HHS Public Access

Author manuscript

Addict Neurosci. Author manuscript; available in PMC 2022 June 10.

Published in final edited form as:

Addict Neurosci. 2022 June ; 2: . doi:10.1016/j.addicn.2022.100016.

Challenges and new opportunities for detecting endogenous opioid peptides in reward

Sineadh M. Conway[a,d,e,1], **Marwa O. Mikati**[a,b,c,d,e,1], **Ream Al-Hasani**[a,b,c,d,e,*]

[a]Department of Anesthesiology, Washington University, St Louis, MO, USA

[b]Department of Psychiatry, Washington University, St. Louis, MO, USA

[c]Division of Biology and Biomedical Sciences, Washington University, St. Louis, MO, USA

[d]Washington University Pain Center, Washington University, St. Louis, MO, USA

[e]Center for Clinical Pharmacology, University of Health Sciences and Pharmacy & Washington University School of Medicine, St. Louis, MO, USA

Abstract

The endogenous opioid peptide system, comprised of enkephalins, endorphins, dynorphins, and nociceptin, is a highly complex neurobiological system. Opioid peptides are derived from four precursor molecules and undergo several processing events yielding over 20 unique opioid peptides. This diversity together with low *in vivo* concentration and complex processing and release dynamics has challenged research into each peptide's unique function. Despite the subsequent challenges in detecting and quantifying opioid peptides *in vivo*, researchers have pioneered several techniques to directly or indirectly assay the roles of opioid peptides during behavioral manipulations. In this review, we describe the limitations of the traditional techniques used to study the role of endogenous opioid peptides in food and drug reward and bring focus to the wealth of new techniques to measure endogenous opioid peptides in reward processing.

Keywords

Opioid peptides; Enkephalin; Dynorphin; β-endorphin; Drug reward; Food reward

Introduction

While opiates, substances derived from opium, have been used for their analgesic and rewarding properties for centuries, the endogenous opioid system was not discovered until the 1970s [1–8]. The endogenous opioid peptide system, consisting of endorphins, enkephalins, dynorphins, and nociceptins, is a highly complex neurobiological system acting

*Corresponding author at: Center for Clinical Pharmacology, University of Health Sciences and Pharmacy & Washington University School of Medicine, Campus Box 8054, 660 South Euclid Avenue, St. Louis, MO 63110-1093, USA. al-hasanir@wustl.edu (R. Al-Hasani).

[1]These authors contributed equally to this work.

Declaration of Competing Interest

We (SMC, MOM, and RA) have no conflicts of interest or limitations on distribution of data or materials to disclose.

through four opioid receptors systems, μ, δ, κ-opioid receptors, (MOR, DOR, and KOR) and the nociceptin opioid peptide receptor (NOPR). Endogenous opioids are derived from four precursors: proenkephalin (PENK), proopiomelanocortin (POMC), prodynorphin (PDYN), and pronociceptin (PNOC) [9,10]. Interestingly, the precursor molecules do not produce a single ligand that is specific to each of the receptors. Rather, all four precursors consist of long amino acid sequences that can undergo a range of processing events to generate over 20 products of varying lengths from 5 to 30 amino acids [11]. We want to stress that nociceptin and its corresponding receptor are considered 'opioid-like' as the receptor exhibits a high degree of structural homology with the conventional opioid receptors [12] but does not carry the same overlapping structural and binding properties as the canonical opioid peptides [13–15]. Endorphins, enkephalins, and dynorphins all occur in multiple forms and should not be considered single neuropeptides. As shown in Table 1, there are many neuropeptide products that can be generated from the precursors. Furthermore, Table 1 also highlights that all endogenous opioid peptides (except for nociceptin) share a common N-terminal Tyrosine-Glycine-Glycine-Phenylalanine amino acid sequence, reviewed in [16]. All the prodynorphin products share this common N-terminal sequence with the addition of leucine, and many have a similar C-terminal residue conserved with dynorphin$A_{1-1}7$ [17]. This feature allows for short peptides such as leu-enkephalin to be generated from different precursors. Overall, the high similarity across endogenous opioid peptides introduces several challenges. Firstly, it is difficult to determine the origin of the shorter peptide forms as they may be derived from the post-translational cleavage of multiple precursors. Secondly, developing methods that distinguish between the unique peptides is extremely challenging.

Based on their highly similar amino acid sequences, endogenous opioid peptides derived from PDYN, PENK, and POMC can interact with MOR, DOR and KOR with different affinities [18]. The peptide and receptor interactions are also dictated by the expression patterns of the two systems in different regions of the brain. For example, it has been shown that MOR expressing cells in the network of intercalated cells (ITC) in the amygdala are surrounded by cells expressing pDYN mRNA. It was also shown that the same region showed heightened DYN immunoreactivity, suggesting that peptides derived from pDYN are likely activating the MOR receptors in the region [19]. For an excellent and thorough review of endogenous opioid peptide interactions with the different opioid receptors, see [19].

Upon release into the extracellular space, opioid peptides may be rapidly cleaved either for degradation or to alter receptor binding activity, which may lead to enhanced binding affinity for a particular receptor [21,22]. For example, the proenkephalin peptide product BAM18, which contains the N-terminal met-enkephalin sequence, binds to MOR, DOR, and KOR, but shows a slightly higher affinity for MOR and similar affinities for DOR and KOR. Processing of this peptide to met-enkephalin leads to a reduction in its affinity for MOR and KOR but an enhanced affinity for DOR [20]. The POMC product ß-endorphin binds both MOR and DOR, and while dynorphins bind with the highest affinity for KOR, they can also bind to MOR and DOR within physiological ranges [18,23]. The NOPR/nociceptin system is the only exception where nociceptin binds specifically to NOPR and shows extremely low affinity to the other opioid receptors [24–26].

