with the radiolabelled ligand (homologous competition) or represents a chemically different compound that act on the same receptor. Radioligand binding studies are still fundamental methods investigating ligand–receptor interactions. Early studies defined a saturable binding site, which bound opioids with nanomolar affinity. The sites were the first in which the binding of agonists and antagonists could be distinguished by sodium ions (Pert et al. 1973), divalent cations (Pasternak et al. 1975), and protein modifying reagents (Wilson et al. 1975). Evidence for heterogenous opioid receptors or receptor multiplicity came from the demonstration of different pharmacological profiles observed on chronic spinal dogs by the use of three prototype opioid agonists: morphine, ketocyclazocine and SKF-10,047 (Martin et al. 1976). Three types of opioid receptors were proposed named \(\mu\), \(\kappa\), and \(\delta\). The presence of distinct \(\delta\)-opioid receptors was suggested to explain the naloxone-sensitive inhibitions of Met- and Leu-enkephalins on the electrically stimulated mouse vas deferens preparations (Lord et al. 1977). The \(\sigma\)-receptor is targeted by phencyclidine (PCP) and its analogues (Monassier and Bousquet 2002), and it is no longer regarded as an opioid receptor. To date there are three well-defined or ‘classical’ types of opioid receptor, MOP, DOP and KOP, but other designations are also accepted (see Table 1).

Opioid receptors are widely distributed throughout the brain and periphery. In the periphery they are found in certain smooth muscles: ileum, vas deferens; myenteric plexus of the gut, and in adrenal medulla, heart, retina, placenta (Cox 1988). Opioid receptor activation by ligands results in a multitude of effects. Generally, MOP or DOP receptors selective agonists are analgesic and rewarding. KOP receptor selective agonists are dysphoric (Waldhoer et al. 2004). DOP receptors have a role in analgesia (Stewart and Hammon 1993), motor integration, gastrointestinal motility, respiration, olfaction, cognitive function and mood driven behaviour. Medullary DOP receptors are important in cardiovascular regulation (Arndt 1987). KOP receptors have a role in nociception, diuresis, feeding and neuroendocrine secretions (Hansen and Morgan 1984), control of immune function, thermoregulation (Handler et al. 1992) and modulation of cardiorespiratory function (Hassen et al. 1984). KOP receptor agonists can produce dysphoria in humans (Pfeiffer et al. 1986). MOP receptor agonists are potent antinociceptive drugs (Paul et al. 1989). MOP receptors have a role in respiration, cardiovascular functions, intestinal transit (Fox-Threlkeld et al. 1994), feeding, learning and memory, locomotor activity, thermoregulation (Handler et al. 1994), hormone secretion, and immune functions, all of which, except hormone secretion, are depressed by MOP receptor stimulation. The respiratory depressant effects of MOP receptor agonists result from a decrease in sensitivity of respiratory centers to hypercapnia (Butelman et al. 1993).

Opioid receptors: molecular structure

Opioid receptors belong to the superfamily of the G-protein coupled receptors (GPCRs) constituting one of the largest family of the receptors (Fredriksson et al. 2003). The GPCRs are located in the cell membrane and most of them are activated by molecules outside the cell to trigger signal transduction pathways inside the cell (Rosenbaum et al. 2009; Kobilka 2007). However, there are a large number of these receptors lacking known ligands denoted as orphan receptors. Some of them supposed not to need a ligand for activation but they are self-activated by heterodimerization (Levoye et al. 2006). GPCRs are found only in eukaryotes, including animals, choanoflagellates and yeast. The importance of the GPCRs in human may be highlighted by their abundance. More than 800 GPCR coding genes have been identified.

<table>
<thead>
<tr>
<th>Current IUPHAR name proposal</th>
<th>Previous IUPHAR name proposal</th>
<th>Structure</th>
<th>Size (human)</th>
<th>Endogenous agonist</th>
<th>Typical agonist</th>
<th>Selective agonist</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOP, (\mu) or mu (MOR)</td>
<td>OP(_1)</td>
<td>heptahelical</td>
<td>400 aa</td>
<td>endomorphins</td>
<td>morphine</td>
<td>cyprodine</td>
</tr>
<tr>
<td>DOP, (\delta) or delta (DOR)</td>
<td>OP(_2)</td>
<td>heptahelical</td>
<td>372 aa</td>
<td>enkephalins</td>
<td>Leu-enkephalin</td>
<td>naltrindole</td>
</tr>
<tr>
<td>KOP, (\kappa) or kappa (KOR)</td>
<td>OP(_3)</td>
<td>heptahelical</td>
<td>380 aa</td>
<td>dynorphin</td>
<td>U-69,593</td>
<td>norbinaltorphimine</td>
</tr>
</tbody>
</table>

Table 1. Heterogeneity of the opioid receptors
so far in the human genome which is ca. 4% of the entire protein-coding genome. From the medicinal point of view, 40-50% of the existing drugs target some of the GPCRs. Due to their involvement in the pain pathways and the drug abuse, opioid receptors have a long history of medical treatments. The GPCR family can be divided to five main classes with no noticeable sequence homology between the classes. Opioid receptors belong to the class A GPCRs (the rhodopsin sub-family), which comprise ca. 85% of the whole family. Despite the high sequence diversity within the GPCRs, their modular structure is highly conserved (Fig. 3).

