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Overview and Historical Perspective of Four Papers Presented on Research Related to the Endogenous Opioid System

Mary Jeanne Kreek

The Laboratory of the Biology of Addictive Diseases, The Rockefeller University, New York, NY 10065 USA

1. Introduction and Background

The endogenous opioid system is now known to play a major role in many aspects of normal physiology and neurobiology, as well as a central role in many of the addictive disease disorders. There are many areas of ongoing exciting research regarding this diverse system, including some new approaches to understanding both the brain localization and specific molecular mechanisms underlying behaviors. These include the use of small interfering RNA directed against the mu opioid receptor system, which when instilled in the substantia nigra-ventral tegmental area has been shown to result in both an ablation of heroin-induced locomotor activity and conditioned place preference. Further, a wide variety of human molecular genetics studies identify the mu opioid receptor as an important contributor to the vulnerability to develop an addiction, as well as a gene whose functional variants may alter physiological and pathological states in a wide variety of ways (e.g., Levran et al., 2008; Nielsen et al., 2008; Zhang et al., 2009). When research first began at The Rockefeller University in 1964 to attempt to develop a pharmacological treatment for opiate addiction, it was conceptualized that a therapeutic agent should be targeted to a specific site of action of opiate drugs like heroin and its active metabolites, primarily morphine (Dole et al., 1966). Further, the probable existence of specific “opiate (later opioid) receptors” was postulated, a concept that had been introduced by a few different groups, including that of the late Martin in Kentucky, and Collier and colleagues in England. Thus, in the selection of a pharmacological agent, criteria were set that it should be orally effective, long-acting, and targeted at what was then simply called “the opiate receptor,” and, of course, methadone was the agent chosen (Dole et al., 1966).

In 1970, Dole and Ingolia published the first paper that gave the scientific methodological approach that ultimately led to the discovery of specific opiate receptors, that is the approach of using stereoselective binding of an opiate-like compound, in this case, the binding to brain membranes of active l (or R) enantiomer of methadone and the inactive d (or more correctly S) enantiomer (Ingolia and Dole, 1970). The labeled enantiomers of methadone had too low specific activity to allow precise differentiation between binding of the active and inactive enantiomers in brain membranes (Ingolia and Dole, 1970). Goldstein built on this technology; using the Dole protocol, he also looked for stereoselective binding of a compound theoretically targeted at the mu opioid receptor. This work also suffered from the

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Corresponding author: Mary Jeanne Kreek, M.D., Laboratory of the Biology of Addictive Diseases, The Rockefeller University, 1230 York Avenue, Box 171, New York, NY 10065, USA, Tel: 212-327-8490, Fax: 212-327-8574, kreek@rockefeller.edu.

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lack of availability of a high enough specific radioactive ligand (Goldstein et al., 1971). Success was achieved in early 1973 when, in a very short period of time, three groups working independently, but all using the technique of stereoselective binding of a presumably opiate-directed agonist or antagonist (but using adequately high specific activity compounds to detect a significant difference) were reported by the groups of Snyder, Simon, and Terenius, respectively (Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1973). Over the next years, several groups using a variety of different chemical probes delineated the existence of not one single opiate receptor, but rather three classes, which were named mu, delta, and kappa.

Over the next several years, the genes for each of the three classes of endogenous opioids, proopiomelanocortin (POMC) gene (yielding beta-endorphin and many other biologically important peptides), the enkephalin gene, and the dynorphin gene were cloned. In 1992, two independent scientific groups, each using the technique of expression cloning and the same cell line, working in Los Angeles and Strasbourg, France, respectively, successfully cloned the gene of the delta opioid receptor (Evans et al., 1992; Kieffer et al., 1992). In rapid succession, the mu and kappa opioid receptors were subsequently cloned, first in rats and mice, and then in humans. In 1994, research began in collaboration with Lei Yu, who headed one of the two groups that first cloned the mu opioid receptor in rodents and in humans, to do extensive sequencing of this coding region of the gene (using the then-available laborious gel sequencing and running the gels both forward and in reverse directions for appropriate identification of any variants) (Bond et al., 1998).