Due to the complex nature of the endogenous opioid peptide system, it has been challenging to develop tools for their reliable detection *in vivo*. Here, we present both the limitations of traditional techniques and more recent advances in detecting endogenous opioid peptides, specifically within the context of reward. There are many excellent reviews on opioid systems in food and drug reward and we refer to them here for additional reading [27–30]. While the wealth of literature on opioid peptides provides insight into their potential roles in food and drug reward, it can be appreciated that there are still many unknowns with respect to the function of each peptide in the different aspects of reward processing. Novel techniques have started addressing some of the existing challenges, and we posit that they can be utilized to answer long-standing questions about opioid peptide function. For ease of reading, a glossary of terms used in the review can be referenced in Table 2.

Traditional opioid peptide experimentation and their challenges

Our current understanding of the role of endogenous opioid peptides in reward and addiction is limited by the complexity of this neuropeptide system and experimental techniques at our disposal. The studies using administration of opiates, stabilized peptides, and selective opioid receptor agonists and antagonists suggest a modulatory role for ß-endorphins, enkephalins, dynorphin, and nociceptin in different aspects of reward behavior. A major area of study of opioid peptides is their role in modulating drug reward. This is unsurprising since the discovery of opioid peptides was prompted by the investigation of endogenous analogues to morphine. Many of the studies that inferred a role for each peptide class relied on radioimmunoassays or the measurement of transcript levels of peptide precursor genes. Fig. 1 summarizes the brain regions in which changes in peptide levels in response to drug exposure have been reported. However, the endogenous peptide promiscuity at the different receptors makes it challenging to conclude specific roles for each class of peptide using these techniques. Another major area of focus on opioid peptides is their role in food reward. Much of what we know regarding the role of the opioid system in food reward has relied on opioid receptor pharmacology (Fig. 2) [31–53]. However, blocking one specific receptor does not necessarily eliminate action of an endogenous opioid peptide [22,54]. The use of agonists and antagonists to probe the role of the opioid system in behavior provides valuable information on the target receptor rather than which specific endogenous opioid peptide may be involved. Here, we highlight the canonical methods by which opioid peptide function is studied and how this research informs our understanding of endogenous opioid peptides in food and drug reward. We also clarify the gaps in our knowledge based on the traditional experimental tools available to study the endogenous opioid systems and present a role for novel techniques.

Opiate and exogenous peptide administration—Much of what we know about how endogenous opioids regulate behavior is through systemic and central administration of opiates and exogenous administration of opioid peptides. One of the first studies implicating the opioid system in feeding were observations of increased food intake after repeated injections of morphine [55]. While this study implicates opioid activity at MOR in feeding, it does not provide insight into which endogenous opioid peptide is responsible for this behavioral effect. Thus, instances of exogenous peptides administration are important. A potential role for the endogenous opioid system in food intake was initially

demonstrated by Grandison and Guidotti who showed that administration of ß-endorphin directly into the ventromedial hypothalamus stimulated food intake [56]. Others have shown that intraventricular administration of dynorphin [57–61], and intracerebral microinjections of dynorphin in the ventromedial hypothalamus (VMH), paraventricular nucleus (PVN), ventral tegmental area (VTA), and the nucleus accumbens (NAc) [57,62,63] increases food intake. This technique has also been used in the drug reward field. For example, it has been shown that pretreatment with nociceptin abolishes both morphine and cocaine place preference. The results of these studies have aided in the conclusion that nociception counteracts the acute rewarding effects of drugs [64]. Still, exogenous peptide administration may not accurately reflect the normal function of the endogenously released peptides. On one hand, the concentration of injected peptide is usually much higher than expected under endogenous conditions. Additionally, there is far less spatial and temporal regulation of exogenously administered peptides compared with endogenous release. Furthermore, the injected exogenous peptides are unstable and likely cleaved into shorter forms upon administration thereby complicating interpretation. To overcome this, many have tested the stabilized form of the peptides in animal models to probe their physiologic and behavioral effects. Dynorphin $A_{1–13}$ and Dynorphin $A_{1–17}$ have both been shown to stimulate operant-controlled feeding in pigs whereas the shorter dynorphin fragments did not [65]. Such biochemical modifications often lead to their own complications, as biochemical modifications often alter the relative affinities of the peptides at opioid receptors. For example, DAMGO is a modified enkephalin molecule that is very stable in *ex vivo* and *in vivo* conditions, but unlike endogenous enkephalin, it is very selective for MOR [66]. Though DAMGO provides a useful way to study MOR signaling, it may not mimic endogenous enkephalin action. Overall, careful consideration should be made when making interpretations of endogenous opioid peptide function from exogenous administration of opioid peptides and opiates.

Opioid receptor agonists/antagonist pharmacology—In the same way that exogenously administered opiates often lead to complications in the interpretation of results, the use of selective opioid receptor agonists and antagonists (Table 3) in the study of endogenous opioid activity can be convoluted. It can be difficult to discern which opioid peptide is responsible for receptor pharmacology effects because nearly all endogenous opioid peptides interact with more than one opioid receptor. For example the role of ß-endorphins in food reward has been widely explored using pharmacological studies by targeting MOR [67]. These studies conclude that ß-endorphins likely act via MOR to stimulate feeding (including saccharin, salt, ethanol, and highly palatable foods) [31–35,37,38,40,41,44,56,68]. However, we know that enkephalins also act as a MOR ligand, so we must be cautious when interpreting pharmacological data as it relates to opioid peptide activity. Furthermore, effects observed with antagonist treatment may be due to the block of ongoing actions of the peptides under baseline conditions, or it may be a result of the action of peptides released specifically in response to a particular behavioral stimulus. The non-selective opioid antagonist, naloxone was a major tool in early research investigating the role of the opioid system in feeding behavior. Early research showed that using naloxone to block opioid receptors significantly decreased food intake in food deprived [69] and non-deprived rats and mice [70,71], suggesting a role for endogenous opioid peptides in feeding.

However, these data do not provide any information about which discrete endogenous opioid peptide may mediate these effects. Arguably more important, pharmacological studies do not provide information regarding which peptides are released. Rather, they provide information of the target receptor at which activity is altered. To complicate things further, when considering the possibility of heterodimerization, an antagonist at one receptor may enhance binding or signaling at a different receptor [72–74], making it possible for the antagonist to function as a positive allosteric modulator in a relevant circuit. Thus, careful consideration must be taken when interpreting results from experiments utilizing pharmacological agonists and antagonists in the study of endogenous opioid signaling.