The N- and C-terminal chains show the highest diversity within GPCRs. Also, the intra- and extracellular loops, ILs and ELs, respectively, are highly diverse. In contrast, the seven transmembrane helix region and the eighth shorter helix at the intracellular side of the membrane contain numerous conserved structural elements maintaining uniform signal transduction machinery. There are conserved single residues in the helices, among them the most conserved ones serve the starting points in the Ballesteros-Weinstein nomenclature for GPCR residue numbering being given a serial number 50 in each helix (Ballesteros and Weinstein 1995). The three conserved sequence motifs, (D/E)RY, NPxxY and CWxP, serve as molecular switches during the receptor activation, namely the ionic lock switch (together with E6.30), Tyr toggle switch and transmission switch (or rotamer toggle switch) switch, respectively (Trzaskowski et al. 2012). Also, there is a conserved hydrogen bonding network connecting the helices which is supposed to change by protonation of D2.50 and (D/E)3.49 leading to activation (Ratnala et al. 2007; Hofmann et al. 2009; Dror et al. 2011a). The protonation of (D/E) 3.49 both triggers the ionic lock and affects the interaction of the receptor with the membrane potential (Zhang et al. 2013). The protonation state of D2.50 influences the helix-helix interactions and has interplay with the allosteric sodium effect on agonist binding as shown both experimentally and theoretically (Selent et al. 2010; Fenalti et al. 2014; Shang et al. 2014). The glycosylation in the N-terminal tail and the palmitoylation at helix 8 as well as the existence of the cistein bridge between C3.25 and EL2 are mostly conserved structural elements, however with greater variation and in some GPCRs they are missing. Cholesterol and palmitoylation may play a role in the interactions with lipid rafts and/or in receptor oligomerization (Chini and Parenti 2009). The same features can be seen in Figure 4 on a 3D model of human MOP receptor by the same color coding except that only protonable carboxyl oxygens are shown in red.

Two independent groups, Kieffer and co-workers (1992)
and Evans (1992), cloned the first (mouse DOP) opioid receptor using similar methods. The cloned receptor consists of 372 amino acids. Hydropathy analysis suggests the presence of seven transmembrane (TM) domains within the amino acid sequence. Opioid receptors consist of a single polypeptide chain. Sequential organisation of the domains in the receptor protein is as follows: extracellular N-terminus, seven hydrophobic TM segments interconnected with 3-3 intracellular and extracellular loops ended with a cytoplasmic C-terminal tail (serpentine model). The N-terminus contains N-linked glycosylation sites, while multiple phosphorylation sites may exist in the cytoplasmic loops and C-terminal.

The rat (Fukuda et al. 1993), human (Knapp et al. 1994) and amphibian (Rana pipiens) (Stevens 2007) δ-receptor has also been cloned. Although only one DOP receptor gene has been cloned, on the basis of in vitro and in vivo pharmacological characteristics a variety of DOP receptor subtypes have been proposed as δ₁-, δ₂-, δ₃-, and δ₆x-receptor (Jiang et al. 1991; Portoghese et al. 1922; Vanderah 1994).

Chen isolated the first MOP receptor cDNA clone (Chen et al. 1993). The rat MOP receptor consists of 398 amino acids, while the human one contains 400 amino acids (Raynor et al. 1995). Five potential N-linked glycosylation sites are present in the N-terminal. To date, MOP receptor genes have also been cloned from mouse (Min et al. 1994) human (Wang et al. 1994; Raynor et al. 1995), rhesus (Macaca mulatta) and crab-eating monkey (Macaca fascicularis) (Miller GM and Madras: direct GenBank deposit), pig (Sus scrofa) (Pampusch et al. 1998), cow (Bos taurus) (Onoprishvili et al. 1999) guinea pig (Cavia porcellus) (Smith et al.: direct GenBank deposit), white suckerfish (Catostomus commersoni) (Darlington et al. 1997), zebrafish (Danio rerio) (Barrallo 2000), and frog (Rana pipiens) (Stevens 2007). Weakly homologous sequences are also present in the genomes of Caenorhabditis elegans and Drosophila melanogaster. A variety of alternative mRNA splice variants have also been cloned and/or suggested for the MOP receptor gene (Pasternak 2001a, 2001b) and some of the resulting proteins differ in their trafficking properties, although their pharmacology does not appear to differ in ligand binding assays (Koch et al. 2001). Thus, it is unlikely that these splice variants explain the observation of pharmacologically distinct MOP receptor subtypes. The μ₁ / μ₂ subdivision was proposed based on that [³H]-labelled, MOP, DOP and KOP selective ligands displayed biphasic binding characteristics (Wolozin and Pasternak 1981; Krizsán et al. 1991). Each radioligand appeared to bind to the same very high affinity site (μ₁) as well as to the appropriate high affinity site (μ₂, δ, κ) depending on the radioligand used. Naloxone and naloxonazine were reported to abolish the binding of each radioligand to the μ₁ site. While both μ₁ and μ₂ receptors are involved in producing analgesia, euphoria is produced mainly through μ₁, and respiratory depression through μ₂ receptors.