In the early sequencing work (1995–1996) studying subjects from three different ethnic-cultural groups, two quite common variants were found, “the C17T” variant and “the A118G” variant (Bond et al., 1998). Functional studies were performed on the A118G variant because it was at a site of putative glycosylation, and, like the C17T variant, resulted in an amino acid change in the N-terminus of the mu opioid receptor. But in the case of the A118G variant, the change of amino acid was from asparagine to aspartic acid, and thus undoubtedly changing the physical properties of the receptor terminus (Bond et al., 1998). While functionality of the A118G variant was being studied, the existence of each of these two variants was reported with no studies of their possible altered functionality. The group of Goldman described the A118G variant but found in their populations that there was no association of this variant with alcoholism (Bergen et al., 1997). In another independent study, reported by the groups of Berrettini in the U.S. in collaboration with Hoehe in Germany, the C17T variant was identified in a primarily African-American population, but no association was identified with substance abuse (Berrettini et al., 1997). What was found in the next two years of study was that the receptor resulting from the A118G variant bound beta-endorphin, the longest (31 amino acids) and longest half-life of the endogenous opioids, three times more tightly than the prototype receptor, but binding of no other peptide nor natural or synthetic mu opioid receptor ligand was altered (Bond et al., 1998). It was further found that signal transduction through the G protein inwardly rectifying potassium channel was increased three-fold with the A118G variant-related receptor. Again, this happened only when beta-endorphin was the ligand. It has been demonstrated that this A118G variant alters objectively measured stress responsivity as well as response to pain and other physiological events modulated by the mu opioid receptor (Bond et al., 1998; LaForge et al., 2000; Kreek et al., 2005; Krosiak et al., 2007; Kreek and LaForge, 2007; Kreek, 2008). Further, as was predicted initially, it was found in a central Swedish population with only modest admixture that the A118G variant was very significantly associated with opiate addiction and also with alcoholism, with high attributable risk to this one variant in each of these complex disorders (Bart et al., 2004; Bart et al., 2005).

2. Commentary on Papers

With this background, it was especially exciting for us to have CPDD focus on some of the most recent advances in research on the endogenous opioid system.

The first paper herein, entitled “Opioid Pharmaceuticals and Addiction: The Issues and Research Directions Seeking Solutions” is by Chris Evans, Ph.D., of the University of California, Los Angeles, and one of the first two scientists to clone any opioid receptor. In his review article, Dr. Evans and his colleagues (Evans et al., 2010) discuss the effective, and sometimes less effective, use of opioid analgesics in the management of acute and chronic pain. He also discusses the phenomenon of tolerance and the related (though clinically relatively rare) phenomenon of opiate-induced hyperalgesia. He also discusses the clinical problems of both heroin addiction and the recent and still increasing enormous problem of illicit abuse of prescription opiates. Then he rigorously addresses the increasing information about the mechanisms involved in the perception of pain, including such diverse related topics as opioid receptor complexes and signal transduction mechanisms. He then discusses manipulation of the cell surface presence of mu opioid receptors. Dr. Evans clearly makes the point that an upregulation of mu receptor constitutive activity, such as he has documented can be manipulated to occur in dorsal root ganglion cells, increases the pain threshold and, at the same time, increases the sensitivity to morphine. He has shown that, in beta-arrestin 2 knock-out mice, there are increased levels of cell surface constitutive activity of mu receptors, and that some approach of increasing such activity might increase the ability to tolerate pain. Further, he suggests that use of an inverse mu receptor agonist might be useful in analgesia management, and further, that manipulation of entirely unrelated receptor systems could be conducted effectively to improve mu opioid receptor agonist-induced analgesia. Looking to the future and in attempting to reduce mu opioid agonist reward, while providing adequate analgesia, he suggests pursuing identified and yet-to-be-identified novel molecular and cellular targets as well as manipulating the location and constitutive activity of mu opioid receptors. His vision is to develop pharmacological agents to effect these kinds of cellular changes and actions at a systems level. However, this work is not yet at a point of looking at chronic versus acute effects of manipulation of these systems, nor is it close to the point of looking at what now is known to be profoundly important in human analgesia management, and in the prevention of reward, that is long-acting steady-state action at mu opioid receptors as opposed to the “on-off” effects, such as conferred by short-acting opiate narcotics used commonly in pain management and by the illicit drug of abuse, heroin.