Peptidase blockade—Another pharmacological method used to investigate the role of opioid peptide signaling in neural activity and behavioral processes has been the blockade of peptidases that break down endogenous opioid peptides. With this approach, the extracellular concentration of opioid peptides is elevated via inhibition of their degradation. However, peptidases that degrade endogenous opioid peptides also break down many non-opioid peptides. Thus, blocking these enzymes does not necessarily result in a behavior exclusively driven by opioids. Some of these enzymes are often described as ‘enkephalinases’ suggesting that these enzymes are selective for the degradation of enkephalin. However, they are responsible for the biosynthesis and cleavage of many neuropeptides. For example, enkephalin convertase was renamed carboxypeptidases E [75] and enkephalinase has since been renamed neprilysin [76]. Despite these more recent publications, these enzymes are often still referred to as ‘enkephalinases’.

Measurement of mRNA expression and peptide levels—Other studies using mRNA expression to quantify opioid peptides based on the presence of their opioid precursors provide some evidence for their role in reward and addiction, but they provide no insight on the contribution of specific opioid peptides or where peptide release occurs. For example, the role of dynorphin in drug reward diverges from enkephalins, endorphins, and nociceptins. It is thought that dynorphins play more of a regulatory role after chronic exposure and during withdrawal from drugs of abuse. This conclusion has relied on a number of studies that showed increases in PDYN mRNA transcript levels in the NAc during abstinence from morphine and methamphetamine [77–79]. These data support the conclusion proposed by Wee and Koob in [80] that dynorphin counteracts the rewarding effects of drugs and that dynorphin is especially active during drug abstinence. Despite the utility of measuring mRNA expression, a simple precursor/product relationship does not exist within the opioid peptide system, and multiple active forms can be generated depending on the degree of proteolytic processing and post-translational processing events. Thus, quantification of mRNA expression does not necessarily provide information on which peptide is produced. Overall, this level of analysis provides no direct detection of endogenous opioid peptides in a given experiment. Despite these challenges, mRNA quantification has been supplemented by direct measurement of peptide levels which has helped determine the identity of the peptide in question.

Genetically modified rodent models—The generation of and experimentation with genetically modified mice has provided a vast amount of evidence implicating the

endogenous opioid system in reward and addiction. However, because each precursor can generate several different peptide products, results from these types of studies carry some limitations as they do not provide information about the function of individual peptides. For example, in PDYN-KO mice, other PDYN-derived peptides and their metabolites are depleted. These data provide information on the collective function of all peptides produced from the PDYN precursor that is eliminated. Thus, we cannot exclude that some of the observed effects are only partially mediated by dynorphin and KOR. While most of these peptides bind to KOR, there are exceptions. For example, leumorphin has been shown to generate effects independent of KOR binding [81].

Additionally, compensatory changes in these KO animal models may obscure the peptides' function or falsely implicate the system. For instance, the role of both ß-endorphins and enkephalins in feeding have also been explored using transgenic KO mice. Using a progressive ratio (PR) task in KO mice of enkephalins, ß-endorphins, or both suggested that both peptide classes contribute to the incentive motivation to acquire food reinforcers [43,82]. Furthermore, KO of enkephalin, ß-endorphin, or both only decreased operant responding in a non-food deprived state, suggesting that these opioids play a role in mediating the hedonics of feeding rather than energy homeostasis. However, follow-up studies utilizing the same methodology compared the role of enkephalins and ß-endorphins in mice of different genetic backgrounds. In these studies, only mice lacking enkephalin, regardless of sex and background strain, showed a decrease in motivation to bar press for palatable food reinforcers in the PR task. Overall, the authors conclude that enkephalins may play a larger role than ß-endorphins in motivation for food reward in PR than ß-endorphin [83]. It is important to highlight the lack of enkephalin specificity in these studies. The precise role of both leu- and met-enkephalin in food reward and motivated feeding remains elusive. Additionally, the findings were based on comparisons of KO mice of two different genetic backgrounds and highlight the complexity and careful consideration needed when studying the role of endogenous opioid peptides when interpreting results for KO animal studies. Similar studies have been conducted for the investigation of the effects of genetic deletion of peptide precursor genes on drug reward. It was shown that ß-endorphin deficient mice showed more robust conditioned place preference to morphine as shown by an increase in the time spent in the drug-paired chamber than enkephalin-deficient and wildtype mice [84]. This suggested that ß-endorphin may play a role in the regulation of opioid-induced reward. Though these studies yield valuable information and insight into the role of a specific peptide family, in this example it is not possible to determine which opioid peptide is responsible for the modulation of drug reward. It also opens up the possibility of other regulatory functions that the precursor gene may have especially in the periphery. Finally, findings from knockout studies could have misleading conclusions on the roles of the peptides unless corroborated with other techniques.

Current advances in opioid peptide investigation

In the sections to follow, we highlight the current techniques and advances that have been developed for the investigation of endogenous opioid peptides and peptidergic neurons. Still, we want to stress that the traditional techniques discussed above have provided a vital foundation for understanding the endogenous opioid peptide systems and have been

essential in guiding the development of these novel tools. Fig. 3 provides a summary of the advantages and major considerations of each technique. It is important to note that we select examples from the literature for the use of the novel techniques and do not provide exhaustive detail of all instances when these techniques were used. Additionally, it should be noted that there are cost considerations and personnel training that apply to each of these techniques.

