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Lee-Yuan Liu-Chen
Saadet Inan *Editors*

The Kappa Opioid Receptor

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Preface

I am very grateful to all the contributors for their efforts and time in contributing to this volume. In the process of organizing and completing this volume, I learned a great deal from all of them. There are many more researchers in the field who could have contributed to this book; however, there are limitations for number of chapters and pages in a book and some researchers declined invitations because of time constraints. Writing the chapters by the contributors and editing of the chapters by Dr. Saadet Inan, Co-Editor of this volume, and me were accomplished during the 2020–2021 COVID-19 pandemic period. This attests to the resilience and strengths of human spirits. Working from home day in and day out allowed me to get deeply immersed in the richness and beauty of science, which was a unique experience in my work life.

The progress of science depends on the prior research and experience of those who came before us. We all stand on the shoulders of giants. In 1973, three groups (Simon, Snyder, and Terenius) independently demonstrated the presence of opioid receptors in brains by radioligand binding. Subsequently, multiple opioid receptors were observed in in vivo and in vitro studies. Dr. W. Martin coined the term "kappa receptor" as the receptor for ketocyclazocine. In 1979, Dr. A. Goldstein's group isolated dynorphin, which was found to be an endogenous ligand for the kappa opioid receptor (KOR) and had extraordinarily potent antinociceptive effects. Subsequently, the precursor of dynorphin was cloned, and in the prodynorphin (pDyn) sequence, there were several dynorphin peptides. Immunohistochemistry and in situ hybridization were performed to map the distribution of pDyn and dynorphins. In the 1980s, U50,488 and norBNI were synthesized and characterized as the first nonpeptide selective KOR agonist and antagonist, respectively, which greatly advanced KOR pharmacology. KOR autoradiography using [^{3}H]U69,593 and [^{3}H] CI-977 provided information on distribution of the KOR in the brain and spinal cord. After cloning of the delta opioid receptor (DOR), the KOR was cloned from several species, including the mouse, rat, guinea pig, and human. Characterization of ligand–receptor interactions, agonist-induced KOR regulation and trafficking ensued. In the meantime, KOR knockout mouse lines were created to elucidate in vivo functions of the receptor.

KOR agonists were thought to have potentials to be non-addictive analgesics; however, their side effects such as dysphoria and psychotomimetic effects caused

termination of clinical trials. Until recently, the only KOR agonist used clinically is nalfurafine, which is used in Japan for treatment of pruritus associated with kidney dialysis and chronic liver diseases. Recently, difelikefalin (formerly CR845) (trade name Korsuva), a peripherally acting KOR agonist, was approved by FDA for treatment of moderate to severe pruritus associated with chronic kidney disease in adults undergoing hemodialysis in the US. Studies on KOR-mediated G protein and β-arrestin signaling suggest that G protein-biased KOR agonists may be useful as analgesic and anti-pruritic with fewer side effects, and this area has been under intense investigation. The KOR plays an integral part in stress responses, substance abuse, and mood disorders. The neuronal circuitries in which the KOR is involved in these responses and disorders are hot topics in the field. KOR antagonists are being investigated for treatment of depression and substance use disorders. Elucidation of KOR crystal structures will enhance understanding of ligand-dependent signaling and capability of structure-based drug design.

The book covers a range of topics on the KOR: the crystal structures, in vitro signaling and regulation, in vivo pharmacology of various chemical classes of agonists and antagonists, potential application of agonists and antagonists, brain imaging of the KOR, and clinical use of nalfurafine (TRK-820), until recently the only KOR agonist used in humans.

We believe that this book covers most of the contemporary issues in the field of KOR research and we hope this book will become a valuable resource for those who are interested in KOR pharmacology and neurobiology. In addition, we wish that this book will provoke deliberations about the questions in the field. Will biased KOR agonists be developed into analgesics and/or anti-pruritic drugs with fewer side effects? If so, what degree of bias and what characteristics are needed? Will KOR antagonists fulfill their potentials to become antidepressants and anti-anxiety drugs and for treatment of drug addiction (cocaine, opioid, alcohol, nicotine, etc.)? Will the crystal structures of the KOR lead to the development of agonists and antagonists with different chemical scaffolds which may be developed into drugs? Mapping of KOR-expressing circuitries and investigation of the functions of the circuitries by optogenetic and chemogenetic approaches will greatly enhance our understanding of KOR neurobiology. Will it ultimately be possible for different agonists to differentially activate circuitries in the brain? Basic biology and pharmacology are inherently iterative processes. The more we know about the biology, the more we will be able to translate that knowledge into better drugs.

We are indebted to all the researchers who have contributed to the KOR field over the decades to bring us to the current state of knowledge and particularly to those who have contributed to the book. We would like to thank Dr. James Barrett, Editor-in-Chief of *the Handbook of Experimental Pharmacology*, for inviting us to edit this book. I really appreciate his entrusting us with this responsibility and the opportunity to work on this project. I am very thankful to my co-editor, Dr. Saadet Inan, who provided valuable help and kept me going in this long process. We would also like to thank Susanne Dathe, Alamelu Damodharan, Arunkumar Kathiravan, Gerit Rother of Springer Publishing Co. for help with the production process of this book.

Last, but not the least, we would like to dedicate this book to Dr. Alan Cowan, who passed away on June 30, 2020. He was a dear friend and a highly knowledgeable opioid pharmacologist.

Philadelphia, PA, USA Lee-Yuan Liu-Chen

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Fundamentals of the Dynorphins/Kappa Opioid Receptor System: From Distribution to Signaling and Function

Catherine Cahill, Hugo A. Tejeda, Mariana Spetea, Chongguang Chen, and Lee-Yuan Liu-Chen

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Abstract

This chapter provides a general introduction to the dynorphins (DYNs)/kappa opioid receptor (KOR) system, including DYN peptides, neuroanatomy of the DYNs/KOR system, cellular signaling, and in vivo behavioral effects of KOR activation and inhibition. It is intended to serve as a primer for the book and to provide a basic background for the chapters in the book.

Keywords

In vivo pharmacology · KOR/dynorphins · Neuroanatomy · Review · Signaling

1 Historical Perspectives

Opium, the dried latex or gum obtained from lancing the outer surface of the seed pods of the opium poppy *Papaver somniferum*, has been used for centuries for medicinal and recreational practices to relieve pain, cough, and diarrhea, and to cause euphoria (Presley and Lindsley 2018). The euphoria produced by opium and iatrogenic-induced positive mood effects have been the basis for its abuse. It was in the twentieth century when there were major advances made in understanding how the active constituents of opium, such as morphine, act to produce their beneficial and harmful effects.

Opioid receptors were first hypothesized in 1954 by Beckett and Casy (1954) and stereospecific saturable binding of levorphanol was proposed to be the opiate receptor (Goldstein et al. 1971). In 1973, the existence of specific receptors for opioid drugs was demonstrated in brain preparations by radioligand binding assays (Pert et al. 1973; Simon et al. 1973; Terenius 1973). Multiplicity of opioid receptors was reported, with different classes of opioid drugs having distinct pharmacological activities (Lord et al. 1977; Martin et al. 1976). Following the identification of opioid receptors, an intense search for the endogenous ligands ensued and cumulated in the discovery of enkephalins, β-endorphin, and dynorphins (DYNs) as ligands for the delta, mu, and kappa opioid receptors (DOR, MOR, and KOR) (Chavkin et al. 1982; Goldstein et al. 1979; Hughes et al. 1975; Loh et al. 1976). Subsequently, the precursors of these peptides, proenkephalin, proopiomelanocortin, and prodynorphin (pDYN), were cloned and their amino acid sequences deduced [see (Höllt 1986) for a review]. These precursors are synthesized as large proteins in cell bodies by the protein synthesis machinery, transported via axonal transport to nerve terminals and cleaved by peptidases to produce the final active peptides during the transport process. In the 1980s, great efforts by chemists and pharmacologists resulted in synthesis and characterization of selective MOR, KOR, and DOR agonists and antagonists, which facilitated in vitro and in vivo opioid pharmacological studies. The first prototypical selective KOR agonist U50,488H and antagonist nor-binaltorphimine (norBNI) were reported by von Voigtlander et al. (1983) and Portoghese et al. (1987), respectively. Roth et al. (2002) demonstrated that

salvinorin A, a compound isolated from the mint family plant *Salvia divinorum,* was a selective KOR agonist and also the first non-nitrogenous KOR agonist.

In 1992, the laboratories of Evans and Kieffer independently cloned the DOR (Evans et al. 1992; Kieffer et al. 1992). Subsequently, the KOR was cloned from several species, along with the MOR and a highly homologous receptor named opioid receptor-like (ORL) receptor [which subsequently renamed as nociceptin/orphanin FQ receptor (NOP receptor)] was cloned [see (Knapp et al. 1995) for a review]. Following cloning of the receptors, many studies demonstrated the structural bases of ligand binding selectivity using chimeric receptor and site-directed mutagenesis approaches. In addition, genetic deletion of the KOR or pDYN in mice revealed the in vivo physiological and pharmacological roles of the DYNs/KOR system and in sustaining drug and alcohol abuse (Chavkin 2013; Simonin et al. 1998). In the meantime, deletion of the MOR, DOR, and NOP receptors was used to demonstrate their functional roles [see (Gaveriaux-Ruff and Kieffer 2002) for review]. In 2012, the X-ray crystal structure of an inactive state of the human KOR complexed with the selective antagonist JDTic was in complexed with the non-selective opioid agonist MP1104 and the nanobody 39 revealed (Wu et al. 2012), and in 2018, the structure of an active state of the KOR was reported (Che et al. 2018). Subsequently, Che et al. (2020) demonstrated the structure of an inactive state of KOR in complex with JDTic and the nanobody Nb6, which has remarkable similarity to that reported by Wu et al. (2012). During this period, structures of active and inactive states of the MOR, DOR, and NOP receptors were also published [see (Marino et al. 2018) for a review].

2 Dynorphin Peptides

Endogenous opioid peptides are processed from three precursors, proopiomelanocortin, proenkephalin, and pDYN. Endogenous activation of the KOR primarily occurs via the opioid peptides derived from pDYN (Chavkin et al. 1982). Dynorphins such as DYN A (1–7, 1–8, 1–13, and 1–17), DYN B, big DYN (DYN A + DYN B), α-neoendorphin, and β-neoendorphin are derived from the processing of pDYN (Fig. 1) by various non-selective proteases, including cathepsin L, prohormone convertases 1, 2, and 3, and carboxypeptidase E [see (Chavkin 2013; Fricker et al. 2020; Schwarzer 2009) for reviews]. Dynorphins and their endogenous target, the KOR, constitute the DYNs/KOR system (Chavkin et al. 1982). In addition, several peptides derived from proenkephalin, such as metorphamide and BAM-18, have high affinity for the KOR (Hurlbut et al. 1986). Although most peptide products elicit opioid-like effects, whether the different peptides have different physiological functions remains unclear. In addition, there is differential processing of pDYN throughout the brain as suggested by early work showing that the ratios of DYNs and other pDYN derivative levels varied across different regions of the mesocorticolimbic and nigrostriatal dopamine (DA) systems (Zamir et al. 1984). Further, pDYN and DYNs are colocalized mostly on presynaptic sites of hippocampal and striatal neurons, but more evenly distributed throughout the

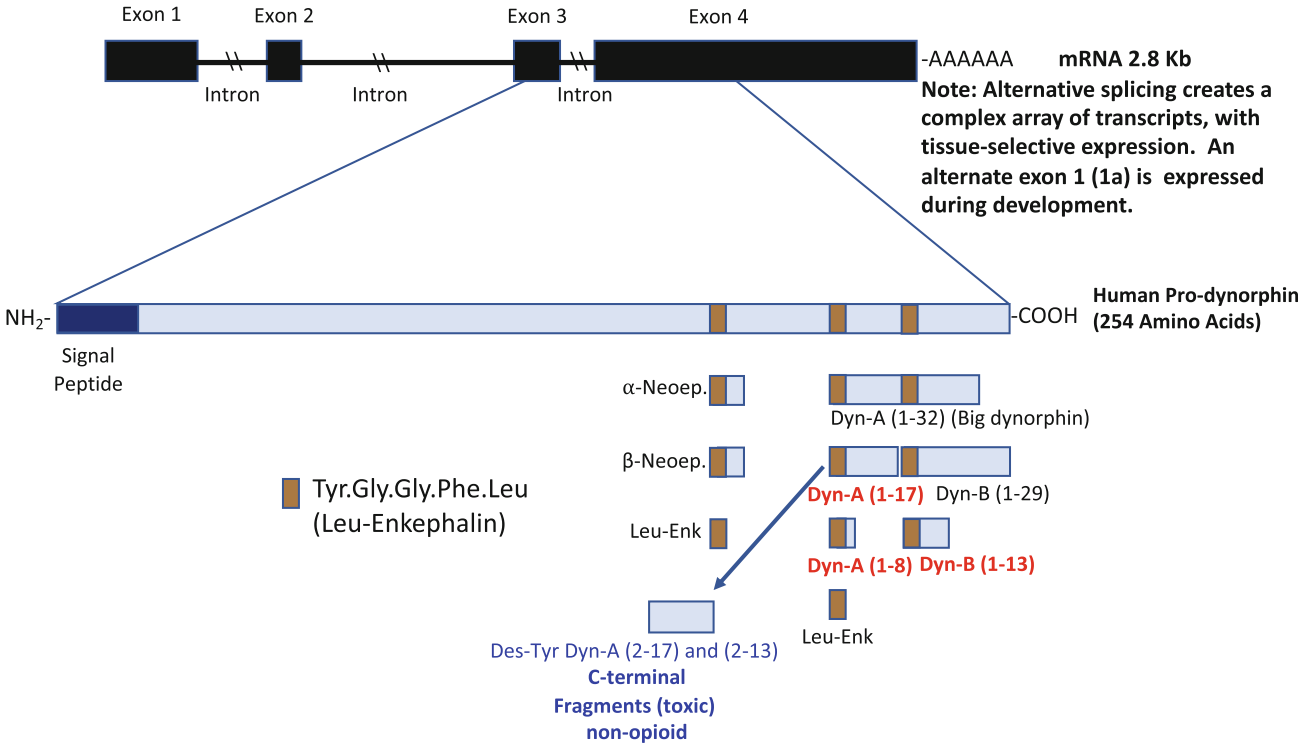

Fig. 1 Summary of post-translational PDYN products, changes in chronic pain and effects on nociception, and expression patterns. A. Dynorphin peptides are up-regulated in chronic neuropathic and inflammatory pain models (marked in red). Spinal (intrathecal) administration of DYN peptides that produce acute antinociception include Dyn A (1-8), Dyn A (1-17), Dyn B (1-13). Spinal administration of DYN peptides, Dyn A (1-17), Dyn B (1-13), Dyn A (2-17), Dyn A (2-13), produces pain-like (allodynia and hyperalgesia) responses via activation of NMDA or bradykinin receptors. [Reviewed in (Podvin et al. 2016)]. Dyn A (2-17 or 2-13) have no activity at KORs

soma of amygdala and cortex neurons. Of the different DYN peptides, DYN A(1-17) is considered the primary KOR ligand having higher potency at the KOR than other DYN peptides [see (Chavkin 2013; Fricker et al. 2020; Schwarzer 2009) for reviews]. Notably, DYNs have affinity for other opioid and non-opioid receptors in addition to the KOR. For example, DYNs bind to the MOR and DOR in brain tissue, with equal or lower affinities than to the KOR [see (Chavkin 2013; Fricker et al. 2020; Schwarzer 2009) for reviews]. Des-Tyr^1 DYNs have direct effects on N-methyl-D-aspartate (NMDA) receptors in the spinal cord and brain (Caudle and Dubner 1998; Shukla and Lemaire 1994). Dynorphins also show activity at bradykinin receptors, which have been implicated in pain (Lai et al. 2006). Thus, DYN peptides, typically considered a monolithic entity in the field, may be more diverse in their processing and actions than has been appreciated.