The cloning of the mouse KOP receptor occurred during efforts to obtain mouse somatostatin receptor subtypes (Yasuda et al. 1993). The mouse KOP receptor consists of 380 amino acids. The KOP receptor has two N-linked glycosylation sites in the N-terminal. A number of KOP receptor subtypes have been described (for review see: Wollmann et al. 1993); κ₁ sites bind to dynorphin 1-17 but not to [d-Ala²,d-Leu⁵]-enkephalin (DADLE; representing the cloned KOP receptors profile), κ₂ sites bind the heptapeptide Met-enkephalin-Arg-Phe (MERF) and DADLE (Benyhe et al. 1997), and the so-called κ₃ site is sensitive to naloxone benzoylhydrazone (Akil and Watson 1994). MOP, DOP and KOP receptor subtypes may result from different posttranslational modifications of the gene product (glycosylation, palmytoylation, phosphorylation, etc), or from receptor oligomerization (Cvejic and Devi 1997).

The cloning of opioid receptors in the beginnings of the 90s was soon followed by the cloning of a novel G-protein coupled receptor that displayed very high homology with
opioid receptors but did not bind opioid ligands (Mollereau et al. 1994; Meunier et al. 2000). This receptor was then deorphanized by discovering its endogenous ligand the heptadecapeptide nociceptin/orphanin FQ (N/OFQ) (Meunier et al. 1995; Reinscheid et al. 1995). This was the first successful example of the reverse pharmacology approach (Civelli et al. 1998). The N/OFQ and its N/OFQ peptide receptor (NOP receptor), (Cox et al. 2000) are widely distributed in the central nervous system, but also in the periphery and in the immune system (Mollereau and Mouledous 2000). Accordingly, N/OFQ elicits a broad range of biological effects such as modulation of pain transmission, of anxiety and response to stress, learning and memory, food intake, locomotor activity. Gene structure and chromosomal localization have also described both for the N/OFQ peptide and the NOP receptor, moreover knock-out animals lacking the neuropeptide gene have been generated and studied (Mollereau et al. 1996; Nothacker et al. 1996; Reinscheid et al. 2000).

The activity of known genes can be modified in vivo using gene-targeting technology. Mice lacking opioid peptides or opioid receptors have been generated by homologous recombination (Kieffer 1999). NOP receptor deficient mice have been generated by disrupting exon 1 (Sora et al. 1997), exon 2 (Matthes et al. 1996) or exons 2 and 3 (Loh et al. 1998). All morphine responses investigated so far are nullified in NOP receptor knock-out (KO) mice: analgesia after acute s.c. administration in tail immersion-1 (Matthes et al. 1996), tail-flick- (Sora et al. 1997) and hotplate tests (Sora et al. 1997); analgesia after intrathecal or i.c.v. administration, respiratory depression (Matthes et al. 1998); constipation (Roy et al. 1998); acute morphine treatment induced modification of locomotor activity (Tian et al. 1997); euphoria (Matthes et al. 1996); chronic morphine treatment induced physical dependence, withdrawal symptoms (Matthes et al. 1996); chronic morphine treatment induced immunosuppression (Gaverieaux-Ruff et al. 1998). The generation of mice deficient in DOP receptors was reported by Zhu et al. (1999), and Filliol et al. (2000). Homologous recombination led to exon 1- or exon 2 deleted mice. Simonin et al. reported mice lacking KOP receptors (Simonin et al. 1998). Targeting was directed to delete the first coding exon. Mice lacking all three opioid receptor genes have been obtained in a two-step strategy. Breeding of mice lacking NOP receptor exon 2 (Matthes et al. 1996) with mice lacking KOP receptor exon 1 (Simonin et al. 1998) produced MOP-/KOP- double mutants, while breeding of the former with exon 1 DOP deficient mice (Filliol et al. 2000) produced MOP-/DOP- double mutants. Triple mutants were obtained from MOP-/KOP x MOP-/DOP matings. These animals showed no $[^3H]$DAMGO, $[^3H]$CI-977 and $[^3H]$DPDPE binding sites, as expected from the parental phenotypes (Simonin et al. 2001). The analysis of responses of MOP, DOP and KOP receptor-deficient mice to prototypic opiates has clarified the role of each opioid receptor in the main actions of opiate drugs. The MOP receptors are essential for the activity of morphine and trigger the large set of well-known opiate activities that are clinically useful or relevant to opiate addiction. The KOP receptors mediate both dysphoric and analgesic activities of classical KOP agonists. DOP receptors produce analgesia, do not mediate opioid reward and dependence and may have other roles, such as improving mood states. Finally, MOP and DOP receptors may interact functionally while KOP receptors act independently.