Photostimulation of peptidergic neurons—To address the limitations detailed in the previous section, several techniques to investigate the role of opioid peptides have been developed. One such technique combines the use of transgenic mouse lines that express cre recombinase under the control of opioid peptide precursor gene promoters with optogenetic manipulations. A light sensitive opsin is delivered using a viral injection and transfects peptide expressing cells in a region-specific manner. This technique has successfully been used by several studies to investigate the role of opioid peptide expressing neurons. Using the PDYN-cre mouse line, it was shown that dynorphin expressing neurons perform distinct roles in reward and aversion in the subregions of the NAc shell where dorsal neurons were involved in reward while ventral neurons were involved in aversion [85]. This technique has also been used for the investigation of the role of enkephalins in the regulation of appetitive behavior using PENK-Cre mice. Photostimulation of PENK expressing neurons was employed to learn about enkephalinergic projections from the Dorsal Raphe Nucleus to the medial NAc shell and their role in appetitive behaviors [86]. Although these techniques offer both regional and cell-type specificity, the activation of opioid peptide precursor gene expressing neurons often leads to the release of multiple types of opioid peptides, some of which may be uncharacterized. This can often confound the interpretations of these studies. For example, the activation of PENK expressing neurons leads to the release of both met- and leu-enkephalin, so the downstream effects may not be attributed to one of the two peptides. Similarly, it has been shown that the photostimulation of PDYN-expressing neurons in the dorsal NAc shell leads to the co-release of dynorphin, met-enkephalin, and leu-enkephalin while the photostimulation of the ventral NAc shell leads to the co-release of dynorphin and met-enkephalin [87]. In this study, the authors challenge the assumption that leu-enkephalin is exclusively derived from PENK neurons due to the co-release of leu-enkephalin during PDYN photostimulation. Importantly, using optogenetic manipulation of peptide precursor expressing neurons offers insight about release properties and enhances spatiotemporal resolution.

Microdialysis coupled with LC-MS—As mentioned above, specificity remains a concern when employing tools that rely on the opioid peptide precursor genes such as optogenetics in transgenic mouse lines. To address this, several groups have piloted sensitive techniques for the detection of opioid peptide release. *In vivo* microdialysis has been widely used, and it has previously been coupled with radioimmunoassays or enzyme-linked immunosorbent assay (ELISA) that rely on the selectivity of antibodies to distinguish between the different opioid peptides. Despite the issue of antibody selectivity, this pioneering work laid the foundation for endogenous opioid peptide detection using microdialysis. The early work systematically investigated different types of custom microdialysis membranes and flow rates to optimize peptide recovery and detection [88].

In 2005, the first report of the use of microdialysis coupled with liquid chromatography/mass spectrometry for the detection of enkephalins was introduced, without the use of antibody-based techniques. The study demonstrated the ability to measure real-time changes in met- and leu-enkephalin release in the rat striatum at the picoMolar (pM) range [89]. The same method was slightly modified and used for the detection of met-enkephalin and leu-enkephalin in the globus pallidus of rats [90]. Furthermore, this technique has been developed and used to measure dynorphin$_{1–8}$ in addition to met- and leu-enkephalin in the dorsal striatum in rats and showed that met- and leu-enkephalin act as signals to eat [91]. More recently, LC-MS detection was further improved through the use of isotopically labeled internal standards of the opioid peptides to aid in more accurate quantification of met- and leu-enkephalin in the rat hippocampus [92]. It has also been demonstrated that it is possible to couple photostimulation with microdialysis and nano-LC-MS for the detection of met-enkephalin, leu-enkephalin, and dynorphin$_{1–8}$ simultaneously in the mouse NAc shell in the pM range [87]. Despite the accuracy of quantification this technique could be further improved with increased temporal resolution. Improved temporal resolution would specifically aid in the simultaneous measurement of both neuropeptides and neurotransmitters from the same samples. An important consideration is how neuropeptides differ from classical neurotransmitters and how that can impact sample collection time. For example, neuropeptides are released at much smaller concentrations compared to neurotransmitters, they have slower dynamics, and are packaged in dense core vesicles rather than synaptic vesicles [93], which together result in longer sample collection times, less than ideal for faster neurotransmitters.

Voltammetry—Another promising advancement in detecting endogenous opioid peptide release *in vivo* utilizes the electrochemical technique of voltammetry. More commonly used for the detection of monoamines like dopamine [94–100], modified multiple-scan-rate voltammetry has been used for real-time detection of enkephalins in adrenal tissue and in the dorsal striatum of anesthetized and free moving rats [101,102]. By applying an electrical potential to a microelectrode, electroactive components of opioid peptides like tyrosine and methionine oxidize at unique potentials that are detected as current. This electrochemical technique is an invaluable advancement in opioid peptide detection because it improves the sampling rates to a subsecond temporal resolution, which is especially important in the monitoring of endogenous opioid peptides that are rapidly cleaved and/or degraded upon release. Furthermore, the small carbon fiber electrodes used in voltammetry generate little to no gliosis, enabling detection much closer to release sites than previously possible with microdialysis probes [101,102]. While these studies have been able to distinguish met- and leu-enkephalin, accurate identification of a specific peptide may be less certain compared with post-microdialysis sample processing. Electrochemical techniques like these alongside microdialysis provide faster and slower indices of endogenous opioid peptide release dynamics, respectively. Furthermore, both may be employed beyond preclinical studies and may prove to be useful in the analysis of clinical samples to further our understanding of endogenous opioid peptide profiles in different patient populations.

Using both microdialysis and electrochemical techniques, the dorsomedial striatum has been identified as a key sight of endogenous enkephalin release during palatable food

consumption. Increases in enkephalin release are seen in the dorsomedial striatum when rats consume chocolate [91], and met-enkephalin specifically, has been electrochemically detected in the dorsomedial striatum of rats consuming sweet palatable food [101]. Future studies like these will provide greater insight into the precise opioid peptide signaling dynamics governing reward behaviors.

