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MINIREVIEW—SPECIAL ISSUE IN MEMORY OF AVRAM GOLDSTEIN

Recent Developments in the Study of Opioid Receptors

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ABSTRACT

It is now about 40 years since Avram Goldstein proposed the use of the stereoselectivity of opioid receptors to identify these receptors in neural membranes. In 2012, the crystal structures of the four members of the opioid receptor family were reported, providing a structural basis for understanding of critical features affecting the actions of opiate drugs. This minireview summarizes these recent developments in our understanding of opiate receptors. Receptor function is also influenced by amino acid substitutions in the protein sequence. Among opioid receptor genes, one polymorphism is much more frequent in human populations than the many others that have been found, but the functional significance of this single nucleotide polymorphism (SNP) has been unclear. Recent studies have shed new light on how this SNP might influence opioid receptor function. In this minireview, the functional significance of the most prevalent genetic polymorphism among the opioid receptor genes is also considered.

Introduction

Avram Goldstein was already an established investigator when he became interested in the actions of opiate drugs in the late 1960s. An early goal was the identification and characterization of the opiate receptor (then always referred to in the singular). This required a reliable assay. Avram's strategy was to use two criteria, the well defined stereoselectivity of the opioid receptor (known as opioid peptide receptors; OPr) and the sensitivity of opiate analgesic action to antagonism by naloxone, to identify that component of total binding of the radiolabeled opiate that represented binding to the receptor (Goldstein et al., 1971). In this initial study, the fraction of opiate binding attributable to the receptor was rather small, but the same basic strategy was used later, together with opiate ligands with much higher radiochemical specific activity and a more efficient method of elimination of nonspecific binding, by Lars Terenius and the Snyder and Simon groups (Pert and Snyder, 1973; Terenius, 1973; Simon et al., 1973) to show the presence in the brain and gastrointestinal tract of binding proteins with high specificity for opiate drugs. This unambiguous demonstration of the binding to OPrs from three independent laboratories triggered continuing studies of the properties of these receptors, and also the search for an endogenous agent (again always discussed in the singular at this time) that was presumed to be the physiologic regulator of the opiate drug receptor. This minireview summarizes recent developments in our understanding of opiate receptors following the publication in 2012 of the crystal structures of all four members of the OPr family, and recent studies evaluating the role in μ-OPr (MOPr) function of the most prevalent genetic polymorphism among the OPr genes.

Insights from Structural Studies of Opioid Receptors

Forty years after the initial demonstration of the presence in brain of receptors for opiate drugs, crystal structures for all four members of the OPr family have now been reported. Avram would be particularly pleased that one of the two responsible groups, the Kobilka group, is based in the Department of Pharmacology at Stanford University, a department that Avram established in 1955. He would also be delighted that the Nobel Prize Committee has recently recognized Dr. Kobilka and his mentor, Robert Lefkowitz, for their contributions to the elucidation of the structures and functions of all G-protein-coupled receptors (GPCRs). Crystal structures for the mouse MOPr (Manglik et al., 2012) and δ-OPr (DOPr) (Granier et al., 2012) were reported from the Kobilka laboratory, while crystal structures for the human κ-OPr (KOPr) (Wu et al., 2012) and nociceptin-orphanin FQ receptor (NOPr) (Thompson et al., 2012) were reported by the Stevens laboratory at the Scripps Research Institute in La Jolla, CA. The reported structures

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ABBREVIATIONS: β_2-AR, β_2-adrenergic receptor; C-24, 1-benzyl-*N*-{3-[spiroisobenzofuran-1(3*H*),4′-piperidin-1-yl]propyl} pyrrolidine-2-carboxamide; DOPr, δ-opioid receptor; ECL, extracellular loop; β-FNA, β-funaltrexamine; GPCR, G-protein-coupled receptor; ICL, intracellular loop; JDTic, (3*R*)-1,2,3,4-tetrahydro-7-hydroxy-*N*-[(1*S*)-1-[[(3*R*,4*R*)-4-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidinyl]methyl]-2-methylpropyl]-3-isoquinolinecarboxamide; KOPr, κ-opioid receptor; MOPr, μ-opioid receptor; NOPr, nociceptin-orphanin FQ receptor; OPr, opioid peptide receptor; SNP, single nucleotide polymorphism; TM, transmembrane.

provide a number of insights into the actions of opiate drugs, and a few surprises. A comparison of the major features of the reported crystal structures of the four receptors making up the OPr family is contained in Table 1.