Very recently the high resolution (2.8 Å) crystal structures of all four opioid receptors have been resolved (Mollereau et al. 2012; Wu et al. 2012; Thompson et al. 2012; Granier et al. 2012). Compared to the buried binding pocket observed in most G-protein-coupled receptors published so far, the morphinan ligand binds deeply within a large solvent-exposed pocket. Of particular interest, the NOP receptor crystallizes as a two-fold symmetrical dimer through a four-helix bundle motif formed by TM segments 5 and 6 (Mollereau et al. 2012). The crystal structure of the human KOP receptor in complex with the selective antagonist JDTic, arranged in parallel dimers, at 2.9 Å resolution. The structure reveals important features of the ligand-binding pocket that contribute to the high affinity and subtype selectivity of JDTic for the human KOP receptor. Modelling of other important KOP receptor-selective ligands, including the morphinan-derived antagonists nornaltorphimine and 5'-guanidinonaltirindole, and the diterpene agonist salvonarin A analogue RB-64, reveals both common and distinct features for binding these diverse chemotypes (Wu et al. 2012). The X-ray structure of the NOP receptor also shows substantial conformational differences in the pocket regions between NOP and the classical opioid receptors NOP and KOP receptors and these are probably due to a small number of residues that vary between these receptors. The NOP–compound-24 structure explains the divergent selectivity profile of NOP and provides a new structural template for the design of NOP ligands (Thompson et al. 2012). The crystal structure of the mouse DOP receptor complexed with naltrindole reveals that the binding pocket of opioid receptors can be divided into two distinct regions. Whereas the lower part of this pocket is highly conserved among opioid receptors, the upper part contains divergent residues that confer subtype selectivity. This provides a structural explanation and validation for the ‘message–address’ model of opioid receptor pharmacology, in which distinct ‘message’ (efficacy) and ‘address’ (selectivity) determinants are contained within a single ligand. Comparison of the address region of the DOP receptor with other GPCRs reveals that this structural organization may be a more general phenomenon, extending to other GPCR families as well (Granier et al. 2012). The four opioid receptor structures reveal several evolutionarily conserved ligand–receptor interactions in the receptors’ binding pockets, which are contained within the seven transmembrane helices (designated TM1–7) of the receptors. For instance, several
The idea of GPCR dimerization goes back to the 1980s (Birdsall 1982), however even afterwards the most accepted model was, which proposed that a single GPCR interacts with a single G-protein (González-Maeso 2011). GPCR dimerization gain much more attention after the development of chimeric muscarinic M3/α2-adrenergic receptors (Maggio et al. 1993), which demonstrated the first indirect evidence for GPCR dimerization (González-Maeso 2011). Also the evidence that GPCR heteromerization can alter ligand binding and receptor signaling (Jordan and Devi 2000; Bowery and Enna 2000) led to the widely acceptance of the GPCR hetero-and homomer concept.

Among the opioid receptors, firstly δ- and κ-opioid receptors were found to form heteromers (Jordan and Devi 2000). The δ-κ heteromer showed distinct ligand binding and functional properties from either receptor and synergistically binds highly selective agonists and enhances signaling (Jordan and Devi 2000). According to Grupta and co-workers, chronic morphine treatment induced μ-δ heteromer formation in brain regions which are important in drug addiction (Grupta et al. 2010). There is also evidence for dimerization between opioid and non-opioid receptors, for instance μ-opioid and α2-adrenergic receptors can directly communicate with each other through the receptor complex (Jordan et al. 2003; Vilaradaga et al. 2008). Opioid and cannabinoid receptor type 1 (CB1) heteromers have also a great literature: μ-CB1 (Hojo et al. 2008) and δ-CB1 (Rozenfeld et al. 2012) receptor heteromers were described by FRET and BRET studies.