Fluorescent sensors—The ability to study opioid peptide dynamics *in vivo* in real time is essential to determine their role in behavioral processes. The development of fluorescent sensor technology has enabled the study of peptidergic neuronal activity and peptide release in real-time. Here, we focus on the sensors developed for the study of opioid peptides and peptide-expressing neurons, however, several fluorescent sensors have also been developed for opioid receptor localization and to enable microscopic evaluation of endogenous receptors [103]. The use of fluorescent calcium indicators has allowed for real-time investigation of neuronal cell activity in a cell-type specific manner. The genetically encoded GCaMP was first introduced as a calcium indicator in 2001 [104]. The sensor functions through a calmodulin protein with a green fluorescent protein tag that binds Ca^{2+}molecules. Binding to calcium induces a conformational change in the calmodulin protein which consequently causes a conformational change in the GFP tag, thereby altering its fluorescence intensity. To measure the fluorescence intensity, the sensor is packaged in a virus that transfects the cell population of interest, and a fiber photometry probe is surgically implanted above the site of viral injection. Conveniently, this approach allows for the measurement of cell activity in any brain region regardless of depth. It has since been used for the investigation of opioid peptide precursor expressing neurons. For instance, a group used the PDYN Cre transgenic line to investigate PDYN expressing neurons in the parabrachial nucleus and their role in ingestive behavior. They showed that these neurons gate feeding upon receiving mechanosensory inputs of food intake behaviors from the digestive tract by monitoring their calcium activity using fiber photometry [105]. Although this technique does not allow for the direct measurement of opioid peptide levels, the calcium signal acts as a proxy measure of neuronal activation and potential release of peptides.

This technology has since been expanded to include sensors for neurotransmitters such as dopamine [106,107], norepinephrine [108], and ligands that bind the KOR [109]. Recently a study used kLight, a fluorescent sensor composed of an inert form of KOR and a green fluorescent protein tag, to measure kLight activity as a proxy of dynorphin release in the PFC during precipitated morphine withdrawal [110]. The sensorkLight functions similarly to GCaMP, whereby ligand binding to the inert KOR induces a conformational change that ultimately alters fluorescence intensity. The authors concluded that the increase in kLight activity as measured by fiber photometry correlated with withdrawal-induced dynorphin release. As a positive control for the sensor's activity, they also showed that U50–488H (a KOR agonist) shows an increase in kLight activity.

This study offers a tremendous advance in the real-time investigation of opioid peptide release and the ability to couple it with behavioral assays. However, in line with our earlier discussions of the lack of opioid peptide selectivity to one receptor, it is possible that the kLight signal occurs due to its activation by other endogenous opioid peptides and not

exclusively dynorphin. This challenge may be addressed if fluorescent sensor experiments are coupled with *in vivo* detection techniques such as microdialysis coupled with LC-MS. Another group has also introduced a fluorescent opioid sensor, MSPOTIT2, which has been validated to increase in fluorescence upon the application of leu-enkephalin and β-endorphin in HEK cell culture [111]. The sensor also showed increased fluorescence in rat cortical neurons upon fentanyl application *in vitro*. Therefore, using fluorescent indicators addresses the issue of temporal resolution seen in other techniques with the caveat that they may not be selective for a certain peptide. However, coupled with other detection techniques, the findings from studies using the fluorescent indicators could be further validated.

Positron emission tomography—The methods outlined so far have been used in preclinical models. The investigation of endogenous opioid peptide release using Positron Emission Tomography (PET) offers a technique that can be used in humans. PET allows for the indirect measurement of endogenous opioid peptides by measuring changes in opioid receptor occupancy using radiolabeled agonists [112]. Similar to the issue outlined in using fluorescent sensors, the use of a receptor-based technique does not provide information about the specifics of the opioid peptide being released. It provides a general idea of endogenous opioid peptides that bind to the receptor which is targeted by the radiolabeled ligand. This method has been used in humans to investigate the effects of a genetic variant on the release of opioid peptides following alcohol use. The study shows that individuals carrying an allele associated with alcohol use disorder have greater release of opioid peptides in the right NAc following alcohol consumption and decreased release in the medial orbitofrontal cortex. The measurements were done using radiolabeled carfentanil and changes in its binding to MORs were measured [113]. In another study, it was shown that a 3-day cocaine binge regimen led to a decrease in radiolabeled ligand binding to KORs in the striatum. The authors concluded that the result may be due to an increase in the release of dynorphins in the striatum following cocaine use [114]. In another human study using PET imaging, it was shown that participants who suffered from cocaine use disorder had elevated levels of NOPR particularly in the midbrain, ventral striatum, and in the cerebellum. This effect was shown by using a radiolabeled Nociceptin to determine changes in binding to NOPR [115]. In conclusion, this technique offers the potential to translate investigations of opioid peptide changes from preclinical models to humans.

Summary

In this review, we present the technical approaches, both old and new, used to study the role of endogenous opioid peptides in reward. Traditional techniques have provided foundational knowledge about the roles for the four classes of opioid peptides (enkephalins, dynorphins, endorphins, and nociceptins) in food and drug reward. However, little is known about the distinct roles of the opioid peptide fragments. Additionally, we found it particularly challenging to describe studies that inferred peptide function from receptor manipulations or peptide precursor gene expression. Thus, we provide schematics to summarize the existing literature as it relates to critical brain regions for opioid peptide action in drug and food reward (Figs. 1 and 2). Critical assessment of that literature led to the conclusion that many studies equate receptor activation with endogenous opioid peptide release. We highlight the limitations of these studies when the inferences specified a role for a unique peptide

without following the study with direct measurements of the peptide in question. However, as techniques improved, the early findings have since been coupled with direct measurement of opioid peptide release at different temporal resolutions. This has allowed for specific measurement of opioid peptide subtypes. To address the gaps in our knowledge, we shed light on the wealth of new techniques that offer a variety of spatial and temporal resolution for the study of opioid peptide release (Fig. 3). The new techniques offer the groundwork for future investigations of specific peptides in reward processes.

Funding

This work was supported by the National Institutes of Health R00 DA038725 (RA), R21DA048650 (RA), F32DA053093-01A1 (SC), NARSAD Young Investigator Grant from the Brain and Behavior Research Foundation, grant no. 28,243 (RA), Cognitive, Computational, and Systems Neuroscience Fellowship from The McDonnell Center of Systems Neuroscience at Washington University School of Medicine (MM).