Achieving crystallization of these GPCRs is a major technological achievement. It required substantial molecular engineering of the receptors during which highly disordered regions of the receptor were replaced with fragments of another protein known to assist in structural stabilization; residues 2–161 of T4 lysozyme were inserted into the third intracellular loop (ICL) of the OPrs to facilitate their crystallization. The highly flexible N- and C-terminal regions of the wild-type receptor protein sequences were also truncated to aid crystallization, and a FLAG tag and a poly-His sequence with cleavage sites were inserted on the truncated N terminus or the truncated C terminus, respectively, to aid purification of the expressed engineered receptors. Despite this extensive engineering, each receptor when expressed in cells in culture retained the ability to bind highly selective ligands with only modest changes in affinity and was capable

TABLE 1

Comparison of the reported crystal structures for the four OPrs complexed with antagonist drugs

Specific amino acids are indicated by their single-letter amino acid code, with numbers indicating their position in the receptor sequence; numbers in parentheses indicate their position within the TM α-helices (using the Ballesteros-Weinstein nomenclature); e.g., H297(6.52) indicates a His residue in sequence position 297, located in the sixth TM α-helix at position 52 within the helix; position 52 refers to the residue location relative to the most conserved amino acid within the helix, which is arbitrarily given the locator 50, so that position 52 is 2 residues toward the C terminus from the most conserved amino acid; a position number of <50 indicates a location toward the N terminus relative to the most conserved amino acid. Note the conservation across the receptor types of the positions within the α-helix structure of amino acid residues critical for ligand binding; e.g., D147(3.32), D128(3.32), D138(3.32), and D130(3.32) in MOPr, DOPr, KOPr, and NOPr, respectively.

Feature	MOPr	DOPr	KOPr	NOPr
Receptor engineering to enable crystallization	Mouse receptor, with N- and C-terminal truncations; inserted N-terminal FLAG tag and C-terminal poly-His to aid purification; lysozyme T4L residues 2–161 inserted in ICL3; crystallized using lipidic cubic-phase technique with cholesterol	Mouse receptor, with N- and C-terminal truncations; inserted N-terminal FLAG tag and C-terminal poly-His to aid purification; lysozyme T4L residues 2–161 inserted in ICL3; crystallized using lipidic cubic-phase technique with cholesterol	Human receptor, with N- and C-terminal truncations; inserted N-terminal FLAG tag and C-terminal poly-His to aid purification; lysozyme T4L residues 2–161 inserted in ICL3; single point mutation I135L; crystallized using lipidic cubic-phase technique with cholesterol	Human receptor; replaced N terminus with a stabilized apocytochrome *b*-RIL fragment and a FLAG sequence; truncation of C terminus; crystallized using lipidic cubic-phase technique with cholesterol
Cocrystallized ligand	β-FNA: MOPr-selective irreversible antagonist	Naltrindole: DOPr-selective reversible antagonist	JDTic: KOPr-selective reversible antagonist (Ki, 0.32 nM)	C-24: NOPr-selective reversible antagonist (Ki, 0.27 nM)
TM domains and ECLs/ICLs (sequence homology data from Granier et al., 2012; Thompson et al., 2012)	7TMs with similar placement to rhodopsin, with Pro-related bends in α-helices	7TMs; 76% homology to MOPr; similar placement to rhodopsin, with Pro-related bends in α-helices	7TMs; 73% homology to MOPr; ECL2 forms a β-hairpin	7TMs; 67% homology to MOPr; similar placement to rhodopsin, with Pro-related bends in α-helices; ECL2 forms a β-hairpin; ECLs enriched in D, E residues; acidic relative to other OPrs; ICL2 forms a short α-helix
Disulfide bridge	C140–C217; links ECL2 to end of TM3	Not reported	C131–C210; links ECL2 to end of TM3	C123(3.25)–C200(ECL2)
Opioid ligand-binding pocket	"Open" binding pocket deep in cell membrane; should facilitate rapid dissociation of reversible ligands	"Open" binding pocket deep in cell membrane; similar to binding pocket in MOPr and KOPr	"Open" binding pocket deep in cell membrane; similar to binding pocket in MOPr and DOPr	Binding pocket is "relatively large" and capable of binding large peptides
Critical ligand-binding residues	D147(3.32): charge-charge interaction with ligand; H297(6.52)+2H_2O: hydrogen bonding to phenolic OH and aromatic ring of morphinans [ligand specific: K233(5.39): covalent link to β-FNA]	D128(3.32): charge-charge interaction with ligand; H278(6.52) +2H_2O: hydrogen bonding to phenolic OH of naltrindole [probable ligand-specific roles for W274(6.48), Y308(7.43), M132(3.35), I277(6.51), Y129(3.33), V281(6.55), L300(7.35), W284(6.58)]	D138(3.32): charge-charge interaction with ligand; W287(6.48), H291(6.52): hydrophobic interactions with ligand [probable ligand-specific roles for V118(2.63), V134(3.28), L135(3.29), Y139(3.33), M142(3.36), V230(5.42), K227(5.39), I294(6.55), I290(6.51), Y312(7.35), I316(7.39), G319(7.42), V108(2.53), Q115(2.60), T111(2.56)]	D130(3.32): charge-charge interaction with ligand; other binding pocket residues show reduced homology with KOPr or MOPr, reflecting low affinity for classic opioids; H(6.52) replaced by Q280(6.52); M134 (3.36) reoriented relative to M142(3.36) in KOPr; A216(5.39) replaces K; T305(7.39) replaces I in other OPrs
Oligomerization	Crystallizes as parallel dimers; tightly associated through TM5, TM6	Crystallizes as antiparallel dimers, possibly reflecting energetically favorable interactions unique to crystallization conditions	Crystallizes as parallel dimers; structures of the two molecules in the dimer are similar but not identical, for example in ICL2	Not reported
Reference	Manglik et al., 2012	Granier et al., 2012	Wu et al., 2012	Thompson et al., 2012