**Endogenous opioid peptides as natural ligands for opioid receptors**

**Endogenous opioid peptides in general**

Natural ligands for multiple opioid receptors are endogenous opioid peptides. Opioid peptides are short sequences of amino acids that specifically bind to opioid receptors in the brain; opiates and opioids mimic the effect of these peptides. Opioid peptides may be produced by the body itself, for example endorphins. The effects of these peptides vary, but they all resemble those of opiates. Brain opioid peptide systems are known to play an important role in motivation, emotion, attachment behaviour, the response to stress and pain, and the control of food intake. Two related pentapeptides, Leu- and Met-encephalin (Hughes et al. 1975) were the first endogenous opioid peptides identified in different mammalian species including the human. Enkephalins have relatively high affinities for DOP receptors, two- to fivefold lower affinities for MOP receptors and substantially smaller affinity for KOP receptors. The discovery of enkephalins was soon followed by the discovery of β-endorphin (Li and Chung 1976). Later a potent opioid peptide with KOP receptor selectivity and very high affinity was described and named dynorphin 1-17 (Goldstein et al. 1979, 1981).
Endogenous opioid peptides are derived from three precursors referred to as proopiomelanocortin (POMC), proenkephalin (PENK) and prodynorphin (PDYN) (Nakanishi et al. 1979; Noda et al. 1982; Kakidani et al. 1982). Each polypeptide precursor is subjected to sequential enzymatic cleavages, transport and other posttranslational modifications resulting in multiple bioactive peptides. These endogenous opioids share the common N-terminal sequence of Tyr-Gly-Gly-Phe-Met/Leu (YGGFM/L), which has been termed either “enkephalin motif” or “message sequence” (Schwyzer, 1986); this pattern can be followed by various C-terminal extensions yielding other mature peptides ranging from 6 to 31 residues in length. Classical opioid peptides starting with tyrosine moiety (Tyr<sup>1</sup>) are specifically interacting with the MOP, DOP and KOP receptors. According to phylogenetic analyses the PENK gene is considered to be the primordial opioid pro-peptide gene (Dores et al. 1993). This is the ‘proenkephalin hypothesis’. Gene duplication is a recurring theme in the evolution of vertebrate polypeptide hormones and neuropeptides. It has been proposed that during the evolution of the opioid peptides at least two duplication events occurred in the most ancestral PENK gene (Dores et al. 1993). Consistent with the proenkephalin hypothesis, the first gene duplication event would have produced the POMC gene, which is the common precursor for the non-opioid adrenocorticotrop hormone (ACTH) and the three melanocyte stimulating hormones (α, β, γ MSHs) and the opioid untriacontapeptide β-endorphin. A further duplication event would have given rise to the PDYN gene. The proenkephalin hypothesis and the evolution of the opioid peptide precursor genes have been described in more details (Khalap et al. 2005; Roberts et al. 2007; Komorowski et al. 2012). Another scenario for gene duplications has been proposed more recently. Opioid peptide gene family members were investigated using a combination of sequence-based phylogeny and chromosomal locations of the peptide genes in various Vertebrates (Sundström et al. 2010). The results showed that the ancestral PENK gene gave rise to two additional copies in the genome doublings. The fourth member was generated by a local gene duplication, as the genes encoding POMC and PNOC are located on the same chromosome in several teleost and bird genomes that have been studied. In conclusion, the system of opioid peptides and receptors was largely formed by the genome doublings that took place early in vertebrate evolution.

**Enkephalins**

Among multiple opioid peptides the enkephalins have the widest tissue distribution. The structural organization of PENK includes an N-terminal signal peptide sequence followed by a cysteine rich domain and then the coding region for the mature oligopeptides interrupted by short non-processing segments. Mammalian PENK contains seven copies of enkephalins bordered by basic dipeptide repeats, such as Arg-Arg, Lys-Lys or Lys-Arg (RR, KK, KR). These dibasic residues represent recognition sites for the processing endopeptidase enzymes, called prohormone convertases (Hook et al. 2008). Upon cleavage terminal basic amino acids are typically removed. Seven opioid core motifs exists within PENK and Met-enkephalin units are present as repetitive sequences with a copy number of six, while one single copy of Leu-enkephalin is also present at the sixth core position. Beside the pentapeptides, two extended Met-enkephalin sequence forms exist: the fourth opioid motif is the octapeptide Met-enkephalin-Arg-Gly-Leu (MERGL) and the PENK C-terminal is indeed a heptapeptide with the structure of Met-enkephalin-Arg-Phe (MERF).

**Nociceptin/orphanin FQ**

The neuropeptide nociceptin/orphanin FQ (N/OFQ) was simultaneously identified as endogenous ligand for an orphan opioid-like receptor (Mollereau et al. 1994) by pioneer applications of the reverse pharmacology approach (Meunier et al. 1995; Reinscheid et al. 1995). N/OFQ is a heptadecapeptide (FGGFHTGAR KSARKLANQ) sharing some sequence similarities with the endogenous kappa-opioid receptor (KOPr) agonist peptide dynorphin A. Likewise, the highest primary sequence identity/homology of the NOP receptor are observed with the KOPr. A unique and distinguishing difference between opioid peptides and N/OFQ is that the N-terminal message motif is YGGF in the opioids (Schwyzer 1986), while the corresponding N-terminal tetrapeptide segment in N/OFQ is composed of FGGF. This difference may be sufficient to prevent N/OFQ binding to opioid receptors. The address domain of N/OFQ is relatively long and involves centrally located positively charged amino acids (Tancredi et al. 2005).