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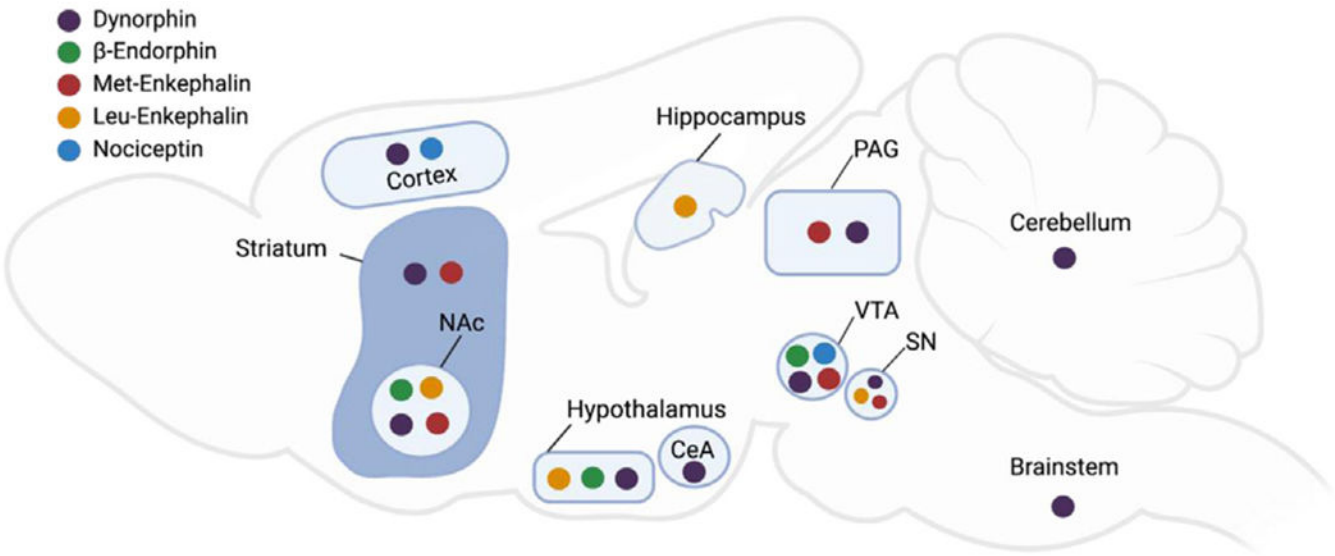

Fig. 1. Endogenous opioid peptide expression and levels following exposure to drugs of abuse.

Drug-induced changes in opioid peptide levels have been measured in multiple brain regions. A region of focus is the Nucleus Accumbens and striatum due to their role in reward processing. NAc = nucleus accumbens, CeA = Central Amygdala, VTA = ventral tegmental area, SN = substantia nigra, PAG = Periaqueductal Gray. Figure created using Biorender.com.

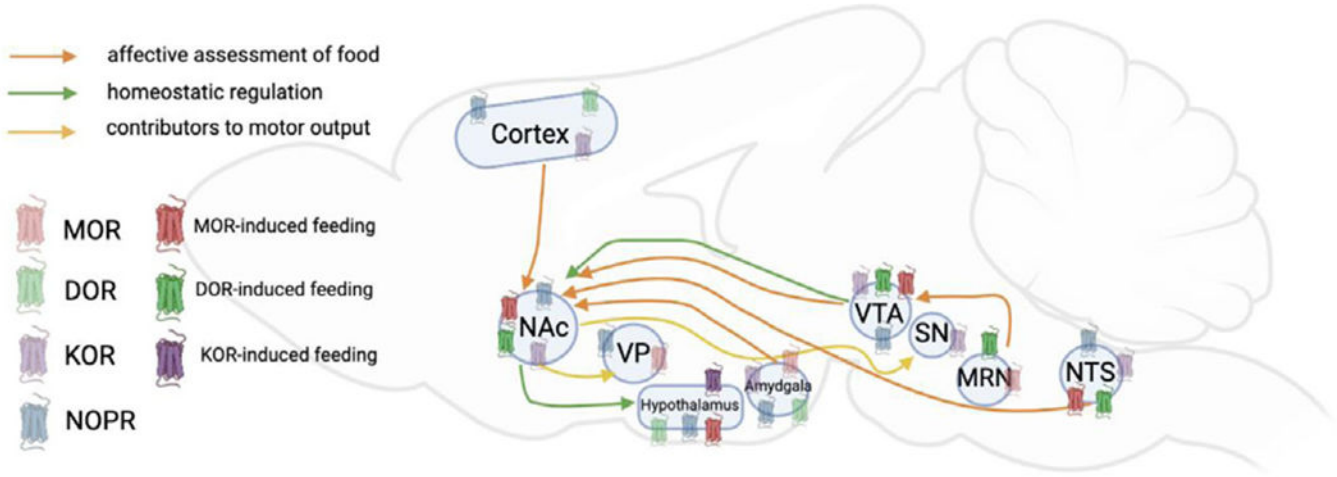

Fig. 2. Brain regions implicated in opioid receptor-mediated feeding behaviors.

The NAc acts as a central integrator of affective assessments of food (orange arrows), homeostatic regulation of feeding behavior (green arrow), and sends efferent projections to brain regions that contribute to motor output (yellow arrows), all of which show diverse opioid receptor expression (shown in faded color). Region-specific, opioid-induced increases in feeding have been shown using opioid receptor specific agonists (shown in full color). NAc = nucleus accumbens, VP = ventral pallidum, VTA = ventral tegmental area, SN = substantia nigra, MRN = medial raphe nucleus, NTS = nucleus tractus solitarius. Figure created using Biorender.com.