of supporting agonist-induced changes in signal transduction pathways.

Antagonist Ligands in the Receptor Complexes

To further aid in the crystallization process, each receptor was bound to a tightly binding selective antagonist drug. The irreversible selective ligand β-funaltrexamine (β-FNA) was bound to the MOPr (Manglik et al., 2012); a covalent link between β-FNA and the ε-amino group of a lysine (K233) residue in the fifth transmembane (TM) domain of MOPr was identified. The DOPr was crystallized in complex with the high-affinity DOPr-selective reversible antagonist naltrindole (Granier et al., 2012). The engineered human KOPr was crystallized in complex with the high-affinity KOPr-selective reversible antagonist (3*R*)-1,2,3,4-tetrahydro-7-hydroxy-*N*-[(1*S*)-1-[[(3*R*,4*R*)-4-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidinyl]methyl]-2-methylpropyl]-3-isoquinolinecarboxamide (JDTic) (Wu et al., 2012), while the engineered human NOPr was crystallized in complex with the novel high-affinity NOPr-selective antagonist 1-benzyl-*N*-{3-[spiroisobenzofuran-1(3*H*),4′-piperidin-1-yl]propyl} pyrrolidine-2-carboxamide (C-24), from Banyu Pharmaceutical (Tokyo, Japan) (Thompson et al., 2012). The C-24 structure is analogous to the first four amino acid residues of the endogenous ligand, nociceptin/orphanin FQ; C-24 has a *K*i of 0.3 nM for the wild-type receptor and about 2 nM for the engineered NOPr. The use of antagonists as the cocrystallized ligands facilitates the formation of crystals by freezing the receptors in their relatively stable inactive conformations.

TM Domains

Each receptor has seven α-helical TM domains (7TM) that are aligned around a central ligand-binding pocket as anticipated from earlier studies comparing analogous sequences in rhodopsin with the OPr sequences, and considering the placement of the α-helical TM domains of rhodopsin. There appears to be considerable similarity in the overall orientation of the 7TM helices between the four members of the OPr family, although the spatial alignment of NOPr differs in places from the more conserved orientations of the MOPr, DOPr, and KOPr TM domains (Thompson et al., 2012). All four receptors have bends in some of the TM helices (TM2, TM4, TM5, TM6, and TM7) induced by the presence Pro residues roughly centered in each TM domain (Thompson et al., 2102). These Pro residues are highly conserved across most GPCRs; their presence is emphasized in the description of the crystal structure of the β_2-adrenergic receptor (β_2-AR), the first GPCR to be crystallized (Cherezov et al., 2007). The bends in the TM domains contribute to the shape of the ligand-binding pocket for each receptor. In contrast to the conserved TM domains, the extracellular loops (ECLs) and the ICLs show more extensive variation between members of the OPr family. The ECL2 domains of KOPr and NOPr differ from those of MOPr and DOPr by the increased frequency of acidic amino acid residues (Asp, Glu), making the entrance to the ligand-binding pocket in these receptors highly acidic (Thompson et al., 2012). This may be related to the highly basic nature of dynorphin A and nociceptin/orphanin FQ, the endogenous ligands for KOPr and NOPr, respectively. The overall structure of GPCRs is also supported by the presence of one or more conserved Cys-Cys bonds. In the OPr family there is just one conserved Cys-Cys disulfide bond in a similar location in each receptor, linking the second extracellular loop (ECL2) to the intracellular end of TM3.