In contrast to classical neurotransmitter systems whereby an action potential results in presynaptic transmitter release from the synaptic active zone, neuropeptide signaling parameters may vary widely in terms of spatio-temporal release, target activation, and receptor fidelity (Gomes et al. 2020). Following sustained neuronal activity, DYNs are released from large dense core vesicles (Cho and Basbaum 1989; Drake et al. 1994) and decrease cellular activation following binding to KOR. Dynorphins can be released from presynaptic neurons onto postsynaptic neurons or act in an auto-inhibitory manner by activating presynaptic KORs in cells that release DYNs, or from be released from dendrites to produce retrograde inhibition of KOR-sensitive presynaptic inputs [see (Chavkin 2013; Schwarzer 2009) for reviews].

Dynorphin peptides are widely expressed throughout the brain and spinal cord, with only the cerebellum and dorsal thalamus having no DYNs (Fallon and Leslie 1986; Mansour et al. 1994; Mansour et al. 1988) (Fig. 2). For example, expression of pDYN is high in the substantia nigra pars reticulata, various hypothalamic nuclei, nucleus of solitary tract, hippocampus, globus pallidus, spinal trigeminal nucleus, and substantia gelatinosa of spinal cord. Medium to low pDYN levels are found in the amygdala, nucleus accumbens, olfactory tubercle, caudate putamen, bed nucleus stria terminalis, preoptic area, periaqueductal grey, parabrachial nucleus, raphe nucleus, cortex, periventricular nucleus of thalamus, substantia nigra pars compacta, ventral tegmental area and superior and inferior colliculus. Although it is important to consider that DYN peptides are released from the terminals of these neurons and rarely from the soma of the neurons. Thus, low pDYN levels do not mean that DYN projection neurons may not release peptide in these locations.

Insights into the role of pDyn-derived peptides in behaviors have been gained utilizing mutant mice lacking pDyn-derived peptides (Schunk et al. 2008; Sharifi et al. 2001). Mice with genetic deletion of pDyn have been demonstrated to (1) show impaired extinction learning of contextual fear (Bilkei-Gorzo et al. 2012); (2) have diminished impairment in cognition associated with aging and stress (Carey et al. 2009; Ménard et al. 2013; Nguyen et al. 2005); (3) exhibit increased alcohol and drug self-administration and decreased stress-induced reinstatement of drug preference (Femenía and Manzanares 2012; Galeote et al. 2009; Redila and Chavkin 2008); (4) display anxiolytic phenotypes relative to controls (Ménard et al. 2013;

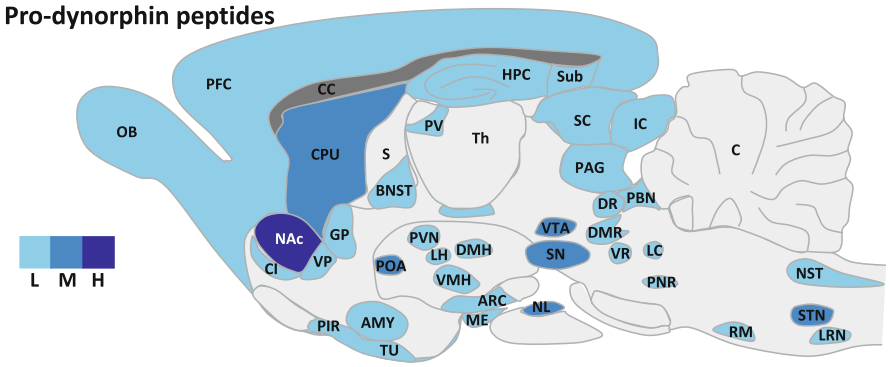

Fig. 2 Dynorphin peptide expression in the rodent brain. *L* low, *M* moderate, *H* high expression. *ARC* arcuate nucleus, hypothalamus, *AMY* amygdala, *BLA* basolateral nucleus, amygdala, *BNST* bed nucleus of the stria terminalis, *C* cerebellum, *CC* corpus callosum, *CeA* central nucleus, amygdala, *CI* claustrum, *CL* centrolateral thalamus, *CM* centromedial thalamus, *COA* cortical nucleus, amygdala, *CPU* caudate putamen, *DMH* dorsomedial hypothalamus, *DMR* dorsal and medial raphe, *DR* dorsal raphe, *DTN* dorsal tegmental nucleus, *EN* endopiriform cortex, *GP* globus pallidus, *HPC* hippocampus, *IC* inferior colliculus, *IP* interpeduncular nucleus, *LC* locus coeruleus, *LD* laterodorsal thalamus, *LH* lateral hypothalamus, *LRN* lateral reticular nucleus, *ME* median eminence, *MeA* median nucleus, amygdala, *NAc* nucleus accumbens, *NST* nucleus tractus solitarius, *NL* neuronal lobe, pituitary, *NRGC* nucleus reticularis gigantocellularis, *OB* olfactory bulb, *OCX* occipital cortex, *PAG* periaqueductal gray, *PBN* parabrachial nucleus, *PCX* parietal cortex, *PFC* prefrontal cortex, *PIR* piriform cortex, *PNR* pontine reticular, *POA* preoptic area, *PV* paraventricular thalamus, *PVN* paraventricular hypothalamus, *RE* reuniens thalamus, *RM* raphe magnus, *S* septum, *SC* superior colliculus, *SN* substantia nigra, *STN* spinal trigeminal nucleus, *Sub* subiculum, *TCX* temporal cortex, *Th* thalamus, *TU* olfactory tubercle, *VMH* ventromedial hypothalamus, *VP* ventral pallidum, *VR* ventral raphe, *VTA* ventral tegmental area, *Zi* zona incerta. [Adapted from Le Merrer et al. (2009)]

Wittmann et al. 2009) or show increased anxiety-like behavior relative to controls (Femenía et al. 2011); (5) have decreased pain in models of chronic pain [see (Tseng and Hoon 2020) for a review]. Therefore, endogenous dynorphins likely play a pivotal role in regulating cognition, fear-related behaviors, reward, anxiety, and nociception.

3 Cloning of the KOR

The KOR has been cloned from several species including humans (Mansson et al. 1994; Simonin et al. 1995; Zhu et al. 1995). The KOR genes were mapped at position q11-12 in human chromosome 8 (Simonin et al. 1995) and mouse chromosome 1A2-3 (Nishi et al. 1994). Like all other G protein-coupled receptors (GPCRs), the KORs have seven transmembrane domains with a N terminal domain outside the cell and an intracellular C-terminal domain. The sequences of mouse, rat, guinea pig, and human KORs have high homology. The amino acid sequence of the human KOR is 94% identical to those of the rat and mouse and 91% identical to that of the

guinea pig (Simonin et al. 1995). The N- and C-terminal domains have more sequence variations than the transmembrane domains among the KORs of different species. The amino acid sequences of the human KOR, MOR, DOR, and NOP receptors share ~60% overall identity and ~80% identity among the transmembrane domains (Zhu et al. 1995). The N- and C-terminal domains have the highest sequence divergence among the four opioid receptors, hence antibodies are typically generated against the N- and C-terminal domain peptides.

4 Neuroanatomy of the KOR

The distribution of the KOR has been investigated with several different methods, including biochemical techniques, i.e. receptor autoradiography and immunohistochemistry (IHC), and recently with mutant mouse lines expressing KOR-Cre or KOR-tdTomato. The KOR IHC, discussed below, has been plagued by lack of highly specific antibodies. The KOR mRNA distribution has been examined using in situ hybridization.

Receptor autoradiography was performed on sections of fresh frozen tissues with radioactive labeled KOR-selective ligand such as [^{3}H]U69,593 (Mansour et al. 1994; Unterwald et al. 1991; Wang et al. 2011) or [^{3}H]CI-977 (Slowe et al. 1999), and the sections were exposed to ^{3}H-senstive films or screens. The most important advantage of receptor autoradiography is its high specificity because of the use of highly selective ligands and anatomical definition. However, it takes a relatively long exposure time to obtain the results due to the low energy of β-particles emitted from ^{3}H-labeled ligands. In addition, autoradiography has low resolutions, making it difficult to visualize small brain regions.

Immunohistochemistry of the KOR has been carried out with antibodies against synthetic peptides corresponding to partial sequences of N- and C-terminal domains of the KOR [for example, (Appleyard et al. 1997; Arvidsson et al. 1995; Drake et al. 1996; Mansour et al. 1996)]. Because these studies yielded significant different results among themselves and also from those of receptor autoradiography, these studies are not described here. Issues related to KOR antibodies are discussed elsewhere in this book.

Recently, reporter protein approaches were employed to map the distribution of the KOR in the mouse brain. The Ross lab generated a KOR-Cre mouse line, in which the exon 2 coding region of the *oprk1* gene was replaced with that of *Cre* recombinase (Cai et al. 2016). They then bred KOR-Cre mice with mice expressing the Cre-dependent allele $Rosa^{lsl\ tdTomato}$ (also known as *Ai14*), which resulted in tdTomato (tdT) being expressed in KOR-containing cells. The KOR distribution in the KOR-Cre x Ai14 mouse brain was similar to that of receptor autoradiography in most regions, however, there were significant differences, such as the presence of the KOR in the cerebellum, high expression in the striatum, and low expression in the claustrum. These differences are due to constitutive and cumulative expression of Cre from all the developmental stages. This can be circumvented by viral injection of Cre-dependent reporter gene into brain regions of interests.

Liu-Chen's lab produced a knockin mouse line that expresses a fusion protein of KOR conjugated in frame with tdTomato 5′ to the stop codon (KOR-tdT) (Chen et al. 2020). The KOR-tdT has similar distribution as KOR in receptor autoradiography of adult mouse brains. As the expression of KOR-tdT is under the control of KOR promotor like the wildtype KOR, KOR-tdT mice do not have developmental issues as KOR-Cre mice. One unique feature is that these mice display prominent KOR-tdT-containing neuronal fibers not seen with receptor autoradiography, for example, projections from the striatum to substantia innominata and substantia nigra reticulata. The KOR-Cre x Ai14 and KOR-tdT mouse lines provide resolutions at the cellular level and are valuable tools for KOR neuroanatomy studies. Liu-Chen's lab, employing the CLARITY technique (Chung and Deisseroth 2013) to clear mouse brains followed by 3-D imaging, produced the first 3-D images of the KOR in the brain, which is also the first for any GPCRs (Chen et al. 2020) (Video 1).

Results from receptor autoradiography and reporter protein approaches show that the KOR has a widespread distribution in the brain. The claustrum, a brain region implicated in consciousness, has the highest level, followed by the endopiriform nucleus (separate regions in rodents that are a single continuous structure in primates); however, the functions of KOR in these areas are not clear. The KOR is present in the areas involved in mood, reward, motivation, and addiction, including the ventral tegmental area, nucleus accumbens, prefrontal cortex, anterior cingulate cortex, amygdala nuclei, bed nucleus of stria terminalis, and raphe nucleus, with the nucleus accumbens shell exhibiting particularly intense signal. Moderate levels of KOR are observed in pain pathways, including the periaqueductal gray, parabrachial nucleus, some nuclei in thalamus, primary and secondary somatosensory cortices. The KOR is found in several nuclei in the hypothalamus, indicating roles of the KOR in neuroendocrine regulation. The KOR is also found in paraventricular nucleus and nucleus reunion of thalamus, but its functions in these brain regions are currently unknown (Fig. 3, for KOR distribution, signaling, and ligands, see https://www.guidetoimmunopharmacology.org/GRAC/ObjectDisplayForward?objectId=318). Discrepancies in KOR binding versus KOR mRNA expression may in part be due to trafficking of KOR to terminals of long-range neuronal projections. The KOR mRNA is localized in cell bodies, whereas KOR binding in any given region is comprised of KOR in local cells and KORs localized on afferent inputs to that region.

The KOR is also found in the dorsal horn of the spinal cord, and is involved in pain and itch regulation. Additionally, the KOR is distributed throughout the body, including in the lungs, heart, spleen, kidneys, liver, and small intestines (Peckys and Landwehrmeyer 1999; Peng et al. 2012), though its precise function in each of these regions is yet to be fully described.

There are species differences in the KOR distribution in the brain, with the receptor distribution in the human brain being more similar to that in the guinea pig than in rat or mouse. For example, in the guinea pig and human, but not the rat or mouse, the KOR is abundant in the cerebellum and deep layers (layers V and VI) of the cortex, and is found in striosomes of the striatum (having patchy distribution) (Mansour et al. 1988; Quirion and Pilapil 1991; Quirion et al. 1987). Transcript for the KOR also shows some divergence between humans and rodents where unlike

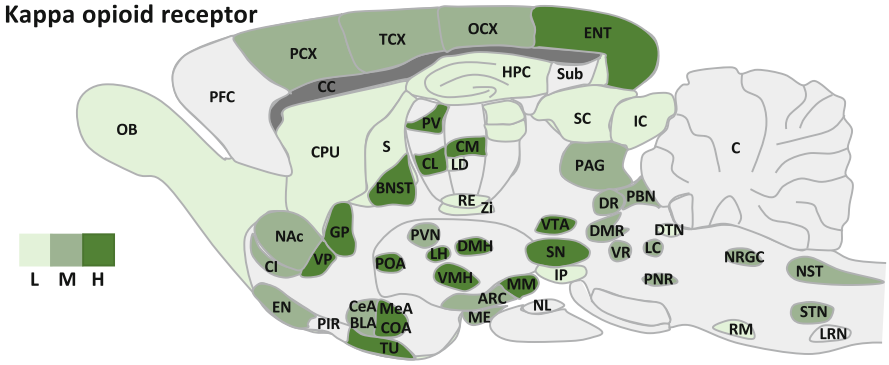

Fig. 3 Expression of the KOR in rat brain. *L* low, *M* moderate, *H* high expression. *ARC* arcuate nucleus, hypothalamus, *AMY* amygdala, *BLA* basolateral nucleus, amygdala, *BNST* bed nucleus of the stria terminalis, *C* cerebellum, *CC* corpus callosum, *CeA* central nucleus, amygdala, *CI* claustrum, *CL* centrolateral thalamus, *CM* centromedial thalamus, *COA* cortical nucleus, amygdala, *CPU* caudate putamen, *DMH* dorsomedial hypothalamus, *DMR* dorsal and medial raphe, *DR* dorsal raphe, *DTN* dorsal tegmental nucleus, *EN* endopiriform cortex, *GP* globus pallidus, *HPC* hippocampus, *IC* inferior colliculus, *IP* interpeduncular nucleus, *LC* locus coeruleus, *LD* laterodorsal thalamus, *LH* lateral hypothalamus, *LRN* lateral reticular nucleus, *ME* median eminence, *MeA* median nucleus, amygdala, *NAc* nucleus accumbens, *NST* nucleus tractus solitarius, *NL* neuronal lobe, pituitary, *NRGC* nucleus reticularis gigantocellularis, *OB* olfactory bulb, *OCX* occipital cortex, *PAG* periaqueductal gray, *PBN* parabrachial nucleus, *PCX* parietal cortex, *PFC* prefrontal cortex, *PIR* piriform cortex, *PNR* pontine reticular, *POA* preoptic area, *PV* paraventricular thalamus, *PVN* paraventricular hypothalamus, *RE* reuniens thalamus, *RM* raphe magnus, *S* septum, *SC* superior colliculus, *SN* substantia nigra, *STN* spinal trigeminal nucleus, *Sub* subiculum, *TCX* temporal cortex, *Th* thalamus, *TU* olfactory tubercle, *VMH* ventromedial hypothalamus, *VP* ventral pallidum, *VR* ventral raphe, *VTA* ventral tegmental area, *Zi* zona incerta. [Adapted from Le Merrer et al. (2009)]

rodents, there was low level of transcript in the human substantia nigra and hippocampus (Simonin et al. 1995).