**Endomorphins**

Endomorphins are a group of lately discovered endogenous opioid peptides consisting of endomorphin-1 (Tyr-Pro-Trp-Phe-NH<sub>2</sub>) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH<sub>2</sub>) (Zadina et al. 1997). They are C-terminally amidated tetrapeptides with the highest known affinity and specificity for the µ-opioid receptor. Endomorphin-1 is found in the nucleus of the solitary tract, the periventricular hypothalamus, and the dorsomedial hypothalamus, where it is found within histaminergic neurons and may regulate sedative and arousal behaviors (Greco et al. 2008). It is assumed that endomorphins are the cleavage products of a larger precursor, but this polypeptide or protein has not yet been identified.
Modelling of opioid receptors

Introduction

Modelling the receptors or receptor-ligand interactions involves a wide range of the computational methods such as quantum chemical (QM), molecular dynamics (MD) and its variants, multivariate statistics (chemometrics) which is the mathematical toolkit for the quantitative structure-activity relationships studies (QSAR), homology modelling (which uses knowledge based statistical potentials) and molecular docking algorithms.

Ligand based modelling

Although the ligand based structure-activity studies are performed to compare the ligands and to make predictions for new drugs without the knowledge anything about the receptor, simultenously they result in assumptions on the complementer receptor structure. Numerous structure-activity studies have been made in the opioid field (e.g., Subramanian and Ferguson 2000; Wong et al. 2002; Pietrzeniewicz et al. 2006; Fürst and Hosztafi 2008; Janecka et al. 2010; Mustazza and Bastanzio 2011). These studies provided quite accurate spatial pharmacophore necessities for both agonists, antagonists on the ligand side (e.g., Feinberg et al. 1976; Loew et al. 1978; Loew and Burt 1978; Misicka 1995; Fournie-Zaluski et al. 1981; Schiller et al. 1983; Brandt et al. 1993; Subramanian and Ferguson 2000). Furthermore, despite the missing protein structure, a great advance was made in the receptor theory. The initial approximation of the enzyme-ligand interactions, the “lock-and-key” theorem (Fischer 1894) implied a rigid protein structure based on the high ligand specificity of the enzymes. Later, recognizing the variations in the pharmacophore arrangement of ligands with very similar activity profile, the “induced fit” theory was introduced assuming flexibility of the binding site of the receptor too (Koshland 1958). Studying the selectivity profiles on a vast number of peptide and non-peptide ligands, another feature of the ligand-receptor interactions was discovered and named as “message-address” concept (Portoghese et al. 1987; Portoghese et al. 1988) arising from the opioid research as seen above. Obviously, to fulfill the immediate need for quantitative estimation and prediction of the effect of the ligands, efficient QSAR methods have been developed. Among many, the 3D-QSAR methods proved to be especially powerful contributing to the development of new drugs as well (e.g., McGovern et al. 2010). Because flexibility has always been a crucial problem in the interaction models, 4D- and 5D-QSAR methods emerged to involve the flexibility of the ligand and the receptor (Duca and Hopfinger 2001; Vedani and Dobler 2002). Alternative methods to address the ligand flexibility were also developed (Loew and Burt 1978; Chew et al. 1993; Martinek et al. 2005; Dervarics et al. 2006). QSAR methods are also suitable for large scale screening of compound libraries which is a common task nowadays.

Receptor based modelling

Modelling the structure of opioid (or any other) receptors is aimed by two mutually fruitful reasons: on one hand, the knowledge of the structure and function of the receptor makes possible the rational (i.e. structure or receptor based) drug design and, in the other hand, the ligands with known effects can help to reveal how the receptor functions. In either case, the molecular interactions between the ligands and the receptor are to be explored, because the response of the receptor upon ligand binding specifically depends on the ligand-receptor complex. The resulting response classifies the ligands by their nature such as agonists, antagonists, inverse agonists or partial agonists and ranks them by binding affinity as well. The bottleneck in the receptor modelling is always to obtain the protein structure. The most valuable way is to start with an experimental structure but, due to the decent number of solved X-ray structures of membrane proteins (now including the opioid receptors), most protein modelling should start with theoretically derived structure, mostly by homology modelling (Vyas et al. 2012). However, good homology models are based on experimental structures of evolutionally related proteins which were not available in the early times of the opioid research. Due to the fact that the orthosteric binding site of the opioid receptors is among the membrane spanning helices it was possible to construct limited receptor models missing the extra- and intracellular loops as well as the N- and the C-terminal tails. These models utilized the quantified lipid- and polar facing propensities of the constituting amino acids obtained by multiple sequence alignment comparison of the already sequenced GPCRs and were able to explain the receptor ligand interactions including the selectivity profiles (Donnelly et al. 1993). It is important to note that the contact regions between the transmembrane helices determined by this method are the same than those obtained by a completely different, statistical-phylogenetic analysis of the GPCR family (Süel et al. 2003). Bera et al. (2011) investigated the quality of the homology models of MOR, DOR and KOR depending on the templates used in modelling. Having the protein structure in hand there are two different modelling methods to continue with, namely docking and molecular dynamics, both of them are based on molecular force fields.