Ligand-Binding Pocket

There are also many similarities in the binding pockets of the OPrs (Fig. 1). In all cases, the binding pocket is located in the center of the receptors, deep within the hollow created by the encircling TM domain regions. The pocket appears more open to the extracellular fluid than is reported for the binding pockets of other GPCRs with small-molecule endogenous ligands. Manglik et al. (2012) suggest that the open nature of the OPr ligand-binding pocket is consistent with the very short dissociation half-lives of highly potent MOPr antagonists; for example, diprenorphine (Ki, 72 pM) has a dissociation half-life of 36 minutes from MOPr. In contrast, the M_3 muscarinic receptor structure displays a much more restricted entry to its ligand-binding site, and the potent M_3 receptor antagonist tiotropium (Ki, 40 pM), has a dissociation half-life of about 35 hours. The amino acid residues within the OPr binding pocket with which the very-high-affinity highly selective antagonists used in these studies interact are in part specific to the unique characteristics of these very specialized ligands. Nevertheless, several similarities across the receptor types are apparent. Conserved Asp residues [D147(3.32), D128(3.32), D138(3.32), and D130(3.32) in MOPr, DOPr, KOPr, and NOPr, respectively] are located in essentially the same location within the third TM helix of each receptor (Fig. 1). Mutation of this Asp residue in each receptor to a noncharged alternative amino acid results in loss of opioid activity. The Asp residue is thought to form a charge-charge interaction with a positively charged group in the ligands binding to each receptor. It has long been assumed than an ionic interaction between the ligand and each OPr is a critical feature in the binding of opiate ligands to their receptors (Beckett and Casy, 1954). The structural basis for this is now apparent. Another common feature of the binding site is the presence of a conserved His residue in three of the four OPrs [H297(6.52), H278(6.52), and H291(6.52) in MOPr, DOPr, and KOPr, respectively]. In NOPr, this His is replaced by a Gln [Q280(6.52)]. The His residues in the three "classic" OPrs are thought to interact by hydrogen bonding through two associated water molecules with the tyrosine-like hydroxyl moieties of the morphinan ligands (Manglik et al, 2012). There are a number of other amino acids located in close contact with the docked antagonist molecules in these receptors (Fig. 1; Table 1). Some of these interactions are probably specific for the unique high-affinity antagonist ligands selected for the crystallization, but many may also be important in the docking and agonist action of physiologic agonists. It should be noted that the Lys residue [K233(5.39)] covalently linked to the β-FNA in the MOPr crystal is likely to be a special case resulting from the covalent nature of this interaction.

Receptor Oligomerization

The MOPr and KOPr crystals formed as parallel dimers tightly associated through TM5 and TM6, and to a lesser extent between TM1 and TM2, although in the KOPr crystal antiparallel dimers were also observed. In contrast, DOPr was reported to crystallize exclusively as antiparallel dimers