There are also differences in the KOR level in the brain among species. Kitchen et al. (1990) reported that B_{max} value of [^{3}H]U69,593 binding to the KOR in adult rat brain was 7.3 fmol/mg protein. The KOR levels in the human frontal cortex and forebrains of animals are in the order of pigeon (8×) > guinea pig (~3×) ~ human (~2.5×) > mouse (~1.5×) > rat brain (1×) (Mansour et al. 1988; Quirion and Pilapil 1991; Quirion et al. 1987). Moreover, the KOR level in the adult rat brain is about 1/20 of that of the MOR, which is second to the NOP receptor that has the highest expression in the brain of all opioid receptors. However, it was reported that the KOR mRNA was the most abundantly expressed over MOR and DOR in the human during development (Wang et al. 2006).

5 KOR Signaling at the Cellular Level

In the brain, DYNs are released upon membrane depolarization and activate the KOR to modulate presynaptic and postsynaptic neural activity (Chavkin et al. 1983; Wagner et al. 1991). Opioid receptors, including the KOR, belong to the rhodopsin sub-family of GPCRs. At the cellular level, activation of the KOR stimulates Gi/o proteins and promotes receptor phosphorylation. Phosphorylated KOR, in turn, recruits β-arrestins, which lead to β-arrestin-mediated signaling (Bruchas et al. 2006; Law 2011; McLennan et al. 2008). It has also been reported that the KOR signals via Gz and G16 proteins (Lee et al. 1998; Tso and Wong 2000).

Activation of KOR results in dissociation of guanosine 5′-diphosphate (GDP) from $G_{\alpha i}$ and association of guanosine 5′-triphosphate (GTP) with $G_{\alpha i/o}$ as well as dissociation of the $G_{\beta\gamma}$ from G_{α} subunits. Activated $G_{\alpha i/o}$ proteins inhibit adenylate cyclase resulting in decreases in cyclic AMP production and a host of effects in downstream targets, and inhibit Ca^{2+}-channel activity and attenuate presynaptic neurotransmitter release. $G_{\beta\gamma}$ proteins activate G protein-gated inwardly rectifying potassium channels (GIRKs) and other potassium channels, which causes a reduction in neuronal or axonal excitability [see (Law 2011) for a review]. $G_{\beta\gamma}$ proteins also enhance ERK1/2 phosphorylation (early phase) via L-type Ca^{2+} channels, phospholipase C (PLC), intracellular Ca^{2+} release, protein kinase C (PKC) (Bohn et al. 2000; Law 2011). β-arrestins-mediated signaling includes activation of ERK1/2 (late phase) and p38 MAPK (Al-Hasani and Bruchas 2011; Bruchas and Chavkin 2010). The KOR also activates c-Jun N-terminal kinase (JNK) and protein kinase B (Akt) pathways. Hence, activation of the KOR results in signal transduction via a variety of intracellular pathways with diverse effects on cells (Bruchas and Chavkin 2010; Pradhan et al. 2012). Biased agonism for the KOR has been demonstrated in vitro, in which agonists preferentially activate G protein- or β-arrestin-mediated pathways [for a review, (Bohn and Aubé 2017)]. Several KOR biased agonists have been reported, which may provide beneficial pharmacological effects with reduced unwanted side effects [see (Bohn and Aubé 2017; Faouzi et al. 2020; Mores et al. 2019) for reviews]. The predominance of one signaling cascade over another may vary by brain region, demonstrating the potential utility of biased KOR ligands that may target one downstream cascade over another (Crowley and Kash 2015). Activation of JNK signaling was reported to inactivate the KOR, which is one of the mechanisms for prolonged durations of action (up to weeks) of KOR antagonists, such as norBNI and JDTic (Bruchas et al. 2007).

6 Agonist-Promoted KOR Phosphorylation and Regulation

Binding of an agonist, such as U50,488H to the mouse KOR, caused KOR phosphorylation at S356, T357, T362, and S369 in the C-terminal domain (Chen et al. 2016) in cultured cells. The KOR phosphorylation at all the residues was mediated by $G_{i/o}$ alpha proteins and G protein-coupled receptor kinases (GRK2, GRK3, GRK5, GRK6), and PKC (Chiu et al. 2017). GRKs-mediated, but not

PKC-mediated, KOR phosphorylation followed by β-arrestin recruitment desensitized U50,488H-induced ERK1/2 response and [^{35}S]GTPgS binding and was involved in agonist-induced KOR internalization (Liu-Chen 2004). PKC activation by phorbol ester induced agonist-independent KOR phosphorylation. Compared with U50,488H, PKC activation induced much higher S356/T357 phosphorylation, much lower T363 phosphorylation, and similar levels of S369 phosphorylation. PKC activation caused a lower level of agonist-independent KOR internalization, compared with U50,488H.

U50,488H promoted KOR internalization in a process that depends on GRKs, β-arrestin, and dynamin proteins (Li et al. 1999). KOR internalization precedes downregulation which, in addition, involves Rab5- and Rab7-dependent process and requires ubiquitination of the KOR (Li et al. 2000). The KOR appears to be internalized into early endosomes then trafficked via late endosomes to lysosomes and proteosomes for degradation (Liu-Chen 2004).

7 X-ray Crystal Structures of the KOR

The understanding of the DYNs/KOR system function and signaling continues to be enhanced by the publication of the high-resolution crystal structures of the receptor. Wu et al. (2012) reported an X-ray crystal structure of an inactive state of the human KOR in complex with the selective KOR antagonist JDTic. Che et al. (2018) provided a crystal structure of an active state of the human KOR in complex with the epoxymorphinan opioid agonist MP1104 and an active-state-stabilizing nanobody (Nb39). The active structure provides significant information for the structural basis of biased agonism (Che et al. 2018) and allosteric modulation (Che et al. 2020). Subsequently, Che et al. (2020) revealed the structure of an inactive state of the KOR bound by JDTic and the nanobody Nb9 stabilizing an inactive state. This structure was found to be very similar to that of Wu et al. (2012). Comparisons between inactive- and active-state KOR structures reveal substantial conformational changes in the binding pocket and intracellular and extracellular regions. The characterization of the crystal structures of inactive and active states of the KOR has provided insight for ligand–receptor interactions allowing new concepts for novel drug design. This structural characterization together with identification of the signaling events that elicit antinociceptive versus dysphoric and psychotomimetic effects, has provided extensive advancement in novel chemical entities that hold promise as new pain treatments with minimal aversive effects including depressive or addictive properties.

8 In Vivo Pharmacology of the DYNs/KOR System

Activation of the KOR in vivo produces many physiological effects and behavioral responses, including analgesia, antipruritic effects, water diuresis, dysphoria/aversion, sedation, motor incoordination, and hypothermia (Mucha and Herz 1985; Pfeiffer et al. 1986; Simonin et al. 1998; Togashi et al. 2002; von Voigtlander

et al. 1983). KOR agonists are effective analgesics without causing respiratory depression and abuse liabilities associated with MOR-selective or preferring analgesics (Paton et al. 2020). In light of the recent opioid epidemic, KOR agonists may be re-examined as analgesics, either alone or as part of pharmacological regimen. Generation of KOR-deficient animals has provided significant knowledge on the in vivo physiological role of the DYNs/KOR system (Ansonoff et al. 2006; Simonin et al. 1998). Dynorphins are released in the central nervous system during pain states and activate KORs to produce analgesia, and KOR-knockout mice exhibit enhanced pain sensitivity (Simonin et al. 1998).

The KOR is also involved in the pathophysiology of pruritus, with KOR agonists as promising antipruritic agents [for a review see Cowan et al. (2015)]. However, to date, nalfurafine is the only KOR agonist approved for clinical use (marketed in Japan). One of the limitations that has hampered the development of KOR agonists as safer analgesics and effective antipruritic agents are the negative effects that emerge following KOR agonist administration. KOR agonists produce dysphoria, depressive-like symptoms and psychotomimetic effects in humans (Pfeiffer et al. 1986; Wadenberg 2003; Walsh et al. 2001), and elicit place aversion and depressive-like behaviors (Mucha and Herz 1985; Shippenberg and Herz 1986) as well as stimulate drug-seeking (Bruchas et al. 2010; Chavkin and Koob 2016; Knoll and Carlezon 2010) in rodents. Activation of the DYNs/KOR system also elicits signs of anxiety and fear in animals and humans (Chartoff and Mavrikaki 2015; Chavkin and Koob 2016; Darcq and Kieffer 2018).

Chavkin and colleagues have proposed that antinociception produced by KOR agonists is mediated by G protein pathways, whereas aversion is mediated by β-arrestin2-dependent p38 MAP kinase phosphorylation [reviewed in (Bruchas and Chavkin 2010)]. However, White et al. (2015) showed that U69,593, salvinorin A and RB-64 (a salvinorin A analog) produced similar levels of aversion in the conditioned place preference (CPA) test in wildtype and β-arrestin2-/-mice, indicating that either β-arrestin2 is not involved in CPA or other pathways besides β-arrestin2 are involved. In addition, βarrestin2 deletion impaired KOR-mediated motor incoordination, but did not affect antinociception or hypolocomotion (White et al. 2015). Morgenweck et al. (2015) demonstrated that β-arrestin2 deletion did not affect the anti-scratch effects of U50,488H or the G protein-biased KOR agonist isoquinone 2.1, indicating that the anti-scratch effect of KOR agonists is not mediated by β-arrestin2.

Several groups have been actively searching for G protein-biased KOR agonists [see (Bohn and Aubé 2017; Faouzi et al. 2020; Mores et al. 2019) for reviews] to avoid the dysphoric and psychotomimetic effects. Whether G protein-biased KOR agonists will fulfill the promise of retaining beneficial analgesic and antipruritic effects with reduced side effects in humans remains to be determined. Another approach is to develop peripherally acting KOR agonists to avoid the central nervous system-mediated side effects. An example is CR845/difelikefalin, which is under clinical trials for treatment of pruritus associated with chronic kidney or liver disease or atopic dermatitis and treatment of pain in post-operative settings (Fishbane et al. 2020; Therapeutics 2020) (https://www.caratherapeutics.com/pipeline-technology/our-pipeline/).

Kappa opioid receptor antagonists have been demonstrated to have antidepressant- and anxiolytic-like activities in rodents. In addition, KOR antagonists were found to reduce stress-induced instatement of drug-seeking for several drugs of abuse in animals. Thus, KOR antagonists may be useful for treatment of depression, anxiety, and drug addiction [see (Al-Hasani and Bruchas 2011; Bruchas et al. 2010; Carroll and Carlezon 2013; Van't Veer and Carlezon 2013) for reviews]. Buprenorphine, having MOR partial agonist and KOR antagonist activities, in combination with the MOR antagonist samidorphan was investigated in clinical trials for treatment of depression, but the combination did not meet the primary endpoint goal (Zajecka et al. 2019). JDTic underwent clinical trials for cocaine use disorder, but failed in phase I due to cardiac side effects (Buda et al. 2015). Aticaprant (formerly JNJ-67953964, CERC-501, and LY2456302) is currently in clinical trials for management of anhedonia (an important symptom of major depressive disorder) (Krystal et al. 2020; Pizzagalli et al. 2020).

9 Conclusion

The KOR is found throughout the peripheral and central nervous systems and participates in a range of physiological functions. The potential therapeutic use of KOR ligands as analgesics and antipruritics and for treatment of mood and substance use disorders is of continued interest and is being pursued by various pharmaceutical companies. The following chapters will provide more thorough and in-depth discussions of KOR functions in various physiological and pathological states, together with recent developments in chemistry and pharmacology of novel KOR ligands and their therapeutic potentials.

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Considerations on Using Antibodies for Studying the Dynorphins/Kappa Opioid Receptor System

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Abstract

Antibodies are important tools for protein and peptide research, including for the kappa opioid receptor (KOR) and dynorphins (Dyns). Well-characterized antibodies are essential for rigorous and reproducible research. However, lack of validation of antibody specificity has been thought to contribute significantly to the reproducibility crisis in biomedical research. Since 2003, many scientific journals have required documentation of validation of antibody specificity and use of knockout mouse tissues as a negative control is strongly recommended. Lack of specificity of antibodies against many G protein-coupled receptors (GPCRs) after extensive testing has been well-documented, but antibodies generated against partial sequences of the KOR have not been similarly investigated. For the dynorphins, differential processing has been described in distinct brain areas, resulting in controversial findings in immunohistochemistry (IHC) when different antibodies were used. In this chapter, we summarized accepted approaches for validation of antibody specificity. We discussed two KOR antibodies most commonly used in IHC and described generation and characterization of KOR antibodies and phospho-KOR specific antibodies in western blotting or immunoblotting (IB). In addition, applying antibodies targeting prodynorphin or mature dynorphin A illustrates the diversity of results obtained regarding the distribution of dynorphins in distinct brain areas.