Molecular docking with opioid receptors

Docking algorithms are designed for fast calculation of optimized ligand-receptor complexes and so forth are widely used for high throughput screening of compound libraries.
They apply simplified molecular force fields with entropic and solvation corrections resulting in score functions to rank the interacting poses of the ligand bound to the receptor (reviewed by e.g., Meng et al. 2011). The usual way is to keep the protein either rigid or flexible while the ligand is flexible with rotatable dihedral angles in its structure. As an exception applied in docking program FRED, the flexibility of the ligands is simulated by pregenerated conformer set of the ligand which than are docked rigidly to the receptor (McGann 2011). Using single rigid receptor conformation corresponds to the “lock-and-key” approximation. Most docking programs have built in algorithms to mimic the flexibility of the receptor, corresponding to the “induced fit” theory, by rotating the side chains of selected residues while keeping the protein backbone rigid. However, the choice of flexible side chains is important and it greatly influences the result of docking calculations (Abreu et al. 2012). The limited receptor flexibility involving only the side chains is overcome by the program DOCK which allows finishing the docking procedure with a molecular dynamics phase using implicit water model for solvation (Moustakas 2006) that now allows the movement of any atom of the protein. Another approach to sample the protein flexibility is to generate conformer ensembles by molecular dynamics and using them rigidly in the docking (Nakajima et al. 1997).

It is also important which conformational state of the receptor is used for the docking calculations (Cui et al. 2013). As an example, screening a database against the crystal structure of β2-adrenergic receptor complexed with an inverse agonist, the hits were also inverse agonists; one of them with high binding affinity (Kolb et al. 2009). As a comparison, docking a database to the crystal structure of the KOR complexed with an antagonist found an agonist, although the affinity was rather weak (Negri et al. 2013). The k selectivity was enforced by sorting out those compounds that showed interactions characteristic to MOR and DOR selective ligands.

Sagara et al. (1996) identified the ligand-binding residues of MOR by docking dihydromorphine derivatives to the active receptor model. Fentanyl derivatives were shown to adopt similar conformations in solution and docked to MOR modelled (Huang et al. 2000). It was found that fentanyl and its analogs interact with the crucial Asp3.32 but other interactions with the receptor are different than those of morphinan opiates (Subramanian et al. 2000; Dosen-Micovic et al. 2006). The different selectivity profiles of the MOR and DOR receptors were modelled by docking cyclic enkephalin analogues, JOM6 and JOM13, to the homology modelled structures and proved by mutagenesis studies (Mosberg and Fowler 2002). It was earlier reported that the constrained peptide JOM13 can bind the DOR with good docking energy, however in a somewhat different manner, than the well known opioid alkaloids (Mosberg 1999). It was also shown that investigating the ligands alone was not able to select the dockable conformation, without the docking calculations. Opioid ligands lacking the classical ammonium ion present a new group. A cyclic endomorphin 1 analogue was shown to bind to MOR differently than JOM6, a high affinity and selectivity µ opioid ligand but it could still activate the mu receptor (Gentilucci et al. 2008). Lesma and co-workers (2013, 2013a) synthesized and investigated endomorphin analogues with beta-amino acid replacements and found that despite the higher steric requirements they could adopt very similar docked positions than the parent compound.

Micovic and co-workers (2009) showed, by docking a series of morphinan derivatives to different rigid DOR conformations, that the ligands prefer different receptor states depending on their agonist or antagonist character and δ, or δ selectivity. Using accurate receptor models, partially based on the crystal structures, an efficient receptor model was developed for Salvinorin A analogues resulting in a quantitative pharmacophor model (Singh et al. 2006). and by docking benzomorphan derivative agonists to KOR showed the conserved salt bridge with D3.32 and both hydrogen bonding and hydrophobic interactions with Y7.35 and H6.52 (Lavecha et al. 2000). A combined ligand- and receptor-based method was developed for Salvinorin A analogues resulting in a quantitative pharmacophor model (Singh et al. 2006). and by docking assisted with molecular dynamics.

Huang et al. (2000a) investigated the binding modes of nociceptin, etorphine and four lofentanil stereomers to nociceptin receptor model. They found a good correlation between the experimental binding constants and docking energies. For the potent and NOP selective agonist Ro 64-6198 and analogues, a common hydrophobic cavity was identified in the homology modelled receptor (Bröer et al. 2003). The pharmacophore arrangement was determined by 3D-QSAR modelling first, then the ligands were docked to the receptor preserving the conserved D3.32 ionic interaction. A similar scenario was perfomed on spiropropiperidine analogues to find the binding interactions (Liu et al. 2010).