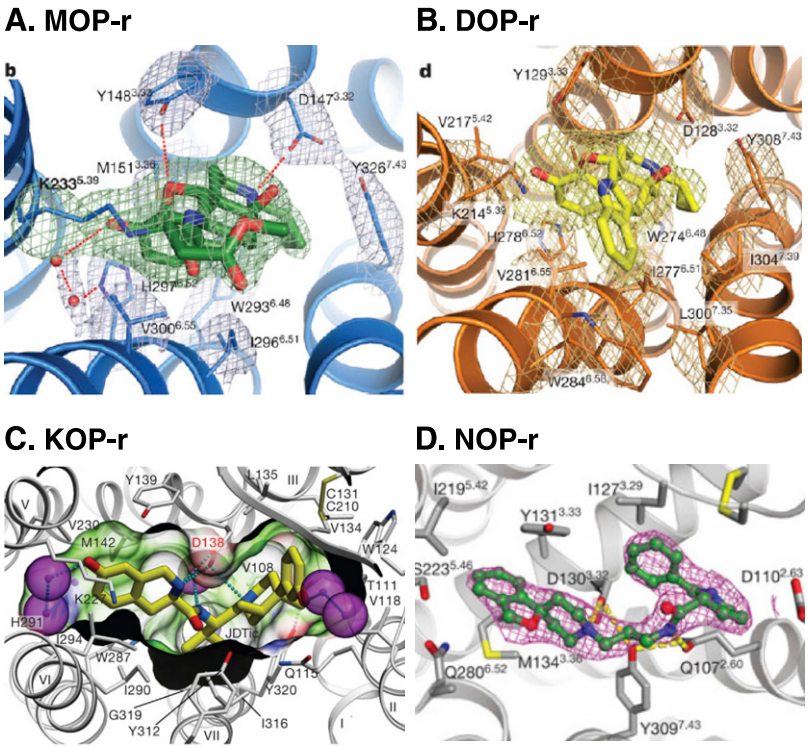

Fig. 1. Comparison of the ligand-binding pockets of the four members of the OPr family, all viewed from the extracellular surface. (A) The ligand-binding site of the MOPr in complex with β-FNA (green) covalently bound to the receptor via K233(5.39). The red spheres indicate water molecules linking H297(6.52) to the phenolic group of β-FNA; polar contacts are indicated with red dotted lines [with D147(3.32) and Y148(3.33)] and hydrophobic interactions are in orange. Light blue mesh indicates the electron density around the receptor protein side chains. (From Manglik et al., 2012, Fig. 3, panel b, with permission from MacMillan Publishers Ltd.) (B) The ligand-binding site of the DOPr in complex with naltrindole (yellow) and the protein chain in brown, showing the close proximity of D128(3.32) and Y129(3.33) to the ligand. H278(6.52) is also strategically located. (From Granier et al., 2012, Fig. 2, panel d, with permission from MacMillan Publishers Ltd.) (C) The ligand-binding site of the KOPr in complex with JDTic (yellow) and the protein chain in blue, with the polar contact of D138(3.32) (highlighted in orange) with the ligand shown as a dotted line. H291(6.52) is also located in close proximity to the ligand. In this panel, water molecules that are part of the crystal structure are shown as magenta spheres; hydrophobic surfaces are indicated in green, hydrogen bond donors in blue, and hydrogen bond acceptors in red. Black indicates the protein interior. (From Wu et al., 2012, Fig. 2, panel a, with permission from MacMillan Publishers Ltd.) (D) The ligand-binding pocket of the NOPr in complex with the peptide mimetic agent C-24 (green, with purple mesh) showing the proximity of D130(3.32), which forms a salt bridge (not shown here) with the ligand. The critical H residue in the other OPrs is replaced in NOPr with Q280(6.52). (From Thompson et al., 2012, Fig. 2, panel d, with permission from MacMillan Publishers Ltd.)

(Granier et al., 2012). The antiparallel form appears unlikely in biologic membranes; the authors argue that the antiparallel arrangement may reflect an energetically favorable arrangement during the crystallization process with naltrindole. It is highly unlikely that the antiparallel arrangement is a reflection of intermolecular associations that occur in vivo (this would require that the binding pocket of one of the receptors in the dimer faced the interior of the cell). The presence of parallel dimers in MOPr and KOPr crystals provides a structural basis for earlier studies reporting the homo- and heterodimerization of OPrs in biologic membranes (Cvejic and Devi, 1997; Jordan and Devi, 1999; George et al., 2000). It should be noted that the observed dimerization in the crystals occurs during crystallization—the engineered receptors were purified as monomers—so there is no certainty that the oligomerization forms found in the crystal represent functional dimer forms present in vivo.

The role of membrane cholesterol in determining the preferred structures of GPCRs and in modulating OPr function also requires consideration. Cholesterol was used to facilitate the crystallization of β_2-AR bound to an antagonist (Cherezov et al., 2007) and when this GPCR was cocrystallized together with G_s in the presence of a β_2-AR agonist (Rasmussen et al., 2011). Cherezov et al. (2007) reported that cholesterol mediates the parallel association of dimers in β_2-AR crystals, raising the possibility that it plays a similar role in facilitating dimer formation in vivo. Crystallization of the four OPrs also required the presence of cholesterol (Granier et al., 2012; Manglik et al., 2012; Thompson et al., 2012; Wu et al., 2012), although the role of cholesterol as a factor determining the observed structures of these receptors is not discussed by the authors. It has long been known that modulating the cholesterol content of OPr-expressing cell membranes can alter the binding and signal transduction properties of the receptors (Lazar and Medzihradsky, 1992; Xu et al., 2006; Gaibelet et al., 2008; Zheng et al., 2012), although the authors differ in their proposed (non–mutually exclusive) mechanisms (e.g., altered membrane microviscosity, receptor partition into lipid rafts, facilitation of association with G proteins, facilitation of dimer formation, modulation of receptor palmitoylation).

Agonism at Opioid Receptors

The elucidation of the crystal structure of all members of the OPr family provides a strong basis for design of selective ligands for each receptor, but the antagonist-bound crystal structures shed less light on the changes in receptor structure and conformation that result in the induction of agonist effects. Like other GPCRs, most of the observed actions of OPr ligands require the activation of a G protein to trigger further downstream events within a cell. OPrs predominantly couple with G_i or G_o to cause dissociation of the G$\alpha\beta\gamma$ complex and trigger downstream cellular processes. Recently, the Kobilka laboratory reported the crystallization of the β_2-AR complexed with the G_s α-subunit (Rasmussen et al., 2011), another extraordinary technical achievement requiring crystallization conditions that maintain the association of an agonist-bound receptor with the G-protein heterotrimer. The agonist form of the β_2-AR with G_s indicates that agonism requires substantial changes in the orientation of β_2-AR-complexed microdomains within the G_s α-subunit. To date there is no report of the crystallization of an OPr or any other $G_{i/o}$-coupled GPCR in complex with the $G_{i/o}$ α-subunit. The Rasmussen et al. (2011) study indicates a pathway toward crystallization of an agonist-form OPr crystal, but many technical challenges will need to be overcome to achieve this. It remains to be determined if G_i and/or G_o activation results

from a reorientation of the C termini of these proteins that is analogous to the agonist-activated β_2-AR-mediated reorientation of the G_s α-subunit C terminus.