Keywords

Antibodies · Dynorphin · Immunoblotting · Immunohistochemistry · Kappa opioid receptor · Prodynorphin

1 Introduction

Antibodies are widely used in biomedical research. They are employed to label specific antigens (most commonly proteins and peptides) via techniques such as western blotting or immunoblotting (IB), immunohistochemistry (IHC), immunocytochemistry (ICC), enzyme-linked immunosorbant assay (ELISA), immunoprecipitation (IP) and fluorescence-activated cell sorting (FACS). For any antibody to be useful, its specificity for the antigen in the intended application is of utmost importance. Well-characterized antibodies that consistently perform as expected are essential for rigorous and reproducible research. However, problems with validation of antibody specificity or lack of validation have been cited as one of the important factors for the "reproducibility crisis" in biomedical research (Freedman et al. 2015). It was estimated that 36.1% of irreproducible research was attributed to biological reagents and reference materials (Freedman et al. 2015), of which antibodies constitute a large portion. For the Human Protein Atlas (HPA), Berglund et al. (2008) examined 6,120 antibodies for 5,067 proteins in the human genome and showed that only 7% and 15% of antibodies achieved high and medium validation scores for IHC of proteins, respectively. Many journal editors and researchers have raised concerns

about the lack of validation of antibody specificity in research (Baker 2015a, b; Bordeaux et al. 2010; Gautron 2019; Pillai-Kastoori et al. 2020; Rhodes and Trimmer 2006; Saper 2005; Saper and Sawchenko 2003; Uhlen et al. 2016). In 2003, *Journal of Comparative Neurology* was the first journal to introduce the requirements of validation of antibody specificity for publishing research work in the journal. Many journals followed, including *Nature*, *Endocrinology*, *British Journal of Pharmacology*, *Journal of Histochemistry and Cytochemistry, Molecular Endocrinology*.

The research community launched several initiatives in attempts to enhance quality and standardization of antibodies used in research, such as antibody evaluation, protocols and documentation. Bourbeillon et al. (2010) developed guidelines called the Minimum Information about A Protein Affinity Reagent (MIAPAR), as an important first step in formalizing standards in reporting the production and properties of protein binding reagents, such as antibodies. It constructed a checklist of required information, including production/purification process, experimental evidence of specificity, updated protocols, and other relevant details. Subsequently, the International Working Group for Antibody Validation (IWGAV) was formed in 2016 and recommended guidelines for raising standards for antibody validation (Uhlen et al. 2016). In 2017 the National Institutes of Health (NIH) issued a notice (NOT-OD-17-068), which required research grant applicants to authenticate key biological reagents, including antibodies.

In addition to improving the standardization of antibodies, antibody performance is another common source of variability, which may vary considerably between suppliers and even batches.

2 General Considerations for Validation of Specificity of Antibodies

2.1 Unique Issues Associated with Antibodies Against G Protein-Coupled Receptors (GPCRs)

GPCR antibodies are typically generated against synthetic peptides corresponding to partial sequences of the N- or C-terminal domains of GPCRs because these two regions have the most divergent sequences and they are accessible in the extracellular and intracellular space, respectively. The peptides are usually coupled to a carrier protein, such as keyhole limpet haemocyanin or thyroglobulin, for use as antigens. Most GPCR antibodies are polyclonal antibodies generated largely in rabbits using conventional methods. GPCR antibodies present unique challenges in that GPCRs are generally present in very low levels in tissues, including the brain. It is thus necessary to have antibodies that have very high affinity for the antigen to allow use of very low concentrations so as to minimize nonspecific interactions. However, such high affinity antibodies are not commonly available for GPCRs. Thus, one drawback of GPCR antibodies frequently encountered is low signal-to-noise ratios, that is low specificity. In IB, it may be necessary to partially enrich the GPCRs by,

for example, IP if appropriate antibodies are available, or lectin affinity chromatography taking advantage of the glycoprotein nature of many GPCRs. Most GPCRs are glycosylated, largely in the N-terminal domain; therefore, they appear as broad and diffuse band(s) in IB with relative molecular weights (M_r's) higher than molecular weights predicted from amino acid sequences [for example, (Huang et al. 2015, 2008; Li et al. 2007; Petaja-Repo et al. 2000, 2002)]. Because of different degrees of glycosylation, M_r's of the same GPCR may have species, tissue and brain region differences (Huang et al. 2008, 2015; Liu-Chen et al. 1993). Many GPCRs appear as two bands: one band represents the fully glycosylated form present on plasma membranes and the other partially glycosylated in the Golgi and endoplasmic reticulum (Huang et al. 2008, 2015; Li et al. 2007; Petaja-Repo et al. 2000, 2002). Because of these issues, it is not possible to use M_r in IB as predicted from amino acid sequences as the first-line characterization criterion of antibodies against a GPCR.

Studies published in 2009 examined several commercially available antibodies against partial sequence of each of D1, D4 and D5 dopamine receptors, β1-, β2- and β3-adrenergic receptors, α1A-, α1B- α1D- and α2B-adrenergic receptors, M1, M2, M3, M4 and M5 muscarinic receptors and GalR1 and GalR2 galanin receptors. The results revealed that none of the commercially available antibodies against these GPCRs showed specificity when tested in IHC, IB or both [(Michel et al. 2009) and other articles in the same issue]. Subsequently similar studies on other GPCRs were published.

2.2 Validation of Specificity of Antibodies

Michel et al. (2009) proposed that specificity of antibodies against GPCRs (or any other proteins) should be validated with at least one of the following methods: (1) Staining should be absent in tissues from knockout mice. (2) Intensity of staining should be reduced following siRNA knockdown of the target protein in cells or in vivo. (3) Closely related receptors expressed in the same cell lines should yield no staining. (4) Antibodies generated against at least two distinct epitopes of the same receptor should yield the same staining. Many labs used haemagglutinin (HA) or FLAG epitope tagged GPCRs and checked the specificity of GPCR antibodies by use of FLAG or HA antibodies as the positive controls. We would suggest that at least two approaches should be taken to more stringently define antibody specificity. The approaches outlined here for validation of antibody specificity are applicable for any immunological techniques. Antibodies suitable for IHC may not be appropriate for IB and vice versa, therefore validation for IHC and IB should be performed separately. This is because in each application samples are treated differently, which may affect the epitopes exposed on the target protein and this, in turn, may profoundly influence binding of antibodies to the target protein. Previously, antibodies pre-absorbed with an excessive amount of the antigen were commonly used as the controls to validate specificity of antibodies, which is now proven to be

inadequate because it eliminated staining of not only the target protein, but also many other proteins which may have similar epitopes.

For GPCRs for which highly selective radiolabeled ligands are available, it is generally accepted that receptor autoradiography results yield the most reliable neuroanatomical distribution of the receptor, albeit with low resolution. Two highly selective KOR agonists, [^{3}H]U69,593 and [^{3}H]CI977, have been used as the radioligands for autoradiography (Mansour et al. 1994; Slowe et al. 1999; Unterwald et al. 1991). IHC staining should produce similar distribution as receptor autoradiography. Yet, it has to be kept in mind that receptor autoradiography only detects receptors with binding activity. Internalized or inactive receptors, which may be targeted by IHC, are not visualized.

3 Antibodies for IHC of the KOR

IHC of the KOR has been carried out with antibodies raised against synthetic peptides corresponding to partial sequences of N- and C-terminal domains the KOR [for example, (Appleyard et al. 1997; Arvidsson et al. 1995; Drake et al. 1996; Mansour et al. 1996)]. A synthetic peptide was conjugated to a carrier protein and polyclonal antibodies were generated with conventional methods. KT2 and KOR1 antibodies, both from rabbits, are discussed here because they are more widely used in IHC. KT2 and KOR1 antibodies were raised against 371–380 and 366–380 peptides in the C-terminal domain of the rat KOR, respectively.

3.1 Characterization of KT2 Antibody and KOR1 Antibodies for IHC

Because the two antibodies were used mostly for IHC, their characterizations for IHC are described. Drake et al. (1996) reported that in ICC, KT2 antibodies label the outer membranes of *Xenopus* oocytes transfected with the rat KOR, but not the untransfected ones. IHC of rodent brain sections with KT2 showed staining in central grey and spinal cord, which was abolished when KT2 antibodies were pre-absorbed with the antigen.

Arvidsson et al. (1995) observed that in ICC, KOR1 antiserum stained Cos-7 cells transfected with HA-conjugated rat KOR (HA-rat KOR) in a similar manner as HA antibodies, but did not stain cells transfected with HA-MOR or HA-DOR. The antigen peptide [KOR(366–380)] blocked the staining with KOR1 antibodies in brain sections and in transfected cells, but shorter peptides [KOR 366–373, 369–376 and 374–380] did not.

At the time of publication, neither antibody was tested in KOR knockout mice in IHC.

3.2 IHC of the KOR in the Brain

IHC of the KOR in the brain was performed on guinea pig brain sections with KOR1 or KT2 antibodies (Arvidsson et al. 1995; Drake et al. 1996) because of higher levels of KOR in this species (Mansour et al. 1988). As discussed by Drake et al. (1996), the KOR1 and KT2 antibodies labeled several brain regions found to have KOR binding by receptor autoradiography, including the substantia nigra, nucleus accumbens, basal forebrain, and endopiriform nucleus. However, neither KT2 nor KOR1 labeled the claustrum, which has the highest level of binding of [^{3}H]U69,593 in the guinea pig brain (Unterwald et al. 1991; Wang et al. 2011), or the cerebellum, which expresses a moderate level of KOR in this species (Unterwald et al. 1991). Distributions of KOR1 and KT2 immunoreactivities (KOR1-IR, KT2-IR, respectively) showed significant differences. KOR1-IR, but not KT2-IR, was present in the diagonal band, suprachiasmatic nucleus, supraoptic nucleus, and VTA. Conversely, KT2-IR, but not KOR1-IR, was observed in the central grey and lateral septum. At the electron microscopy level, KOR1-IR appeared predominantly postsynaptic since it was localized to cell bodies and dendrites, whereas KT2-IR was found mostly in processes with varicosities, which had the appearance of axons (Arvidsson et al. 1995; Drake et al. 1996).

The reasons for the discrepancies in staining patterns of KOR1 and KT2 antibodies are not clear. The findings suggest that antibodies, even raised against similar antigens, may recognize different epitopes, which underscores the difficulties associated with raising specific antibodies against GPCRs.

3.3 Generation of a Mouse Line Expressing a Fusion Protein of the KOR Conjugated with tdTomato (KOR-tdT)

To circumvent the issues associated with KOR antibodies, Chen et al. (2020) generated a mouse line expressing the KOR conjugated in-frame with tdTomato 5′ to the stop codon (KOR-tdT) to facilitate identification of KOR-containing neurons. Clearing of whole brains with CLARITY revealed 3-dimensional (3-D) images of distribution of KOR for the first time. It was also the first 3-D image of a GPCR distribution in the rodent brain. 3-D brain images of KtdT and IHC on brain sections with antibodies against tdTomato show similar distribution to that of autoradiography of [^{3}H]U69,593 binding to KOR in wildtype mice. KOR was visualized at the cellular level, such as co-localization with tyrosine hydroxylase (TH) and agonist-induced KOR translocation into intracellular space in some ventral tegmental area (VTA) neurons. These mice thus represent a powerful and heretofore unparalleled tool for neuroanatomy of KOR at both the 3-D and cellular levels.

4 KOR Antibodies for IB

In this section, antibodies for IB of KOR generated by Chen et al. (2016) are described. They generated antibodies against the rat/mouse KOR peptide (368–380) in rabbits (PA847) and in guinea pigs (5698) and purified each with antigen affinity chromatography (PA847p and 5698p, respectively). The antibodies were fully characterized for IB only.

4.1 Detection of KOR Expressed in Cells

In IB of CHO cells stably transfected with FLAG-human KOR (FLAG-hKOR), rabbit KOR antibodies PA847p recognized a broad and diffuse protein band of Mr. 52 kDa and a less diffuse band of 42 kDa, which were absent in untransfected CHO cells (Fig. 1a). When IB was performed with anti-FLAG antibodies [Purified rabbit anti-FLAG antibody (F7425), Sigma Aldrich], two protein bands with the same M_r's were detected (Fig. 1a). The M_r 52-kDa band is a full glycosylated form

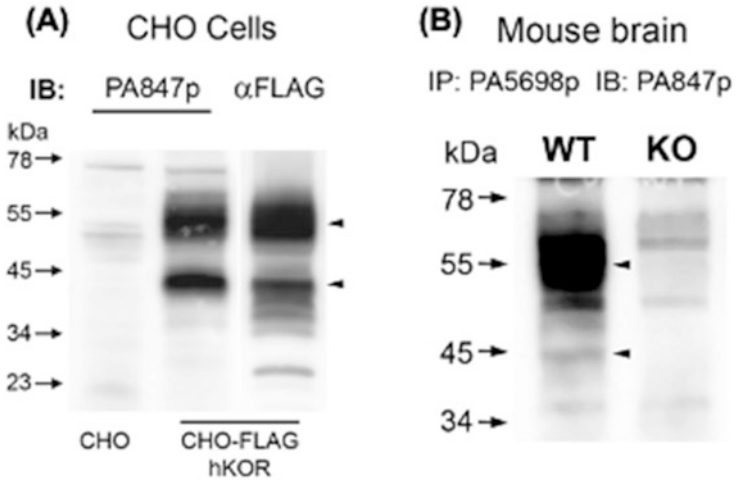

Fig. 1 (**a**) Antigen affinity chromatography-purified rabbit antibodies against mouse KOR (371–380) peptide (PA847p) recognize human KOR expressed in CHO cells in immunoblotting with high specificity. CHO-FLAG-hKOR cells and CHO cells were solubilized with Laemmli buffer and subject to SDS-PAGE. Immunoblotting with PA847p revealed two protein bands (indicated by arrow heads) of Mr 52 kDa and Mr 42 kDa in CHO-FLAG-hKOR cells, which were not present in CHO cells. Anti-FLAG antibodies and PA847 recognized the same two protein bands of Mr 52 kDa and Mr 42 kDa in CHO-FLAG-hKOR cells. (**c**) Immunoblotting of KOR in mouse brains with rabbit antibodies against mouse KOR(371–380) peptide (PA847p) revealed two bands (indicated by arrow heads) of Mr 55 kDa and Mr 45 kDa in wildtype (WT) brains, but not in KOR−/− (KO) brains. Mouse brains (with cerebella removed) were solubilized and KOR was immunoprecipitated with guinea pig antibodies against mouse KOR(371–380) (5698p) followed by SDS-PAGE and immunoblotting with rabbit antibodies PA847p. Each experiment was performed twice with similar results

of the KOR, whereas the M_r 42 kDa band represents immature forms of the KOR (Li et al. 2007). The broad and diffuse nature of the 52-kDa band is due to heterogeneity of glycosylation (Li et al. 2007).