The message-address concept was directly addressed in an
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early study comparing the selectivity features of naltrexone, naltrindole and norbinaltorphimine by docking these ligands to opioid receptor homology models (Metzger et al. 1996); recently a more exhaustive investigation applied a higher number of the ligands and the crystal structures of the receptors (Zaidi et al. 2013). The interacting residues identified by the docking and structure-activity studies are compared with the recent crystal structures (Filizola and Devi 2012) and shown for MOP receptor in Figure 5.

Molecular dynamics with opioid receptors

Molecular dynamics (MD) simulation is able to discover the molecular interactions at atomic level as a series of time events. Its current accuracy can be demonstrated by a computer simulation of an antagonist binding spontaneously to the receptor reproducing the experimental crystal structure (Dror et al. 2011b), although at an extremely high cost of computer time. However, a huge amount of structural information has been collected about the opioid receptors by more limited MD methods.

The investigation of the pharmacophores of the peptides and other highly flexible ligands can only be performed by MD methods and by comparison with other ligands. Overlapping structural elements of enkephalin and naltrindole, i.e. the Phe side chain and the benzene moiety of the fused indol, respectively, were found to be responsible for selectivity (Portoghese et al. 1991). A bent backbone was obtained for [Met]- and [D-Ala²,Met³]-enkephalins and an identical pharmacophore arrangement with that of morphine (Ishida et al. 1988). The energy minimized conformers of the same peptides were also compared to find the most overlapping conformers that are most probably the bioactive conformers (Loew and Burt 1978). These conformers were overlapping with the pharmacophores of a rigid opiate ligand. Interestingly, these conformers were not the lowest energy ones and shared a bent backbone too. For endomorphin 2 and its analogues was also found that the bent backbone is predominant for the more potent analogues (Borics et al. 2012). Numerous other MD studies were performed on peptide ligands to find the active conformation for dermorphins (Mierke et al. 1990; Wilkes and Schiller 1992), deltorphins (Segawa et al. 1994), enkephalins (Chew et al. 1993; Mishra et al. 1994; Bryant et al. 1994; Gussmann et al. 1996; Shenderovich et al. 2000; Shen My and Freed 2002), endomorphins (Podlogar et al. 1998; Borics and Tóth 2010), and dynorphins (Collins and Hruby 1994). Bernard and co-workers (2003, 2005) developed a MD based method to distinguish agonists and antagonists.

Molecular dynamics simulations of the receptors reveal the understanding of their functioning. In early studies only the transmembrane helix bundle was built by homology modeling and subjected to MD simulation. Comparative studies involving all three opioid receptors resulted in stable structures preserving all helix contacts characteristic to the GPCRs but showed differences capable of explaining the selectivities (Strahs and Weinstein 1997; Filizola et al. 1999). With a similarly built model of KOR refined by MD in a phospholipid and water environment, different binding regions were found for the endogenous dynorphin and for synthetic ligands (Iadanza et al. 2002). A more complete DOR model involving the loop regions too was built and refined by MD however, it was not able to explain the sodium effect and ligand binding interactions (Aburi and Smith 2004). A model of MOR refined by MD in lipid and water reproduced the binding mode of naltrexone by docking (Zhang et al. 2005).

A non-opioid type binding mode to KOR was modelled for Salvinorin A, a non-classical opioid ligand, to explain its selectivity (Vortherms et al. 2007). The involvement of EL2 and rotation of TM2 was assumed resulting in a rearrangement of the binding pocket.

The importance of the conserved disulfide bridge between C3.25 and EL2 was investigated by mutagenesis studies for MOR (Zhang et al. 1999) and DOR (Ehrlich et al. 1998). In both cases the receptors lost the specificity and high affinity for high affinity opioid ligands. This is presumably due to the change in the overall conformation of the receptors affecting the ligand binding region too. The activation of the membrane embedded KOR was modelled by simulated annealing method and concluded that only the agonist disrupted the ionic lock (Kolinski and Filipek 2010). Similarly, the activation of MOR was modelled in the presence of morphine (Serohijos et al. 2011). The activation of nociceptin receptor involved movements of transmembrane helices 3 and 6 (Daga and Zaveri 2012). For DOR Collu and co-workers (2012)
simulated the movement of agonists. The allosteric effect of sodium ion ("sodium-effect") was modelled by long MD study in the case of MOR (Yuan et al. 2014) and for all three opioid receptor types (Shang et al. 2014).

The binding mode of endomorphin analogues in the active MOR was modelled by MD starting with docked structures (Liu et al. 2009). The resulting models conformed well with the statements of the message-address concept and the results obtained by mutagenesis studies. Liu and co-workers (2009a) modelled the mu-delta receptor dimerization by all-atom MD concluding that the transmembrane helices 1, 7 of the mu receptor and 4, 5 of the delta receptor are facing in the stabil model.

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