Opioid Receptor Polymorphisms and Receptor Function

The primary sequence of a GPCR is a major determinant of the secondary and tertiary structure of the mature receptor. It is therefore possible that polymorphisms in an OPr gene might result in the expression of a receptor with a modified tertiary structure and altered functional activity. There are numerous single nucleotide polymorphisms (SNPs) in the human MOPr gene, but most are rare and their functional significance, if any, is unknown (see review by Mague and Blendy, 2010). At this time, a polymorphism in an OPr gene that alters the major conformation of the expressed receptor has not been reported. In the MOPr gene, one SNP (rs 1799971) occurs relatively frequently in some human populations. The polymorphism is located in exon 1, where a change in adenosine (A) to guanosine (G) in nucleotide position 118 (A118G) results in a change in amino acid sequence in which Asn40 in replaced by Asp (designated N40D). This SNP has now been studied more extensively than the other SNPs in MOPr or any SNPs in the other OPrs. A118G occurs with variable frequency in different human populations, with the highest reported allelic frequency of 118G being 48.5% in a Japanese population. In contrast, the 118G allelic frequency is 15.4% in European-Americans, 14% in Hispanics, 8% in Bedouins, and 5% in African Americans (Gelernter et al., 1999); other studies show approximately similar relative distributions by population and confirm the high expression of this SNP in Asian populations (Bond et al., 1998; Tan et al., 2009). Initial reports suggested that this SNP was associated with addictive behaviors for several drugs, but more extensive studies have not confirmed this apparent association, and the effect of the A118G polymorphism has been variously reported to be either an increase or a reduction in the risk of substance abuse. There is more consistent agreement that A118G is associated with impaired opioid signaling through MOPr and a need for increased opiate drug doses in patients with the G variant in a variety of painful conditions (see review by Mague and Blendy, 2010).

The N40D (A118G) mutation occurs in the N-terminal extracellular domain of MOPr, a part of the receptor that is highly disordered. Manglik et al. (2012) removed this extracellular domain in their engineered receptor to facilitate its crystallization. It is therefore unlikely that this SNP alters the basic three-dimensional structure of the MOPr protein. Early reports suggested that A118G resulted in increased signaling through MOPr by the endogenous ligand β-endorphin (Bond et al., 1998), but more recent studies found unchanged opioid ligand binding with impaired opioid signaling in the 118G variant (Mague and Blendy, 2010; Oertel et al., 2012). Kroslak et al. (2007) reported that 118G reduced the level of MOPr protein (observed as reduced ligand B_{max} for opioid ligands) and found a lower potency of opiates as inhibitors of adenylyl cyclase in oocytes transfected with this receptor variant. To evaluate the function of this receptor more fully, Mague et al. (2009) generated a mouse analog with nucleotide A112 of the mouse MOPr gene mutated to a G (A112G), resulting in conversion of Asn38 to Asp38 (N38D; corresponding to N40D in the human gene). The mutated mouse receptor displayed essentially unchanged ligand-binding affinities for several ligands, but reduced levels of receptor mRNA and protein expression were observed in most brain regions, suggesting that a reduction in the number of receptors may account for the impaired signaling. One effect of the N40D change is the loss of an N-glycosylation site on the N terminus of the receptor protein. Huang et al. (2012) have confirmed that the N38D (A112G) receptor shows reduced glycosylation in homozygous A112G mice and that the reduced glycosylation is associated with a reduction in the stability of the modified receptor. Thus, one potential explanation of the reduced level of receptor expression is a reduced stability of the less glycosylated MOPr protein.

Other factors also contribute to the reduced levels of receptor protein in those carrying the A118G mutation. Zhang et al. (2009) have shown that a G in position 118 is associated with reduced levels of the MOPr mRNA expression in Chinese hamster ovary cells expressing transfected variant forms of the receptor mRNA and that 118A mRNA was significantly more abundant than the 118G mRNA in human autopsy brain tissue from eight heterozygous subjects. This raises the interesting question of how a change in the gene sequence in the coding region of the gene might affect the levels of the expressed mRNA. Zhang et al. (2009) discuss the possibility that 118G causes reduced mRNA stability, but did not find an allele-specific impaired mRNA stability in transfected Chinese hamster ovary cells.