4.2 Detection of the KOR in Mouse Brains

Detection of the KOR in mouse brains by IB is much more challenging than in cells because of the very low KOR expression level as well as the great complexity of protein constituents in brains. When KOR contents in brain membranes are calculated based on the B_{max} value of [^{3}H]U69,593 binding reported by Kitchen et al. (1990) (7.3 fmol/mg protein) and molecular weight of the protein backbone of ~42 kDa (KOR has 380 amino acids in length), the KOR constitutes only 0.000031% of total brain membrane proteins, making its detection difficult. Liu et al. (2020) thus partially purified KOR from solubilized brain membranes by IP with purified guinea pig anti-KOR (5698p) followed by IB with PA847p rabbit anti-KOR. Compared with brains of KOR knockout mice, wildtype mouse brains had one broad band of high intensity of ~55 kDa and a sharper band of light intensity of ~45 kDa (Fig. 1b), which most likely represent fully glycosylated and immature forms of the KOR, respectively. The differences in Mr's between CHO cells and brains are likely due to variations in the extent of glycosylation, similar to what was observed for the MOR (Huang et al. 2008; Huang and Liu-Chen 2009; Liu-Chen et al. 1993). The much higher level of the immature form of the KOR in CHO cells may be due to the stronger CMV promoter in the KOR plasmid transfected into CHO cells. It is noteworthy that even in the KOR knockout, there are immunoreactive bands, demonstrating that it is crucial to have tissues from KOR knockout mice as a control to discern the truly positive bands.

5 Antibodies for IB of Phosphorylated KOR

Following agonist activation, GPCRs are phosphorylated by G protein-coupled receptor kinases (GRKs) and second messenger-activated protein kinases, such as protein kinase A and protein kinase C. Antibodies specifically recognizing phosphorylated form of GPCRs are useful research tools. Importantly, phospho-specific antibodies should recognize phosphorylated GPCRs, but not unphosphorylated GPCRs. Specificity of phospho-specific antibodies should be validated by at least two of the following experiments: 1) staining of the phospho-specific antibodies should be increased by a prototypic agonist that is known to induce receptor desensitization and internalization. 2) staining of the phospho-specific antibodies is abolished by treatment of samples or IB transfer membranes with phosphatase. 3) staining of the phospho-specific antibodies is abolished by mutation of the phosphorylation site to Ala. 4) in IB, phospho-specific GPCR antibodies should recognize protein bands of similar apparent M_r as GPCR antibodies.

5.1 Detection of Phosphorylated KOR in Cells

Chen et al. (2016) determined the sites of U50,488H-promoted mouse KOR (mKOR) phosphorylation to be S356, T357, T363 and S369 in the C-terminal domain. Antibodies were generated against three phosphopeptides (pS356/pT357, pT363 and pS369) and purified first with phospho-peptide affinity chromatography followed by adsorption with unphosphorylated peptide affinity beads to enhance specificity against the phosphorylated peptide. The antibodies were fully characterized for IB only.

Using mouse neuro2a neuroblastoma (N2A) cells stably transfected with FLAG-tagged mouse KOR conjugated with 6 x His (N2A-FmK6H cells), Chen et al. (2016) demonstrated that following U50,488H treatment and IP of KOR with FLAG antibodies, IB with each of rabbit anti-pS356/pT357, anti-pT363 and ant-pS369 revealed a high-intensity broad and diffuse band of ~52 kDa in U50,488H-treated samples (Fig. 2a, phosphorylated mKOR). In saline-treated samples, there was no staining with anti-pT363 and ant-pS369; however, there was a faint staining with anti-pS356/pT357, suggesting basal phosphorylation (Fig. 2a). Blots were then stripped and re-blotted with rabbit anti-FLAG antibodies to stain total KOR. mKOR was revealed as a diffuse band of 52 kDa (Fig. 2a, total mKOR) and the amounts of total KOR were not different between the saline- and U50,488H-treated groups. In N2A-FmK6H cells, the intensity of the immature mKOR form was low, like in mouse brains.

When transfer membranes were treated with lambda protein phosphatase, which dephosphorylates phosphoserine, phosphothreonine and phosphotyrosine, U50,488H-promoted staining in the 52-kDa protein band by anti-pS356/pT357, anti-pT363 and anti-pS369 was eliminated (Fig. 2a, dephosphorylated mKOR). These results indicate that the immunoreactivity of the 52-kDa band is due to phospho-KOR.

S356A, T357A or S356A/T357A substitution abrogated anti-pSr356/pT357 staining in control and U50,488H-treated mKOR (Fig. 2b). T363A and S369A mutations of the FmK6H eliminated U50,488H-induced staining by anti-pT363 and anti-pS369, respectively (Fig. 2b).

Thus, anti-pS356/pThr357, anti-pT363 and anti-pS369 react with mKOR phosphorylated at S356/T357, T363 and S369, respectively. These results validate further the specificity of antibodies for phosphorylated KOR.

5.2 Detection of Phosphorylated KOR in Mouse Brains

In mouse brains, phosphorylated KOR has to be enriched by IP before IB because of the low level of KOR. KOR in solubilized membranes was immunoprecipitated with guinea pig antibodies against KOR(368–380) (5698p) and IB was performed with the rabbit anti-pT363 or anti-pS369 for detection of phosphorylated KOR or rabbit antibodies against KOR(368–380) for total KOR (PA847p). Guinea pig antibodies were used for IP, whereas rabbit antibodies were used for IB to avoid cross

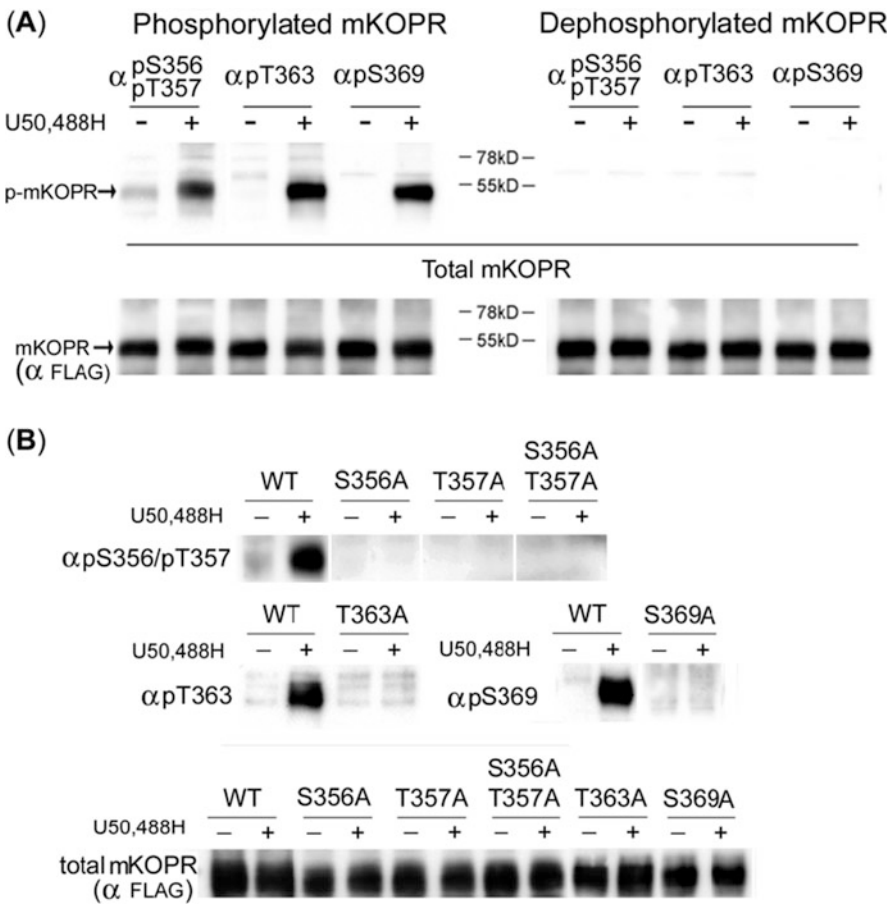

Fig. 2 (**a**) Phospho-peptide antibodies have high specificity for phosphorylated KOR. *left*: *U50,488H greatly enhanced anti-pS356/pT357, anti-pT363 and anti-pS369 immunoblotting intensity of the mKOR.* Murine neuro2A neuroblastoma cells stably transfected with FLAG-tagged mouse KOR conjugated with 6 x His (N2A-FmK6H) were treated with vehicle or 10 μM U50,488H for 30 min. Cells were solubilized and the receptors were partially purified with a Ni-NTA agarose column. Eluates were subject to SDS-PAGE and immunoblotting with indicated antibodies. Membranes were stripped and re-blotted with anti-FLAG antibodies for total KOR. The experiments were performed three times with similar results. Untransfected N2A cells were subjected to similar treatment and immunoprecipitation procedures, and none of anti-pS356/pT357, anti-pT363 and anti-S369 detected a 52-kDa band in immunoblotting (data not shown). *right*: *Immunoblotting intensity was greatly reduced by dephosphorylation*. Experiments were performed as described above, except that PVDF membranes with transferred proteins were incubated with lambda phosphatase and then subject to immunoblotting with indicated antibodies. Membranes were stripped and re-blotted with anti-FLAG antibodies for total KOR. Phosphatase treatment reduced staining of U50,488H-treated samples, indicating phospho-specificity of the antibody. In addition, phosphatase reduced pS356/pT357 staining in the control, demonstrating constitutive phosphorylation of the sites. These experiments were performed four times with similar results. (**b**) Effects of mutations in the mKOR on U50,488H-promoted receptor phosphorylation detected with phospho-KOR antibodies. The cDNA construct of the wildtype, S356A, S357A, S356A/S357A, T363A or S369A mutant of the mKOR was transfected into N2A cells and stable mixed clonal cells were established. Cells were treated with vehicle or U50,488H (10 μM) for

reactivities. Wildtype or KOR knockout C57BL/6J mice were treated with saline or U50,488H (10 mg/kg, s.c.) and killed 30 min later and brains were dissected and frozen on dry ice immediately. Solubilization of brains, IP of KOR and IB of phosphorylated KOR were performed as described (Liu et al. 2020) (see Fig. 3 legend). In wildtype C57BL/6J mice, U50,488H treatment greatly increased the intensity of anti-pT363 and anti-pS369 staining in brains (Fig. 3a). In contrast, in the brains of KOR knockout mice, there is no staining by either antibody following saline or U50,488H pretreatment, indicating specificity for the phosphorylated KOR. Staining with anti-pS356/pT357 was not performed because T363 and S369 are the two primary phosphorylation sites and are phosphorylated before S356/T357 (Chen et al. 2016). Without enrichment with IP, it was not possible to detect phosphorylated KOR with IB.

5.3 U50,488H Promoted KOR Phosphorylation at T363 and S369 in Mouse Brains in a Dose-Dependent Manner

Male CD-1 mice were treated with saline or 1, 2, 4, 6 or 10 mg/kg U50,488H (s.c.) and killed 30 min later and brains were removed and frozen on dry ice immediately. KOR phosphorylation following IP of KOR was detected with IB as described above. As shown in Fig. 3b, U50,488H promoted KOR phosphorylation at T363 and S369 in a dose-dependent manner. The staining intensities of phosphorylated KOR were normalized against that of the total KOR in respective lanes, which were then normalized against that at 10 mg/kg U50,488H (Fig. 3c). The EC_{50} values of U50,488H-induced KOR phosphorylation were estimated to be ~1.5 mg/kg for T363 and ~ 3 mg/kg for S369 (Fig. 3c).

6 KOR Antibodies from Commercial Sources

Many antibodies generated against KOR peptides are available from commercial sources with varying levels of validation, but there have not been systematic studies as for other GPCRs [for example, [(Michel et al. 2009) and other articles in the same issue]. The burden of specificity validation is on researchers, who have to perform experiments to check antibody specificity in their own particular application(s).

Fig. 2 (continued) 30 min, harvested and receptor proteins were purified and resolved with SDS-PAGE. Immunoblotting was performed with the indicated antibodies. The amount of total mKOR was determined with another gel loaded with the same aliquots. S356A, S357A or S356A/S357A substitutions abolished basal and U50,488H-promoted mKOR phosphorylation detected by anti-pS356/pT357. T363A and S369A mutations eliminated mKOR phosphorylation detected by anti-pT363 and anti-pS369 staining, respectively. The experiments were performed two times with similar results (from Chen et al. 2016)

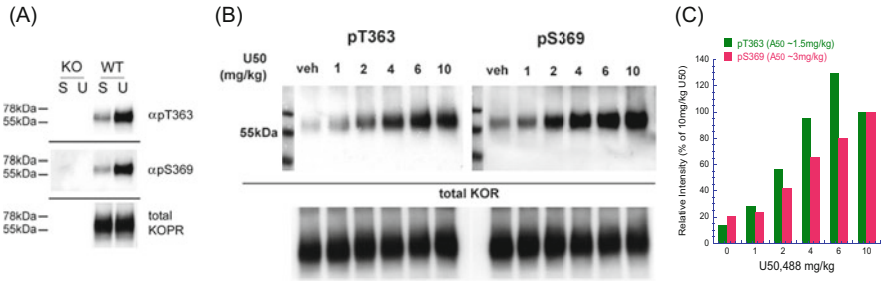

Fig. 3 Detection of U50,488H-promoted KOR phosphorylation in mouse brains by immunoblotting. (**a**) In immunoblotting, anti-pT363 and anti-pS369 antibodies are specific for phospho-KOR in mouse brains. Wildtype (WT) and KOR−/− (KO) C57BL/6 adult mice were injected with saline or U50,488H (10 mg/kg, s.c.) and euthanized 30 min later and brains were immediately removed and frozen on dry ice. Four brains were pooled as one sample because of the low expression level of KOR. Brains were solubilized with 2% dodecyl-β-D-maltoside (DDM) and centrifuged and the supernatant was incubated with Pansorbin to remove proteins interacting with protein A and centrifuged again. KOR in the supernatant was immunoprecipitated with guinea pig antibodies against the KOR(371–380) peptide (custom-generated, Ab5699) (2 μg/ml, overnight, 4°C) followed by goat anti-guinea pig IgG conjugated to agarose. Pelleted agarose beads were washed extensively and KOR was dissociated from agarose with Laemmli buffer. The mixture was resolved with SDS-PAGE followed by IB with rabbit anti-pT363 and anti-pS369 antibodies. In the wildtype, the p-KOR band appeared as a broad diffuse band with a median Mr of 60 ~ kDa and U50,488H treatment greatly enhanced the staining. KOR−/− mice did not show any staining in saline- or U50,488H-treated group. The blot was then stripped and re-blotted for total KOR with purified rabbit antibodies against the KOR(371–380) peptide (PA847p). Thus, anti-pT363 and anti-pS369 are specific for the phosphorylated KOR in the mouse brain. The experiment was performed twice with similar results. S: saline; U: U50,488H; WT, wildtype; KO, KOR−/−. (**b**), U50,488 dose-dependently promoted KOR phosphorylation at T363 and S369 in mouse brains as detected by immunoblotting with phospho-specific antibodies. Male CD-1 mice were injected (s.c.) with saline or an indicated dose of U50,488H (1, 2, 4, 6, or 10 mg/kg) and euthanized 30 min later. Brains were immediately removed and frozen. KOR was partially purified by immunoprecipitation, resolved with SDS-PAGE and IB was performed with rabbit anti-pT363 and anti-pS369 antibodies. The blot was then stripped and re-blotted for total KOR with purified rabbit antibodies against the KOR (371–380) peptide (PA847p). Experiments were performed twice with similar results. (**c**) Quantification of (**b**). Intensity of protein bands were quantified with ImageGauge software. p-KOR staining intensity was normalized against that of the total KOR in the same lane. The resulting data were then normalized against those of 10 μM U50,488H**.** Data are the mean of two samples, each from 4 mouse brains

Anecdotal evidence indicates that researchers often found that the antibodies were not specific after spending money and devoting time and efforts.