The second paper is by Mark von Zastrow, MD, Ph.D., of the University of California, San Francisco, entitled “Regulation of Opioid Receptors by Endocytic Membrane Traffic: Mechanisms and Implications for Understanding Addictive Drug Action.” Dr. von Zastrow focused his paper (von Zastrow, 2010) on mechanisms involved in the regulation of opioid receptors by endocytic membrane trafficking and the possible implications of these phenomena. He particularly focused on endocytosis and sorting and ultimate fate of receptor-ligand complexes within cells after endocytosis. He discusses, on one hand, sorting within the cell to lysosomes, leading to degradation of receptors and thus prolonged downregulation, versus recycling rapidly to cell surface, then providing conserved activity. He further went on to present data on reduced antinociceptive tolerance in a mouse model with enhanced opioid receptor endocytosis and representation of receptors on cell surface, as well as data from a mouse model with enhanced tolerance and dependence due to reduced endocytosis which was, in part, work from other laboratories. Work from his laboratory has been conducted primarily in simplified model cell systems, but from this work, it has been possible to elucidate fine points of mechanisms involved in endocytosis and in determining the fate following endocytosis of opioid receptors, including mu and also other types.

Ultimately he plans to (and has now started to) conduct studies using transgenic mouse models to explore the impact of different mechanisms of endocytotic trafficking on opioid regulation.

The third paper is by Julie Blendy, Ph.D., of the University of Pennsylvania, Philadelphia, entitled "OPRM1 SNP (A118G): Involvement in Disease Development, Treatment Response, and Animal Models." Dr. Blendy, in a very creative way, made a transgenic mouse model that utilizes an analogous type of SNP to A118G at the 112 position and has gone on to do a variety of studies with this model, some aspects of which are covered in this paper. The paper herein (Mague and Blendy, 2010) contains a review of the role of the mu opioid receptor in specific aspects of normal physiology as well as diseases, with emphasis on addictive diseases, and further provides an overview of the many papers (some referred to above) which have been written on the function of the A118G variant, papers which have appeared over the ensuing twelve years since the elucidation of this functional variant of the mu opioid receptor (Bond et al., 1998). There is also a presentation of the rationale for creating a mouse which would have a variant similar to the A118G variant in humans. The authors provide the strategy which they used to create this mouse model, which would have a variant of the mu opioid receptor altering the same amino acids as the human variant, that is, which changes asparagine to aspartic acid, in this case at amino acid position 38 (and not 40, as in the human), also therefore in the N-terminus of the mu opioid receptor. These mice are A112G mice and utilized, among other approaches, the availability of bacterial artificial chromosome (BAC) vectors, one of the few times that these vectors have resulted in developing a mutant mouse that has a variant analogous to a human functional variant, as contrasted to the most common use, which has allowed elegant neuroanatomy studies of gene localization and pathways of trajectory thereof. A very brief discussion of some of the results of their paper (Mague et al., 2009) is presented herein. Similar to what both the group of Kreek and that of Sadee have found with the human A118G variant, they found that the A112G variant resulted in decreased mu opioid receptor mRNA and cell surface receptor expression (Kroslak et al., 2007; Mague et al., 2009; Zhang et al., 2005). However, unlike earlier A118G variant receptor studies, there was no difference in binding by the receptor resulting from the A112G variant of the beta-endorphin, as found in studies of the A118G variant receptor. There were similarly no differences in binding of other ligands, such as morphine, DAMGO or naloxone by either variant receptor. No studies of signal transduction were reported. Further, they found that mice with the 112G allele showed a modest elevation in locomotor activity following acute morphine administration and also failed to develop sensitization. These mice also seem to have reduced morphine antinociceptive responses. Of interest, mentioned briefly in this paper and presented in the PNAS paper, the group of Blendy found an intriguing sex-by-genotype interaction, with the female A112G animal mice not displaying a conditioned place preference for morphine nor any aversion to environments associated with naloxone-precipitated withdrawal. The availability of such mice is enormously exciting, and this group is to be commended for this major step forward.

The fourth paper is by Raphael Maldonado, Ph.D., of the Universidad Pompeu Fabra, Barcelona, entitled "The Endogenous Opioid System: A Common Substrate in Drug Addiction." One of the very important features of this review article is, of course, that the group of Maldonado has over many years conducted in direct collaboration most of the behavioral studies for the numerous and extremely important genetically modified mouse constructs of the group of Kieffer, which she has created, by recombinant technology, systematic deletions of the mu, delta and kappa opioid receptors, and then combinations of two of each of these and finally a mutant with all of these three genes deleted. Maldonado and colleagues (Trigo et al., 2010) include a brief discussion of some of his recent work on the role of the endogenous opioid system in nicotine reward. This discussion includes some of his work on the possible involvement of the opioid system in effects induced by nicotine

on anxiety-like behaviors. He also discusses the early work of Corrigan, who showed that mu opioid receptor antagonists that reduce cocaine self-administration do not alter nicotine self-administration (Corrigan and Coen, 1991). Other studies have shown attenuation of nicotine-induced antinociception and rewarding effects in mu opioid receptor knock-out mice. These studies are highlighted in this summary because of the recent focus by Maldonado working with Kieffer and others in a major collaborative effort on nicotine dependency.