Oertel et al. (2012) now offer an alternative explanation. They have shown that the A118G variant introduces a newly identified methylation site on the *OPRM1* gene (the gene coding for MOPr) at nucleotide position +117. The extent of methylation of the *OPRM1* DNA at +117 and at downstream methylation sites in DNA extracted from the brains obtained post mortem from heroin abusers (who died from an opiate overdose) and in controls, comparing methylation between the 118A and 118G alleles, was dependent on whether A or G was present at position 118. Significant increases in methylation ($P < 0.05$ or greater) were found at positions +117, +145, +150, and +159 in 118G-carrying heroin-abuser subjects, but were not observed in control subjects carrying the 118G allele. The significance of the altered methylation was evaluated by comparing the levels of MOPr mRNA expression in 118A and 118G carriers in both heroin abusers and control subjects. Heroin abusers carrying 118A exhibited significantly higher MOPr mRNA levels in two brain regions (thalamus and S11 cortex) than 118A controls, and an increase in the level of MOPr-binding sites; in contrast, heroin abusers with 118G (either one or two copies) expressed levels of MOPr mRNA and MOPr binding that were very similar to those of 118G controls in both brain regions. These results indicate that the presence of the 118G allele impairs the increased expression of MOPr mRNA that occurs when MOPr signaling efficiency is reduced after chronic opiate drug exposure. The sites showing increased methylation with the 118G allele include two predicted binding sites for the transcription factor Sp1 in *OPRM1* DNA, providing a possible explanation for the reduced ability of chronic opiate drug users with the A118G polymorphism to increase MOPr RNA expression in response to impaired receptor signaling efficiency. The reason that increased *OPRM1* DNA methylation was observed only in heroin-abuser 118G carriers but not in the 118G carrier controls is unexplained at this time. Nevertheless, it is clear

that the A118G polymorphism occurring in a significant fraction of most populations modifies the regulation of MOPr expression and the sensitivity to the actions of opiate drugs. This study points to the complexity of the interaction of genetic, epigenetic, and environmental factors in the regulation of expression of the MOPr gene.

Concluding Thoughts

These recent developments in OPr research demonstrate that the research field in which Avram Goldstein played a prominent founding role 40 years ago is still very active. Increased understanding of the three-dimensional structure of all members of the OPr family will make it possible to design drugs with increased specificity for each receptor type and to address possible allosteric regulation of receptor function. We await with interest the determination of the three-dimensional structures of the receptors when complexed with agonists and effector proteins. Improved understanding of how receptor expression and function is modified by primary sequence variations will contribute to our understanding of the differences between individuals in their responses to opiate drugs. Avram's seminal contributions to the development of this research field continue to bear fruit.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Cox

References

Beckett AH and Casy AF (1954) Synthetic analgesics: stereochemical considerations. *J Pharm Pharmacol* **6**:986–1001.

Bond C, LaForge KS, Tian M, Melia D, Zhang S, Borg L, Gong J, Schluger J, Strong JA, and Leal SM et al. (1998) Single-nucleotide polymorphism in the human mu opioid receptor gene alters β-endorphin binding and activity: possible implications for opiate addiction. *Proc Natl Acad Sci USA* **95**:9608–9613.

Cherezov V, Rosenbaum DM, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS, Choi HJ, Kuhn P, Weis WI, and Kobilka BK et al. (2007) High-resolution crystal structure of an engineered human β_2-adrenergic G protein-coupled receptor. *Science* **318**:1258–1265.

Cvejic S and Devi LA (1997) Dimerization of the delta opioid receptor: implication for a role in receptor internalization. *J Biol Chem* **272**:26959–26964.

Gaibelet G, Millot C, Lebrun C, Ravault S, Sauliere A, Andre A, Lagane B, and Lopez A (2008) Cholesterol content drives distinct pharmacological behaviours of micro-opioid receptor in different microdomains of the CHO plasma membrane. *Mol Membr Biol* **25**:423–435.

Gelernter J, Kranzler H, and Cubells J (1999) Genetics of two mu opioid receptor gene (OPRM1) exon I polymorphisms: population studies, and allele frequencies in alcohol- and drug-dependent subjects. *Mol Psychiatry* **4**:476–483.

George SR, Fan T, Xie Z, Tse R, Tam V, Varghese G, and O'Dowd BF (2000) Oligomerization of mu- and delta-opioid receptors. Generation of novel functional properties. *J Biol Chem* **275**:26128–26135.

Goldstein A, Lowney LI, and Pal BK (1971) Stereospecific and nonspecific interactions of the morphine congener levorphanol in subcellular fractions of mouse brain. *Proc Natl Acad Sci USA* **68**:1742–1747.