7 Antibodies against Dynorphins: Some Considerations

Dyn peptides, like other neuropeptides, are synthesized as large precursors (Watson et al. 1983) and sorted into large dense core vesicles. There they are processed by proteolytic cleavage and subsequent modifications like amidation to yield mature

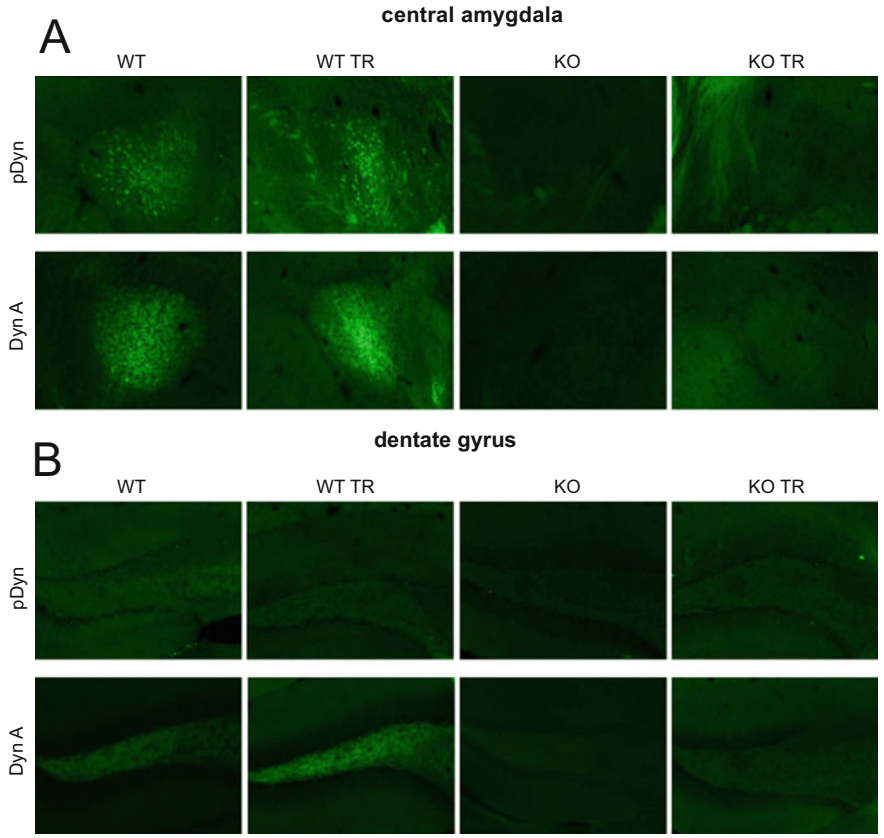

Fig. 4 Immunofluorescence images comparing antibodies against pDyn and Dyn A in central amygdala (**a**) and dentate gyrus (**b**) of WT and Dyn KO mice. For the optional antigen retrieval step, free-floating PFA-fixed 40 μm coronal brain sections were incubated in a 10 mM sodium citrate solution (pH 8.7) in an 80°C water bath for 20 min. After blocking, the sections were incubated with primary antibodies against pDyn 1:1,000 (Neuromics, host guinea pig, GP 10110, lot 100,031) or Dyn A 1:2,000 (Peninsula, host rabbit, T-4268, lot 06613) overnight at room temperature. Following washes, Alexa Fluor488 goat anti-guinea pig 1:1,000 (A11073, lot 1,637,243) was applied as secondary antibody for pDyn and Alexa Fluor488 donkey-anti-rabbit 1:1,000 (A21206, lot 2,156,521) for Dyn A for 2.5 h at room temperature. Images were acquired using a ZEISS Axio Imager M1 wide-field fluorescence microscope with a 20x objective. *WT* wildtype, *KO* pDyn knockout mice, *TR* target retrieval

functional peptides [for a review, see (Schwarzer 2009)]. The precursor prodynorphin (pDyn) is processed to produce dynorphin A, dynorphin B, α-neoendorphin, and β-neoendorphin. For IHC, this provides high antigen density within these vesicles. In contrast to the membrane bound GPCRs, peptides are well protected from a direct influence of fixation of tissue. Still, target retrieval may enhance the permeability of membranes (cells and vesicle) and enhance signal intensity (Fig. 4). Interestingly, different antibodies yield partially contradictive results in IHC, irrespective of the fixation protocol applied. The processing of the

precursor peptides depends on the presence of enzymes needed for maturation within the vesicle. This results in differently sized intermediate and mature peptides along the axon or between different neuronal populations. One example was reported from the pituitary gland. In the posterior lobe, processing to mature peptides appeared almost complete. In contrast, predominantly larger precursor fragments were isolated from the anterior lobe (Day and Akil 1989; Seizinger et al. 1984). Coexistence of pDyn and dynorphins in the same axon, even the same vesicle, was also reported from brain (Yakovleva et al. 2006). Available antibodies target different regions of the precursor and may be affected by the processing in opposite directions. Endo- and exoproteolytic processing may destroy the antigen, resulting in loss of signal. By contrast, some antibodies detect only free ends of peptides, thereby depending on the processing to generate the antigen (Fig. 4). Likewise in the hippocampus, pDyn is highly expressed in granule cells (Hurd 1996), but hard to detect with antibodies against pDyn. By contrast antibodies against mature DynA nicely label the axons of granule cells (Fig. 4). Processing of the propeptide also is reflected in the appearance of differently sized fragments in IB. The specificity of antibodies targeting mature dynorphins can hardly be controlled in IB. Therefore, KO animals are an essential control. Antibodies targeting mature DynA (Peninsula) or DynB (Peninsula and ABD serotec) and those targeting pDyn (Avivasysbio, Neuromics) or the middle segment of pDyn (Acris) yielded similar results, yet with some discrepancies (see Fig. 4b) and clear batch to batch variability.

8 Conclusion

When antibodies against the KOR, Dyn peptides or proDyn are used, it is critical to validate specificity of the antibodies in the intended application using exactly the same conditions for the experiments. Experimental conditions (most importantly antibody dilution) need to be optimized to minimize the background and maximize the signal and, in the process, optimal conditions may be achieved to have no staining in knockout mice. Proper controls have to be performed, including knockout mice, siRNA knockdown, transfected vs. untransfected cells, transfected cells expressing the target vs. closely related molecules. Knockout mouse samples are considered the most important controls for experiments involving animal tissues.

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Conflict of Interest The authors have no conflict of interest.

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Part II

In Vitro Studies

Structural Characterization of KOR Inactive and Active States for 3D Pharmacology and Drug Discovery

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Abstract

The structure of the human kappa opioid receptor (KOR) in complex with the long-acting antagonist JDTic was solved crystallographically in 2012 and, along with structures of other opioid receptors, revolutionized our understanding of opioid system function and pharmacology. More recently, active state KOR structure was also determined, giving important insights into activation mechanisms of the receptor. In this review, we will discuss how the understanding of atomistic structures of KOR established a key platform for deciphering details of subtype and functional selectivity of KOR-targeting ligands and for discovery of new chemical probes with potentially beneficial pharmacological profiles.

Keywords

3D pharmacology · GPCRs · Kappa opioid receptor · Protein structure · Structure-based drug design · Virtual ligand screening

1 Introduction

Deciphering the atomistic structural details of proteins and their complexes has always been a critical aspect in studying signal transduction in many physiological pathways, as it provides a key framework for understanding molecular function, designing new molecular tools to interrogate this function, as well as for discovery on new drug candidates. For the 7-transmembrane G protein-coupled receptors (GPCRs), this structural revolution has started only recently in 2007 (Cherezov et al. 2007; Rosenbaum et al. 2007; Warne et al. 2008), owing to several major technological breakthroughs that include the design of more stable receptors (Chun et al. 2012) and lipidic cubic phase (LCP) crystallization methods (Cherezov 2011). By 2012, only seven GPCRs had their structures solved (Katritch et al. 2012a). Therefore, a publication of a structural portrait of the full opioid receptor family that year, including human KOR (Wu et al. 2012) and nociception receptor (NOP) (Thompson et al. 2012), as well as mouse μ receptor (MOR) (Manglik et al. 2012) and δ receptor (DOR) (Granier et al. 2012) signified the beginning of a new era of comprehensive structural coverage of GPCR families.

The opioid receptor structures were immediately put to use for analysis of GPCR function (Katritch et al. 2013, 2014; Venkatakrishnan et al. 2013), explaining the activity of opioid drugs (Vardy et al. 2013) and discovery of new ligands (Negri et al. 2013; Zheng et al. 2017) The KOR structural study in particular (Wu et al. 2012) was cited more than 900 times according to Google Scholar. The KOR structure was solved in complex with a promising drug candidate for the treatment of cocaine abuse and other neurological disorders, JDTic (Carroll and Carlezon 2013), which is a highly selective and long-acting KOR antagonist, and therefore provided a key template for the design of new KOR antagonists (Negri et al. 2013; Zheng et al. 2017). At the same time, it gave important insights into the plasticity of the binding

pocket in opioid receptors, as JDTic induced a unique conformation of the pocket (Vardy et al. 2013; Zheng et al. 2017), potentially associated with unique functional features of this long-acting ligand (Bruchas et al. 2007). Moreover, KOR structure, along with other opioid receptor structures, revealed highly conserved water interaction networks in the orthosteric pocket, critical for binding of both endogenous ligands and drugs (Goldfeld et al. 2015; Vardy et al. 2013).

In 2018, the structural information on KOR was greatly expanded by the determination of an active-state crystal structure of KOR in complex with the highly potent agonist MP1104 (Varadi et al. 2015) and stabilizing nanobody Nb39 (Che et al. 2018). While exhibiting many common features with MOR-Nb39 complex (Huang et al. 2015, 2021), the KOR active structure revealed a number of important selectivity features, contributed to understanding activation mechanisms, and enabled the design of new series of derivatives with beneficial pharmacological profiles (e.g. Uprety et al. 2021). Most recently, another structure of inactive-state KOR was solved in complex with Nb6 nanobody, selectively stabilizing the inactive state of the receptor and demonstrating the new mode of nanobody binding (Che et al. 2020).

This review will discuss structures available for KOR, their direct impact on the kappa opioid field, and the novel opportunities opened by this 3D structural framework for computational modeling studies of receptor dynamics and for structure-based ligand discovery.

2 Structure Determination of KOR

2.1 KOR-JDTic Structure in Inactive State

Currently, three distinct structures of KOR are publicly available (Fig. 1). The structure elucidation of JDTic-bound human KOR (Wu et al. 2012) (PDB ID 4DJH) solved in 2012 was enabled by several technological breakthroughs in GPCR crystallography. One of the most crucial of them was membrane protein crystallization in lipidic cubic phase (LCP), which allowed crystal formation in a lipid-like environment and has been used for a vast majority of GPCR structures. Similar to adrenergic β2-AR (Rosenbaum et al. 2007) and several other GPCR structures, inactive state human KOR was stabilized by replacing its intracellular loop 3 (ICL3) by soluble T4L phage lysozyme. The flexible N-terminus (42 amino acids) and C-terminus (21 amino acids) were truncated, and a point mutation Ile135$^{3.29}$Leu was introduced to increase protein expression. Interestingly, JDTic itself plays a major role in the thermostabilization of KOR for crystallization, potentially due to its exceptionally long off-rates. No other antagonist has been resolved in complex with KOR yet, though such structure could contribute to the understanding of binding of many short-acting antagonists including morphinans and drug candidates like LY2456302 (Urbano et al. 2014) (see Sect. 7.1).

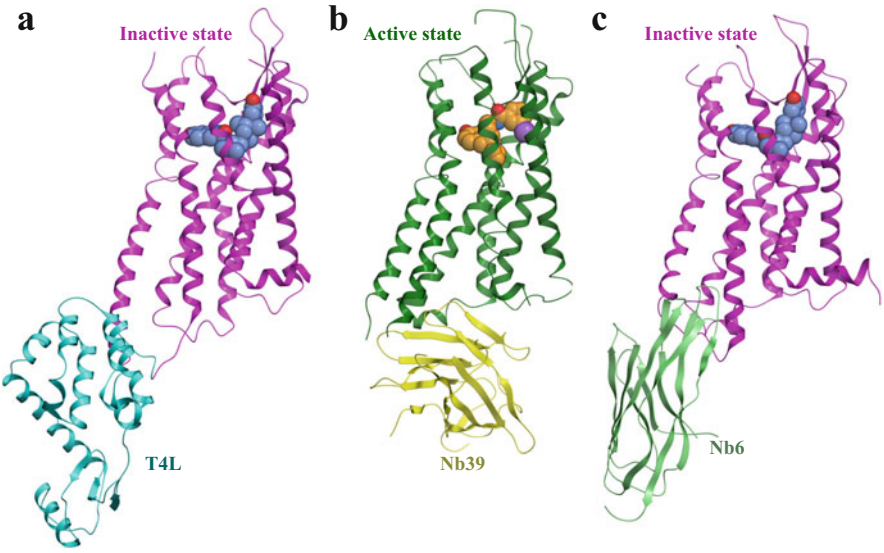

Fig. 1 Crystal structures available for KOR: (**a**) KOR-JDTic complex in an inactive state (PDB ID 4DJH, resolution 2.9 Å), (**b**) KOR-MP1104 complex, stabilized in the active state by Nb39 nanobody (PDB ID 6B73, resolution 3.1 Å), and (**c**) KOR-JDTic stabilized in inactive state by Nb6 nanobody (PDB ID 6VI4, resolution 3.3 Å)

2.2 KOR-MP1104 Structure in Active State

Determination of agonist-bound KOR (Che et al. 2018) (PDB ID 6B73) required further stabilization of the receptor in an active state using nanobody technology. Small single-chain nanobodies have been developed for structural elucidation of several other GPCRs in an active state, for example, active-state structure of MOR used Nb39 nanobody. The same Nb39 nanobody, inserted into the conserved G protein binding cavity on the intracellular side of the receptor was found to stabilize active-state conformations of KOR (Che et al. 2018). In this crystallization construct, the human KOR was also modified by truncation of C-terminal (ΔArg359-Val380) and a single point mutation Ile135Leu. However, instead of T4L fusion in ICL3 in the inactive state construct, the new construct had the N-terminus residues (Met1-His53) replaced with the thermostabilized apocytochrome b_{562} RIL (BRIL) (Chun et al. 2012), thus helping to avoid unwanted interference of ICL3 fusion with nanobody binding. The structure was solved in complex with high affinity/high potency agonist MP1104, based on morphinan scaffold (Varadi et al. 2015).