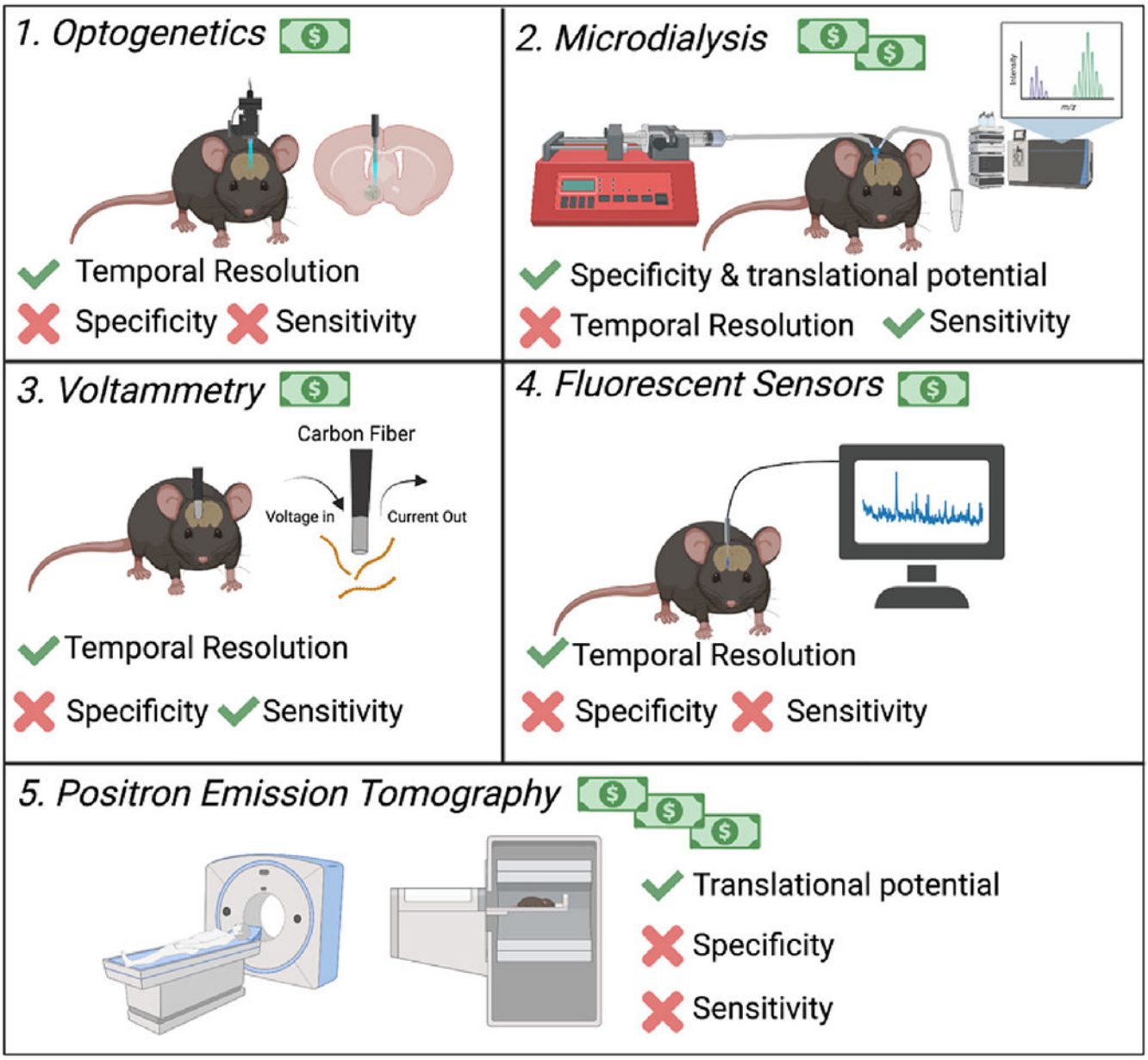

Fig. 3. Detection and experimentation techniques for endogenous opioid peptides and peptidergic neuronal activity.

There are several new techniques for the study of opioid peptide dynamics such as (1) optogenetics which offers good temporal resolution but lacks specificity to one peptide, (2) microdialysis paired with LC/MS which allows the sampling of regional interstitial fluid and offers high sensitivity with limited temporal resolution, (3) voltammetry which offers high temporal resolution with limited specificity to a unique peptide, (4) fluorescent sensors which allow the real-time investigation of peptidergic neurons and peptide release with high temporal resolution and limited specificity, and (5) Positron Emission Tomography which uses proxy measures of radiolabeled ligand displacement for the study of endogenous peptide release offers translational potential with limited specificity. The dollar icon was

used to indicate general costs of each technique relative to the other techniques in the figure. Sensitivity here is defined as the ability to detect concentrations of opioid peptides. Adapted from [118]. Figure created using Biorender.com.

Table 1

Endogenous opioid peptides.

Opioid peptide amino acid sequences derived from proopiomelanocortin, proenkephalin, and prodynorphin share a common N-terminal leu-enkephalin sequence (bolded) and C-terminal residues conserved with dynorphin A 1–17 (italicized). Amino acid sequences are depicted using the letter amino acid notation. Additional amino acids are indicated in plus sign where only a partial sequence is shown.

Peptide	Amino Acid Sequence
Proopiomelanocortin products	
ß-endorphin$_{1–26}$	**YGGF**MTSEKSQTPLVTLFKNAIIKNA
ß-endorphin$_{1–7}$	**YGGF**MTSEKSQTPLVTLFKNAIIKNAY
ß-endorphin$_{1–31}$	**YGGF**MTSEKSQTPLVTLFKNALLKNAYKKGQ
Proenkephalin products	
Leu-enkephalin	**YGGFL**
Met-enkephalin	**YGGF**M
Met-enkephalin-Arg-Phe	**YGGF**MRF
Met-enkephalin-Arg-Gly-Leu	**YGGF**MRGL
BAM 12	**YGGF**MRRVGRPEWW
BAM 18	**YGGF**MRRVGRPEWW +4
BAM 20	**YGGF**MRRVGRPEWW +6
BAM 22	**YGGF**MRRVGRPEWW +8
Peptide E	**YGGF**MRRVGRPEWW +10
Prodynorphin products	
Leu-enkephalin	**YGGFL**
Dynorphin A$_{1–7}$	**YGGFL** *RR*
Dynorphin A$_{1–8}$	**YGGFL** *RRI*
Dynorphin A$_{1–9}$	**YGGFL** *RRIR*
Dynorphin A$_{1–13}$	**YGGFL** *RRIRPKLK*
Dynorphin A$_{1–17}$	**YGGFL** *RRIRPKLKWDNQ*
Big Dynorphin	**YGGFL***RRIRPKLKWDNQ*KRYGGFLRRQFKVVT
Dynorphin B$_{1–13}$	**YGGFL***RR*QFKVVT
Leu-morphin	**YGGFL***RR*QFKVVTR +15
a-Neoendorphin	**YGGFL***R*KYPK
ß-Neoendorphin	**YGGFL***R*KYP
Pronociceptin products	
Nociceptin	FGGFTGARKSARKLANG
Nocistatin	MPRVRSLVQVRDAEPGADAEPGADAE +15
Orphanin FQ2	FSEFMRQYLVLSMQSSQ