Granier S, Manglik A, Kruse AC, Kobilka TS, Thian FS, Weis WI, and Kobilka BK (2012) Structure of the δ-opioid receptor bound to naltrindole. *Nature* **485**:400–404.

Huang P, Chen C, Mague SD, Blendy JA, and Liu-Chen LY (2012) A common single nucleotide polymorphism A118G of the μ opioid receptor alters its N-glycosylation and protein stability. *Biochem J* **441**:379–386.

Jordan BA and Devi LA (1999) G-protein-coupled receptor heterodimerization modulates receptor function. *Nature* **399**:697–700.

Kroslak T, Laforge KS, Gianotti RJ, Ho A, Nielsen DA, and Kreek MJ (2007) The single nucleotide polymorphism A118G alters functional properties of the human mu opioid receptor. *J Neurochem* **103**:77–87.

Lazar DF and Medzihradsky F (1992) Altered microviscosity at brain membrane surface induces distinct and reversible inhibition of opioid receptor binding. *J Neurochem* **59**:1233–1240.

Mague SD and Blendy JA (2010) OPRM1 SNP (A118G): involvement in disease development, treatment response, and animal models. *Drug Alcohol Depend* **108**: 172–182.

Mague SD, Isiegas C, Huang P, Liu-Chen LY, Lerman C, and Blendy JA (2009) Mouse model of OPRM1 (A118G) polymorphism has sex-specific effects on drug-mediated behavior. *Proc Natl Acad Sci USA* **106**:10847–10852.

Manglik A, Kruse AC, Kobilka TS, Thian FS, Mathiesen JM, Sunahara RK, Pardo L, Weis WI, Kobilka BK, and Granier S (2012) Crystal structure of the μ-opioid receptor bound to a morphinan antagonist. *Nature* **485**:321–326.

Oertel BG, Doehring A, Roskam B, Kettner M, Hackmann N, Ferreirós N, Schmidt PH, and Lötsch J (2012) Genetic-epigenetic interaction modulates μ-opioid receptor regulation. *Hum Mol Genet* **21**:4751–4760.

Pert CB and Snyder SH (1973) Opiate receptor: demonstration in nervous tissue. *Science* **179**:1011–1014.

Rasmussen SG, DeVree BT, Zou Y, Kruse AC, Chung KY, Kobilka TS, Thian FS, Chae PS, Pardon E, and Calinski D et al. (2011) Crystal structure of the β2 adrenergic receptor-Gs protein complex. *Nature* **477**:549–555.

Simon EJ, Hiller JM, and Edelman I (1973) Stereospecific binding of the potent narcotic analgesic (3H) etorphine to rat-brain homogenate. *Proc Natl Acad Sci USA* **70**:1947–1949.

Tan EC, Lim EC, Teo YY, Lim Y, Law HY, and Sia AT (2009) Ethnicity and OPRM variant independently predict pain perception and patient-controlled analgesia usage for post-operative pain. *Mol Pain* **5**:32 DOI: 10.1/1186/1744-8069-5-32.

Terenius L (1973) Stereospecific interaction between narcotic analgesics and a synaptic plasma membrane fraction of rat cerebral cortex. *Acta Pharmacol Toxicol (Copenh)* **32**:317–320.

Thompson AA, Liu W, Chun E, Katritch V, Wu H, Vardy E, Huang XP, Trapella C, Guerrini R, and Calo G et al. (2012) Structure of the nociceptin/orphanin FQ receptor in complex with a peptide mimetic. *Nature* **485**:395–399.

Wu H, Wacker D, Mileni M, Katritch V, Han GW, Vardy E, Liu W, Thompson AA, Huang XP, and Carroll FI et al. (2012) Structure of the human κ-opioid receptor in complex with JDTic. *Nature* **485**:327–332.

Xu W, Yoon SI, Huang P, Wang Y, Chen C, Chong PL, and Liu-Chen LY (2006) Localization of the kappa opioid receptor in lipid rafts. *J Pharmacol Exp Ther* **317**: 1295–1306.

Zhang Y, Wang D, Johnson AD, Papp AC, and Sadée W (2005) Allelic expression imbalance of human mu opioid receptor (OPRM1) caused by variant A118G. *J Biol Chem* **280**:32618–32624.

Zheng H, Pearsall EA, Hurst DP, Zhang Y, Chu J, Zhou Y, Reggio PH, Loh HH, and Law PY (2012) Palmitoylation and membrane cholesterol stabilize μ-opioid receptor homodimerization and G protein coupling. *BMC Cell Biol* **13**:6 DOI: 10.1186/1471-2121-13-6.

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