2.3 JDTic-KOR-Nb6 Structure in Inactive State

The most recent structure of KOR used the same construct as KOR/MP1104/Nb39 active-state structure but was stabilized in the inactive state by antagonist JDTic and another antibody Nb6 (Che et al. 2020) (PDB ID 6VI4). The receptor conformation was found practically identical to the original KOR-JDTic complex, despite different constructs, crystal packing, and the presence of Nb6 nanobody. However, it showed the utility of nanobodies selectively binding inactive or active states of GPCRs in both crystallographic and fluorescent imaging studies, the latter allowing to monitor conformational states of the receptor in real-time.

3 Overall Architecture of the KOR in Inactive and Active States

The KOR and other opioid receptors belong to γ-branch of the rhodopsin-like Class A GPCRs, and as such share overall architecture with the large Class A family, as well as some common features attributed to the peptide-binding receptors. The KOR has a canonical serpentine arrangement across the plasma membrane, where seven transmembrane (TM) α-helices are connected by three extracellular and three intracellular loops (ECL and ICL, respectively). Like many other class A receptors, KOR structure has a β-hairpin loop formed in the large extracellular loop ECL2, which is stabilized by a conserved disulfide bond to $C131^{3.25}$ at the top of TM3 (superscript shows Ballesteros-Weinstein numbering for GPCRs (Ballesteros and Weinstein 1995)). The structured ECL2 harnessed to the top of TM3 keeps the orthosteric ligand pocket wide open, allowing binding and specific recognition of bulky endogenous peptides.

The conformation of the truncated N-terminal region in KOR upstream from TM1 remains unknown, however stabilizing effects of its truncation or replacement by BRIL suggest that it is likely to be unstructured, at least in the absence of peptide ligands. It still might play a role in selective recognition of endogenous KOR ligand dynorphins, which have excess positive charges on their N-terminal region potentially complementing the negative charge on the KOR N-terminal domain, though details of this interaction remain to be studied. Supporting mobility of the N-terminal domain, KOR does not have a Cys residue in the N-terminus region proximal to TM1, nor a Cys in ECL3, which often form a conserved disulfide bond in other peptide-binding GPCRs like angiotensin, apelin, chemokine, and endothelin receptors.

On the intracellular side, KOR has relatively short intracellular loops (4, 8, and 5 amino acids, respectively), which are highly conserved among opioid receptors. All three loops were found well-structured in the KOR inactive structure with N-terminal BRIL fusion that keeps ICL3 intact (PDB ID 6VI4). In the Nb39-bound active state structure of the same construct, however, the large movement of TM6 disrupts the stable conformation of ICL3, leaving five residues on the tip of the loop unresolved. The ICL1 maintains its conformations in all three inactive and

active state KOR structures. The ICL2 maintains two α-helical turns in all three structures, however, is shifted by as much as 6 Å in the structure with T4L fusion as compared to structures lacking this fusion. The helix VIII, similar to many other GPCRs has about three α-helical turns in the inactive state KOR structures, while only one turn of this helix was resolved in the active state KOR, suggesting its higher mobility. Overall, both inactive and active state structures of KOR are quite similar to the corresponding structures of MOR and DOR, allowing a robust comparison of those details specific to KOR.

4 Structural Determinants of Long-Acting Antagonist JDTic Binding in Inactive Structure

The inactive state KOR was co-crystallized with JDTic (Carroll et al. 2004), a very high affinity, potent, and selective antagonist with a long duration of action (Fig. 2a). In the structure, JDTic sits deep in the orthosteric KOR pocket and stretches across the receptor, interacting with residues from all transmembrane helices except TM1. The ligand is engaged in extensive ionic, polar, and hydrophobic interactions, including (1) salt-bridges to D138$^{3.32}$ residue, (2) water-mediated hydrogen-bonding interactions with TM5 and ECL2, (3) complementary interactions to hydrophobic residues that form the "floor" of the orthosteric site in TM3, TM5, and TM6, (4) hydrophobic interactions in small sub-pocket between W287$^{6.48}$ and Y320$^{7.43}$, and (5) interactions with residues of a sub-pocket between TM2 and TM3.

The JDTic ligand has two basic amino groups in piperidine and isoquinoline, both predicted to be protonated at physiological pH. Both basic nitrogen atoms interact concurrently with D138$^{3.32}$ residue, as the ligand adopts characteristic V-shaped conformation inside the binding pocket. The isoquinoline moiety of JDTic is tucked in the hydrophobic pocket lined by non-polar side chains M142$^{3.36}$, V230$^{5.42}$, W287$^{6.48}$ in the bottom, and I290$^{6.51}$ and I294$^{6.55}$ in the top. Also, the hydroxy group in isoquinoline position 6 is directed towards the TM5 and forms hydrogen bonds with two conserved crystallographic waters, one bound to H291$^{6.52}$ side chain and K227$^{5.39}$ backbone carbonyl, and another to Y139$^{3.33}$ side chain. At the other end of the ligand, the phenoxy group interacts with structured waters coordinated by ECL2 backbone amides. Yet another water deep in the pocket, coordinated by D138$^{3.32}$ and N141$^{3.35}$, contributes to binding via interactions with the JDTic amide group. All these water-mediated interactions emphasize the importance of structured waters in the analysis of the high-affinity ligand binding and specificity.

Most remarkably, JDTIC inserts itself deep between residues D138$^{3.32}$ and Y320$^{7.43}$, in sharp contrast with MP1104-bound KOR, or DOR and MOR structures characterized with other opioid antagonists, where these two residues form a stable and conserved H-bond. The separation of D138$^{3.32}$ and Y320$^{7.43}$ in the JDTic-bound KOR structure involves ~2 Å outward movement of the TM2 backbone in this region. These induced-fit conformational rearrangements in KOR-JDTic complex and atypical two salt-bridge interactions with D138$^{3.32}$ likely contribute to the very slow off-rates and unique pharmacological profile of JDTic, including potent inverse

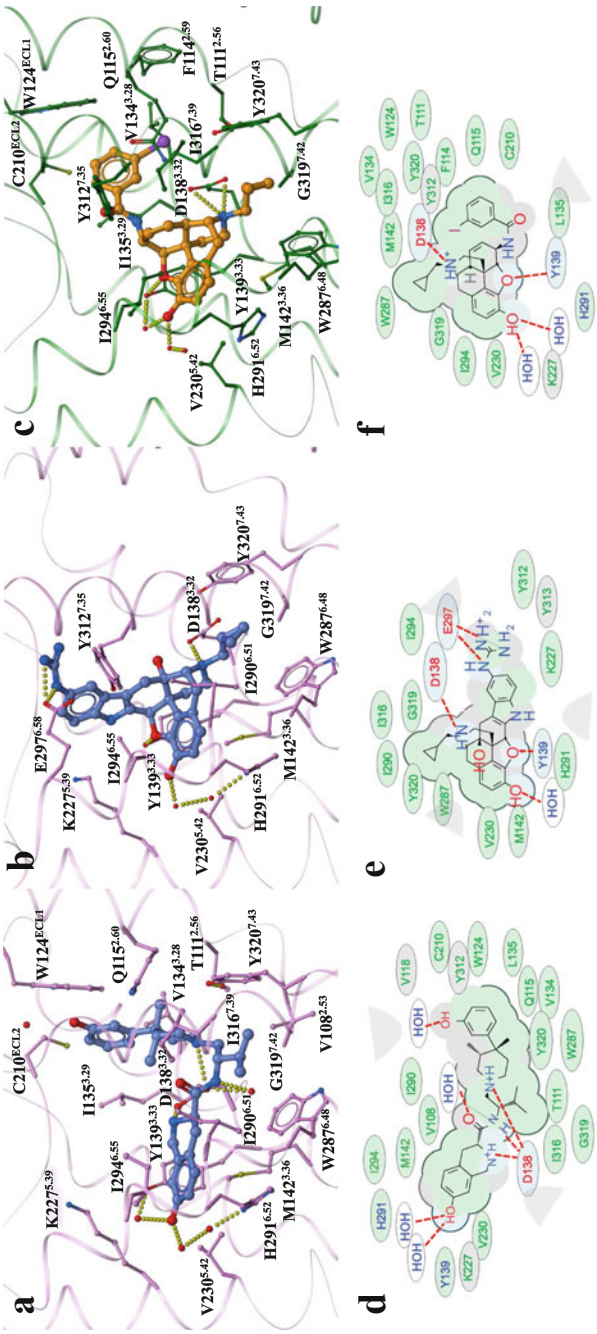

Fig. 2 Details of ligand binding into the KOR orthosteric pocket. (**a**) KOR-JDTic complex in inactive state (PDB ID 4DJH, resolution 2.9 Å), (**b**) KOR-GNTI docking model (Zheng et al. 2017), based on flexible optimization of the model based on DOR homology, and (**c**) KOR-MP1104 complex in the active state nanobody (PDB ID 6B73, resolution 3.1 Å)

agonist activity (Che et al. 2020). Note that more recently solved nanobody-stabilized KOR (Che et al. 2020) (PDB ID: 6VI4) confirms these peculiar features on JDTic binding, even though the structure was obtained with a different construct, a different crystal packing and complexed with Nb6 antibody (Che et al. 2020).

Importantly, the induced fit conformation observed in KOR-JDTic bound pocket, while very useful for docking and analysis of JDTic analogs, e.g.(Schmitt et al. 2015), makes it rather suboptimal for docking of morphinan-based compounds, as well as many other short-acting antagonists. To fill this gap, optimized models of KOR-morphinan binding have been developed and used in docking and structure-based discovery (Zheng et al. 2017) (Fig. 2b), as discussed below in Sect. 7.

5 Structural Determinants of Agonist MP1104 Binding in the Active-State Structure

The active-state KOR structure was obtained in complex with Nb39 nanobody and an epoxymorphinan derivative MP1104, which is a highly potent agonist with a sub-nanomolar affinity at KOR, while also potent agonism at MOR, with lower activity at DOR. The epoxymorphinan scaffold of MP1104 partially overlaps with the isoquinoline moiety of JDTic (see Fig. 2c) and also forms a similar salt-bridge anchor to the $D138^{3.32}$ side chain, which is consistent with other morphinan agonists, e.g. resolved in complex with MOR (BU72) (Huang et al. 2015). Somewhat unexpectedly, the mutation analysis performed in this study showed that D138A mutant of KOR which disrupts the salt bridge to the M1104 amino group maintains its high-affinity binding of MP1104, though reduces its functional potency. This suggested that $D138^{3.32}$, while being an important anchor of binding for many opioids, may be less critical for some agonists like MP1104 or SalA (Vardy et al. 2013). This observation is in good agreement with a slightly larger distance between MP1104 amine and $D138^{3.32}$ in this structure (3.0 Å) as compared to the usual 2.6–2.7 Å observed in the JDTic and other opioid agonists and antagonists. The epoxymorphinan scaffold of MP1104 is also well positioned for water-mediated hydrogen-bonding interactions to TM5, and though slightly lower resolution (3.1 Å) would not allow reliable assignment of waters in this structure, conserved structure in this region suggests a similar water-mediated network as in JDTic bound structure. On the other side of the MP1104 core, meta-iodophenyl moiety is tucked into the TM2-TM3 sub-pocket, which is lined by non-conserved residues $V118^{2.63}$ and conserved $W124^{ECL2}$, which can play a role in subtype and functional selectivity of KOR ligands (see Sect. 8.4).

Similar to the active-state structures of MOR (Huang et al. 2001, 2015, 2021) and DOR (Claff et al. 2019), the KOR-MP1104 structure is characterized by a finite contraction in the volume of the orthosteric site (~10%) as compared to JDTic-bound inactive state. The MP1104-induced contraction is slightly bigger in KOR, as compared to the contraction observed between morphinan-bound inactive and active MOP structures (6%). Another interesting observation is a slightly deeper binding of MP1104, which is likely connected to conformational changes in $M142^{3.36}$ and

W287$^{6.48}$ residues at the bottom of the binding pocket. Interestingly, these residues were also suggested to impact agonist efficacy, thus the strength of these interactions may provide a useful molecular link to the receptor activation mechanism.

6 Large-Scale Conformational Changes in KOR as a Part of the Activation Mechanism

Substantial large-scale rearrangements in KOR conformation can be observed between the JDTic-bound inactive state structure (Wu et al. 2012) and the active state MP1104-bound structure (Che et al. 2018) (Fig. 3). Thus, the orthosteric pocket of active state KOR undergoes considerable contraction due to the finite movements of TM2, TM5, and TM6 helices, as well as ECL2 towards the receptor core. The concerted contraction reduces the volume of the KOR orthosteric pocket from ~1,049 Å^3 in JDTic-bound inactive KOR to ~945 Å^3 in the MP1104-bound active KOR structure. Contraction of the pocket is one of the general features of agonist-induced activation observed in many GPCRs (Katritch et al., 2013; Venkatakrishnan et al. 2013), reflecting fast initial binding of agonists from an extracellular milieu into

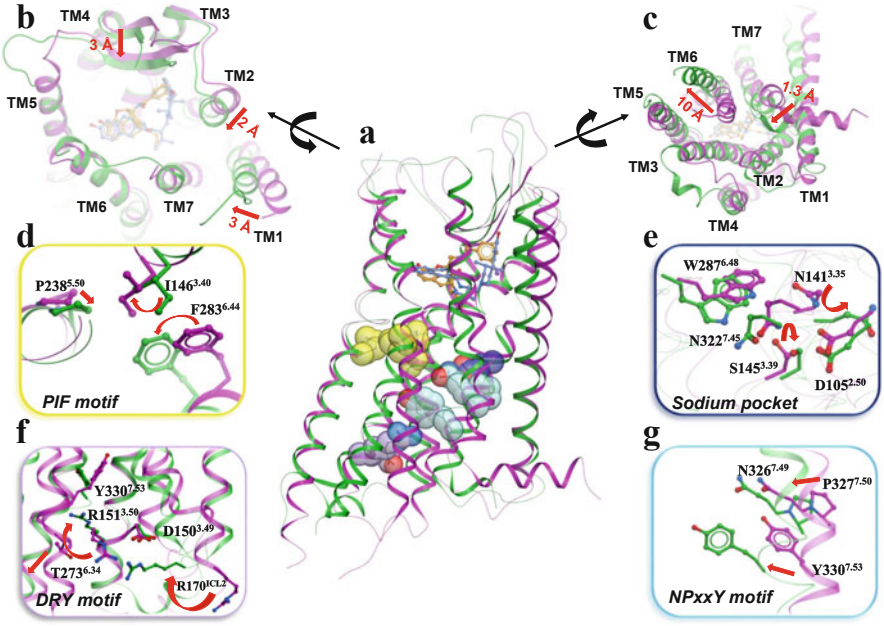

Fig. 3 Structural determinants of activation of KOR. (**a**) Structural alignment of inactive (purple) and active (green) KOR showing the relative position of microswitches, (**b**) Extracellular view of the alignment indicating inward movement of TM2 and ECL2 during activation, and (**c**) Intracellular view of the alignment indicating outward movement of TM6 and inward movement of TM7 during activation, (**d–f**) Conformational changes between active (green) and inactive (purple) for (**d**) P-I-F motif, (e) Sodium binding pocket, (**f**) D-R-Y motif, and (**g**) NPxxY motif

the large orthosteric pocket, followed by more tight interactions upon the pocket contraction coupled with receptor activation on the intracellular side. On the cytoplasmic side of the KOR-MP1104-Nb39 structure, the canonical active-like conformational changes include (1) outward movement in the intracellular end of TM6 by ~10 Å and (2) inward movement of TM7 by ~3 Å at the level of residue $Y330^{7.53}$. A slight inward movement in TM5 about 2.2 Å is also observed. These conformational changes stabilize KOR-Nb39 interactions, mimicking the changes observed in MOR and other active-state GPCR structures in complex with G-proteins (Katritch et al. 2013; Koehl et al. 2018; Venkatakrishnan et al. 2013).