A = Alanine, *R* = Arginine, *N* = Asparagine, *D* = Aspartic acid, C = Cysteine, *E* = glutamic acid, *Q* = Glutamine, *G* = glycine, *H* = Histidine, *I* = Isoleycine, *L* = leucine, *K* = lysine, *M* = methionine, *F* = Phenylalanine, *P* = Proline, *S* = Serine, *T* = threonine, *W* = Tryptophan, *Y* = Tyrosine, *V* = Valine.

Table 2

Glossary of terms.

Term	Definition
Affect	Collective reference to an emotional feeling, can be positive or negative
Agonist	a molecule that activates a receptor upon binding to it.
Antagonist	a molecule that inhibits or interferes with the activity of a receptor upon binding to it.
Appetitive feeding	an active searching process to consume food, indicative of desire for food
Conditioned Place Preference	a classical conditioning behavioral paradigm used to assess the rewarding properties of a drug.
Conditioned Place Aversion	a classical conditioning behavioral paradigm to assess the aversive properties of a drug.
Dysphoria	A negative affective state characterized by generalized discontent
Feeding hedonics	consumption of food just for pleasure. In this condition, a subject will eat when not in a state of energy depletion but rather food is consumed uniquely for its gustatory rewarding properties.
Feeding for regulation of energy homeostasis	consumption of food to alleviate a state of energy depletion.
Food Reinforcer	food that is used to increase desired behavior.
Hedonia	Pleasure, enjoyment, and satisfaction; absence of distress
Hyperphagia	increased appetite for food.
Incentive motivation	Behavior guided by a desire for reinforcement
Incentive value	the perceived value of a motivating stimulus or condition.
Intracerebroventricular injection	A type of injection that requires stereotaxic surgery to either directly inject a drug or place a cannula in the cerebral ventricles to deliver drugs bypassing the blood brain barrier.
Liking	The actual pleasurable impact of reward consumption, separate and distinguishable from wanting
Motivation	Process that initiates, guides, and maintains goal-oriented behaviors
Naloxone	Non-selective opioid receptor antagonist
Opiate	a substance derived from the opium poppy plant.
Opioid	natural or synthetic substances that act on opioid receptors.
Orexigenic	appetite stimulant, describes a substance that increases appetite.
Palatability	the quality of being agreeable to taste; tastiness
Progressive Ratio (PR) reinforcement	schedule of operant reinforcement in which the response requirement increases following each reinforcer. The increase in response requirement is pre-determined by the experimenter as either step-wise or exponential. It is used to measure the incentive value of the reinforcer and the motivation to obtain the reinforcer.
Real-time place preference	a behavioral test of reward/aversion. The animal is placed in a rectangular chamber with two physically identical halves. Optogenetic stimulation is paired with one side of the chamber to test for rewarding or aversive properties of the stimulation dependent on the amount of time the animal spends on each side of the chamber.

Term	Definition
Reinforcement	Anything that increases the likelihood that a response will occur
Self-stimulation	a behavioral test of reinforcement/reward. The animal is placed in an operant chamber with the opportunity to lever press or nose poke for stimulation of a given brain region. When an animal operantly responds for stimulation, it is presumed that the stimulation is rewarding/reinforcing.
Social defeat paradigm	a rodent model of social stress during which a naive mouse is exposed to a pre-determined aggressor mouse.
Sucrose preference test	reward-based test, used as an indicator of ability to experience pleasure and/or palatability
Wanting	A positive shift in the incentive salience state

Table 3

List of commonly used and available opioid receptor agonist and antagonist and their interaction with opioid receptors.

Based on the pharmacology of commonly used opioid receptor agonists and antagonists, it can be appreciated that these ligands can interact with one or more opioid receptors. Thus, it can often be difficult to discern the role of endogenous opioid peptides based simply on receptor pharmacology. All agonist and antagonist information were obtained from Sigma Aldrich (St. Louis, MO) and [9,116,117].

	Mu	Delta	Kappa	Nociceptin
Agonists				
Morphine	Agonist		Weak Agonist	
Codeine	Weak Agonist	Weak Agonist		
Fentanyl	Agonist			
Remifentanil	Agonist			
Methadone	Agonist			
Hydrocodone	Agonist			
Oxycodone	Agonist			
DAMGO	Agonist			
U50,488H			Agonist	
Salvinorin A			Agonist	
DPDPE		Agonist		
SNC80		Agonist		
MCOPPB				Agonist
Ro 64–6198				Agonist
Ro 65–6570				Agonist
Antagonists				
Naloxone	Antagonist	Weak Antagonist	Antagonist	
Naltrexone	Antagonist	Weak Antagonist	Antagonist	
nor-Binaltorphimini (nor-BNI)			Antagonist	
Buprenorphine	Antagonist/partial agonist	Antagonist/partial agonist	Antagonist/partial agonist	Agonist
CTOP	Antagonist			
CTAP	Antagonist			
Naltriben		Antagonist		
Levallorphan	Antagonist/partial agonist	Antagonist/partial agonist		

	Mu	Delta	Kappa	Nociceptin
β-Funaltrexamine	Antagonist		Agonist	
β-Chlornaltrexamine	Antagonist	Antagonist	Antagonist	
AZ-MTAB			Antagonist	
LY255582	Antagonist	Weak Antagonist	Weak Antagonist	
SB-612,111				Antagonist
LY2940094				Antagonist
LY2444296			Antagonist	