3. FUTURE DIRECTIONS

The four presentations were all by leading scientists in our field, each working in various areas of neurobiology, that have profound implications for specific human addictive diseases as well as other areas. The four papers presented herein present some of the individual laboratories' more recent and exciting work, along with some articulated implications for the future. The two major research areas represented by these four papers could be classified as systems neurobiology (Blendy and Maldonado) and molecular cellular biology (Evans and von Zastrow). All four of these pieces of work are dependent upon and utilize modern techniques of molecular biology and, in one case, human molecular genetics as well. The two papers utilizing primarily the techniques of cell biology include very exciting new work on opioid receptor complexes, with emphasis on what peptides and systems are essential for effecting activity, and include the signal transduction systems, as well as systems involved in immediate binding and trafficking of the receptors. Further, this work of Evans addresses the extent to which ion channels versus other types of signal transduction may play a role, as well as the possible interactive role of the immune system, especially the chemokines and cytokines and adhesion molecules in opioid effects.

In the context of deep concern about the increasing abuse of drugs, Evans and colleagues suggest that basic research on reduction of development of tolerance, but also regulation of the reward pathways, to the extent possible, would be desirable in future designs of opioid agonist analgesic medications. In a historic context, it is extremely important to remind ourselves that the College on Problems of Drug Dependence was created in the 1920s, that is over ninety years ago, with the hope series of non-addicting pain medications. This remains a laudable goal. However, the idea that this is going to happen readily or quickly probably is not correct, and both Evans and von Zastrow are aware of this. Certainly, it has been shown repeatedly in scientific research that it is easier to create a genetically modified construct, a peptide or chemical entity, than it is to get any type of pharmaceutical based on exciting findings through the entire pipeline to yield the development of a new medication. Outside of the discussions of Evans and von Zastrow, but very important to consider, is that novel formulation of compounds (for instance, medications that are difficult to bite or chew, in contrast with the current friable medications, and slow onset, long duration medications, whether these are innate pharmacokinetic properties (such as pertain on different mechanisms with methadone and buprenorphine) or by very clever formulations, may, in fact, be able to achieve many of the goals to decrease reward and, yet, preserve and even increase effectiveness in pain management. Nevertheless, the basic work in these areas is very exciting for the future.

Similarly, the innovative work by von Zastrow, which overlaps some of the early and ongoing work with, and now by, Evans, but which focuses very specifically on endocytic membrane trafficking mechanisms using cell systems and also more recently in the intact central nervous system of whole animals, is extremely important. As van Zastrow points out, there are profound differences amongst the three classes of opiate receptors with respect to endocytosis and other differences dependent on size and location of the peptide molecules. Clearly, understanding the mechanisms that influence opioid receptor trafficking and

signaling at the cellular systems level are, as this author suggests, critical for developing new targets for pharmaceutical developments in the future, as well as potentially for selecting compounds that already exist, for instance, for use in the management of pain as opposed to the management of gastrointestinal hypermotility disorders (diarrhea) or the management of long-term opiate addiction. Though outside the focus of this review, some work done by von Zastrow and especially by some of his former trainees have already taught us that different opioid agonists currently used in therapeutics either for pain, for treatment of addiction, or for other indications may, in fact, have very different rates as well as mechanisms involved in endocytotic membrane trafficking of opiate receptors. The mechanisms by which these xenobiotics may have different profiles, as compared to peptides that are more easily created, for instance, with the suggested changes in regions of the cytoplasmic tail of the peptides, may be important for development of future novel pharmaceuticals and also for providing enhanced formulations and recommendations for use of existing agents.

In quite a different domain, the work of Maldonado and colleagues, as well as others, have made superb use of genetically modified animals in both behavioral studies and some mechanistic studies. These have provided critical information as to the types of roles of each of the different six components of the endogenous opioid system (mu, delta, and kappa opioid receptors and beta-endorphin, enkephalin, and dynorphin peptides) in mammalian physiology and pathophysiology. They have also conducted direct studies of the interaction of these opioid systems with other short- and long-acting neurotransmitter systems, such as glutamate and dopamine. The findings of a role, for instance, of the mu opioid receptors in several very different addictive diseases, have been of great importance. Also, it has been important to use these approaches to study both the impacts of chronic and persistent, as well as acute and subacute effects of drugs of abuse, on the endogenous opioid system. This kind of work has become more interactive with the exciting basic cellular and biological work of groups like Evans and Maldonado. It has already been appreciated that knocking out genes has many advantages but also certain limitations. It has been shown that conditional knock-outs may be very helpful by not changing animals during development. Further, there are techniques to enhance gene expression in specific brain regions, primarily through viral construct use, as well as to reduce gene expression selectively in specific brain regions (most recently by use of small interfering RNAs). These approaches may be very effective in further defining the role of specific genes in various stages of development, including adolescents and adults, and the role of not only specific genes and gene products, such as the opioid receptors and peptides, but also the precise localization of various sites of action.

The elegant work of Blendy and colleagues represents another whole domain, which will undoubtedly be a major domain in the future. With progress being made in human molecular genetics, and specifically the human molecular genetics of the addictive diseases and processes known to be involved in the addictive diseases, it is enormously exciting to see this work of Blendy model a well-defined functional polymorphism in the human mu opioid receptor (A118G), documented to be associated with both heroin abuse and alcoholism, and, possibly more importantly, to be involved in response to stress and stressors as objectively measured through regulation of the stress-responsive hypothalamic-pituitary-adrenal axis. The finding that non-human primates have a similar variant, but with a slightly different location has further emphasized the importance of this variant. The very insightful approach of the group of Blendy to place their mutant variant construct not the identical site as the human variant, but at a nearby site in the N-terminus that similarly changes the physical chemical properties of the mu opioid receptor by changing an arginine to an aspartic acid and at a site of putative glycosylation that their rodent model has some similar functional alterations as those which have now been documented to pertain in humans, is very important. Her group has already made some extremely interesting findings, of which

possibly the most novel and unexpected are the gender differences which have not been observed yet in the in the human studies of this functional variant. This type of molecular genetic approach, that is use of a “humanized” mouse (or rat) containing identical specific human variants or, in this case, an analogous functional gene variant, will be one which will invaluablely serve the field of systems neuroscience in the near future. It probably is not wise to make such time-consuming and expensive constructs unless a gene variant has been shown after identification to be functional, in either cellular-molecular or metabolic pathways, or preferably in whole humans. In the case of the A118G variant, there is ample cellular-molecular work, followed by and interspersed with excellent studies documenting the role of this variant in atypical objectively measured response to stressful challenges, and further, association with two specific addictive diseases which are known to affect stress responsive systems. Further, the generosity of Blendy and her group in sharing the model both intellectually and literally is exemplary. The exciting findings in her own laboratory may be anticipated to continue using this “knock-in” mouse model. The complexities of using this model are very much appreciated by this group. For example, they point out that although this variant has been shown by their studies to be a “loss-of-function” phenotype, such loss of function is not observed in all the effects, especially in human studies; rather, “gain of function” is present in some. This type of more thoughtful consideration is superb, and future work will undoubtedly involve further understanding of the mixtures of “gain” and “loss” of function, which can reside contemporaneously as a result of a functional variant of any gene of importance.

In the future, we will continue to need elegant studies of molecular-cellular mechanisms, such as those presented herein, as well as detailed studies of signal-transduction mechanisms, and specific interactions with other neurotransmitters and neuropeptides. Further, it is quite clear that we will need to translate such studies, to the extent possible, into systems neurobiology, both using inbred strains of mice and rats, as well as various genetically altered constructs. Human molecular genetics in many areas, including relationships with specific addictive diseases and possibly more importantly the mechanisms and pathways which we are elucidating that may be involved in addictive diseases, will ultimately yield ideas for further “knock-in” mouse models that can be used effectively in systems neurobiology. The findings from each of these areas may lead in the future not only to a further understanding of specific addictive diseases, but also to the identification of novel targets for therapeutics, either using heterocyclic compounds, or peptides, or by manipulation of gene expression directly or through epigenetic changes. However, it is essential that one recognize that prevention, early intervention, and enlightened treatment using approaches that we currently have, and further acceptance of such treatments, which will require much further destigmatization of the addictive diseases and the persons suffering from these diseases, will be essential in the immediate future.

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