The structural analysis of KOR inactive and active-state structures also gives insight into the mechanisms of allosteric coupling between the changes in the ligand-binding pocket and changes in the G-protein binding interface that lead to receptor activation. This mechanism, largely conserved in class A GPCRs involves a tightly coupled combination of helical motions, conserved microswitches, as well as the collapse of the sodium binding pocket (Katritch et al. 2014) in the middle of the receptor 7TM helical bundle.

First of all, the contraction of the pocket, and specifically the inward movement of TM5 engages the conserved P-I-F motif ($P^{5.50}$-$I^{3.40}$-$F^{6.44}$), which converts the inward movement of $P238^{5.50}$ into a rotameric switch in $I146^{3.40}$ side chain and rotation of $F283^{6.44}$, coupled with the swivel motion of the bottom part of TM6 (Katritch et al. 2013). Moreover, residues $W287^{6.48}$ and $M142^{3.36}$ located at the "floor" of the orthosteric ligand pocket are directly pushed downward by the MP1104 morphinan scaffold, also engaging in the P-I-F motif and facilitating the activation changes.

These conformational changes are also coupled to the changes in the sodium binding pocket, which is highly conserved and known to be involved in the activation mechanism of many of class A GPCRs, including opioid receptors (Katritch et al. 2014). Because detection of sodium itself in the crystal structures requires very high resolution (<2.4 Å), among opioid receptors, it was explicitly resolved only in inactive DOR-naltrindole structure at 1.8 Å resolution (Fenalti et al. 2014, 2015). However, the sodium binding pocket was found to be structurally identical in DOR, KOR, and MOR, with $D105^{2.50}$, $S145^{3.39}$, and $N131^{3.35}$ side chains identified as Na^+ is coordinating triad. Moreover, numerous data show similar dependence of all opioid receptors on this sodium site, and extensive MD studies suggest similar (though not identical) sodium binding properties (Selvam et al. 2018; Shang et al. 2014) in opioid receptors. Importantly, in the active state conformation, the sodium pocket collapses, expelling the sodium ion, as demonstrated by the high-resolution active-state structure of MOR (Huang et al. 2001, 2015, 2021). Instead of coordinating Na^+, in the active state $D105^{2.50}$ and $S145^{3.39}$ side chains form direct hydrogen-bonding interactions. Also, $N131^{3.35}$ side chain, which formed a water-mediated contact with $D138^{3.32}$ of the orthosteric pocket in the KOR inactive state, undergoes a major conformational change, redirecting towards the lipid interface, which also contributes to ligand-induced activation changes.

Other conserved motifs, "NPxxY" in TM7 and "DRY" in TM3 are involved in the KOR activation mechanism. The NPxxY motif (N326$^{7.49}$-P327$^{7.50}$-xx-Y330$^{7.53}$) is closely associated with the sodium pocket, with the Y330$^{7.53}$ backbone undergoing the most pronounced changes in TM7, including its inward shift and rotation. Moreover, the Y330$^{7.53}$ side-chain switches from pointing up towards the sodium pocket, where it is involved in a water-mediated hydrogen-bonding network in the inactive state, to pointing sideways towards TM5 in the active state, and potentially forming a hydrogen bond with R156$^{3.50}$ and Y246$^{5.58}$ side chains. The R156$^{3.50}$ itself belongs to the DRY motif (D$^{3.49}$-R$^{3.50}$-Y$^{3.51}$) and changes its conformation dramatically between inactive and active state KOR structures. Like in many other class A GPCRs, in the inactive conformation the basic R156$^{3.50}$ residue of DRY forms salt-bridging interactions with neighboring acidic D155$^{3.49}$ residue. In the active state conformation, this salt bridge interaction is broken, and the R156$^{3.50}$ side chain adopts an extended conformation pointing into the G-protein binding cavity, where it can directly engage with G-protein (Koehl et al. 2018). The importance of the DRY "ionic lock" was shown in many GPCRs. For example, in rat MOR, mutation of D$^{3.49}$ to H, Y, M, or Q leads to constitutive activation of the receptor (Li et al. 2001). Moreover, R156$^{3.50}$ forms an H-bond with T273$^{6.34}$ in inactive state structures of KOR supplementing the DRY "ionic-lock." In the KOR active state structure, however, T273$^{6.34}$ moves outwards, facing towards the lipid bilayer, comprising another switch conserved in opioid receptors (Huang et al. 2001; Manglik et al. 2012).

While this direct analysis of structural changes between inactive and active states gives a simplified "binary" picture of activation, the structures also provide an important 3D framework for further analysis of the dynamics of activation by molecular dynamics simulations (MD) and other approaches. The inactive-state structure was instrumental in studies of sodium ion dynamics in KOR and other opioid receptors (Selvam et al. 2018; Shang et al. 2014), showing that sodium spontaneously enters the pocket from the extracellular side and analyzing potential pathways for sodium egress. Another MD study employing KOR structure compared ligand-induced activation in KOR and MOR, suggesting that the activation may also involve a flow of water molecules into the interior of the receptor, i.e. into the sodium pocket, where antagonists may be blocking this flow (Yuan et al. 2015a).

One important aspect of KOR function, specifically the details of the receptor interactions with G protein or arrestins remain to be solved, which can be achieved with recent developments in cryo-EM technologies (García-Nafría and Tate 2020). However, key inferences can be already drawn from recently determined MOR active state structures in complexes with Nb39 nanobody (Huang et al. 2001, 2015, 2021) and with Gi (Koehl et al. 2018). Note that both KOR and MOR recruit $G_{i/o}$ and that their active state structures have been resolved using the same Nb39 antibody, revealing almost identical receptor conformations and interactions of MOR and KOR in this region. The sequence alignment of the Nb39 interaction surface between KOR and MOR indicates extremely high sequence identity in the G protein binding region, including key charged residues of ICL2 (D168/179^{ECL2} and R170/181^{ECL2}), with only one non-conserved residue in TM6 (L275/M283). This

conservation, combined with the observed conformational similarity between Nb39-complexed MOR and G_i complexed MOR also suggests that the key interaction of MOR-G_i complex is likely to be transferable to KOR-G_i complex. This similarity was recently exploited to build a model of KOR-Gi complex and evaluate dynamics using MD simulations, suggesting that KOR undergoes similar changes as MOR upon full activation by G protein (Mafi et al. 2020). Nevertheless, direct determination of structures of KOR-Gi and especially KOR-b-arrestin complexes in the future might reveal some unexpected new features and contribute to the understanding of KOR signaling mechanisms.

7 Structure-Based Insights into KOR 3D Pharmacology

The structure of KOR has been so far determined with only 2 ligands – with long-acting inverse agonist JDTic in an inactive state (Che et al. 2020; Wu et al. 2012) and epoxymorphinan agonist MP1104 in an active state (Che et al. 2018). The structures, however, provide an excellent framework for docking of other important ligand chemotypes and analysis of 3D pharmacology of KOR antagonists and agonists.

7.1 KOR Antagonists: Long-Acting and Short-Acting

Most directly, the crystal structure of KOR-JDTic complex (Wu et al. 2012) can be applicable to the analysis of binding, pharmacology, and structure-activity relationships of JDTic-like analogs. Indeed, several papers employed the structure to guide or explain the design of new JDTic-like analogs with variations in different parts of the scaffold, including the development of phenoxybenzamide substitutes (Kormos et al. 2013), substitutions in both hydroxyl groups of JDTic (Kormos et al. 2014), fluoroalkyl derivatives of JDTic (Schmitt et al. 2015), as well as other variations in or around the isoquinoline core including PDTic scaffold (Ondachi et al. 2018).

Docking of the other KOR ligands, including short-acting antagonists proved to be suboptimal in our studies (Vardy et al. 2013). For example, docking of LY2456302 into this structure (Wang et al. 2017) does not seem to engage the important anchor interaction between the tertiary amine in the ligand and the $D138^{3.32}$ side chain. This issue was especially striking when comparing docking of dual-selectivity KOR-MOR ligands or pan-opioid ligands, which consistently showed suboptimal poses and drastically reduced scores of these ligands as compared to docking into MOR or DOR. To resolve this issue, Zheng et al. (2017) employed ligand-guided optimization of the orthosteric pocket using LiBERO approach (Katritch et al. 2012b), which provides a way to sample alternative conformations and select those most suitable for docking a set of representative ligands and separating them from decoys. In the KOR case, the representative ligand set comprised 100 high-affinity KOR ligands from ChEMBL database, mostly based on morphinan scaffolds. One model was generated by LiBERO directly from

KOR-JDTic structure, while the other was based on a KOR homology model built with DOR-naltrindole inactive structure as a template (Fenalti et al. 2014, 2015). The utility of these models was then demonstrated not only in improved molecular docking of non-JDTic KOR ligands, but also in a prospective virtual ligand screening and identification of novel KOR ligands as described in Sect. 8.2 (Zheng et al. 2017).

7.2 KOR Agonists

The active state of KOR was crystallized in complex with an epoxymorphinan, MP1104 (Che et al. 2018). As expected, several other important KOR ligands sharing epoxymorphinan scaffold, including nalfurafine, IBNxTA, 5-′-guanidinonaltrindole (5′-GNTI), and 6′-guanidinonaltrindole (6′-GNTI), all share a similar binding pose. According to docking and SAR analysis of key derivatives in the Che et al. study (Che et al. 2018), the 6th-position amide substitutions such as iodo-phenyl of MP1104 and IBNxTA, and of furyl ring of nalfurafine bind in the hydrophobic region between TM2 and TM3. In other ligands possessing the fused ring systems such as 5′-GNTI and 6′-GNTI, the indole ring is directed towards the extracellular region and is located in the hydrophobic cavity between TM3, TM5, and TM6. Interestingly, the basic guano group of 5′-GNTI and 6′-GNTI has distinct salt-bridging partners, E297$^{6.58}$ for 5′-GNTI, and D223$^{5.35}$ and E209^{ECL2} for 6′-GNTI. This perhaps indicates molecular mechanism of the functional variance observed for 5′-GNTI's antagonism and 6′-GNTI's partial agonism (Uprety et al. 2021; Wu et al. 2012).

Another class of KOR agonists, arylacetamides, includes U50,488 and U69,593, which are the prototypical KOR selective full agonists often used for in vitro and in vivo studies. These compounds also have a positively charged amino group in their 5-membered pyrrolidine ring, which forms an anchoring salt-bridging interactions with D138$^{3.32}$, as predicted by docking and validated by mutation analysis (Vardy et al. 2013). Interestingly, unlike many epoxymorphinans and peptide opioids, the arylacetamides lack any hydrogen bonding to crystallographic waters between TM3 and TM5. The di-chlorophenyl and phenyl of U50,488 and U69,593, respectively, are docked in the hydrophobic sub-pocket between TM2-TM3, which overlaps with the position occupied by iodo-phenyl of co-crystallized MP1104 in the active state KOR structure.

Salvinorin A (SalA), a natural product (Yan and Roth 2004), belongs to a very distinct class of KOR agonists that lacks any basic amines capable of forming the canonical salt bridge interactions with acidic D138$^{3.32}$. Several binding models for salvinorin analogs have been proposed over the years supported by site-directed mutagenesis and structure-activity relationship studies. For example, early docking in the inactive state JDTic co-crystallized structure (Vardy et al. 2013), the Sal A ligand was proposed to interact via hydrogen-bonding interactions to Q115$^{2.60}$, C210^{ECL2}, non-conserved Y312$^{7.35}$, with the furanyl ring pointing towards the extracellular region. A similar pose was also obtained in docking into a model of

active-state KOR, built by homology from the active-state MOR structure (Huang et al. 2015) and optimized with KOR ligands (Roach et al. 2017). The modeling analysis in the latter study also suggested that 20-nor-SalA maintains the conformation and high-affinity binding of SalA, which was validated experimentally and brought about a series of new SalA analogs and derivatives (Roach and Shenvi 2018). Interestingly, an attempt to dock SalA to the active state KOR structure (Che et al. 2018) suggests an alternative docking mode (Zaidi unpublished), with the furanyl moiety pointing down towards the receptor center. Such instability, as well as the fact that some of the mutation and SAR data for SalA covalent derivatives cannot be reconciled together, suggests a potential existence of alternative binding modes for SalA. This might also explain the challenges presented by attempts to co-crystallize SalA or derivatives with KOR, which remains an area of active research.

Finally, structural details of binding of the endogenous agonist dynorphin A are of great interest for the analysis of biological signaling of KOR and design of peptide analogs. The conformation of dynorphin has been studied by docking the truncated dynorphin (1–8) into the inactive KOR structure, and the pose was validated by extensive mutagenesis (Vardy et al. 2013). In this binding mode, the peptidic Y1 adopts a position similar to the phenol-piperidine fused ring system of MP1104, forming hydrogen bonds to the conserved crystallographic waters. The acidic D138$^{3.32}$ side chain interacts with the positively charged N-terminus, as well as with amides of G2 and G3 of dynorphin. This pose is in good agreement with the binding poses of other opioid peptides crystallized with DOR (Fenalti et al. 2015) and other agonists resolved in MOR and DOR structures that mimic the N-terminal "YGGF" part of dynorphin. Another study using liquid-state NMR spectroscopy and molecular dynamics modeling (O'Connor et al. 2015) proposed a similar pose, termed "KOR-1," as an initial binding pose for dynorphin, exhibiting high conformational dynamics in NMR assessment. The authors also proposed a "KOR-2" conformation as a final more rigid pose in the fully active receptor, where the Y1 side chain of dynorphin penetrated down towards the sodium binding pocket, stacking against W287$^{6.48}$ and forming hydrogen bonds with N141$^{3.35}$. The validity of the "KOR-2" pose, though, remains hypothetical, as none of the active state structures of KOR, MOR, and DOR detected a receptor conformation compatible with Y1 "down" position. Note that characterization of the full-length dynorphin A (1–17) binding to KOR also awaits more elaborate modeling or structural studies and might involve additional interactions with the acidic N-terminal domain of KOR.

7.3 Determinants of Opioid Ligand Selectivity at KOR

Overall, the three opioid receptors exhibit a remarkable amino acid sequence conservation, particularly in the orthosteric binding pocket. Consequently, many opioid ligands show limited selectivity amongst the receptors, and the design of highly KOR selective ligands represents a substantial challenge. However, non-conserved regions of the binding pocket can be leveraged to design highly

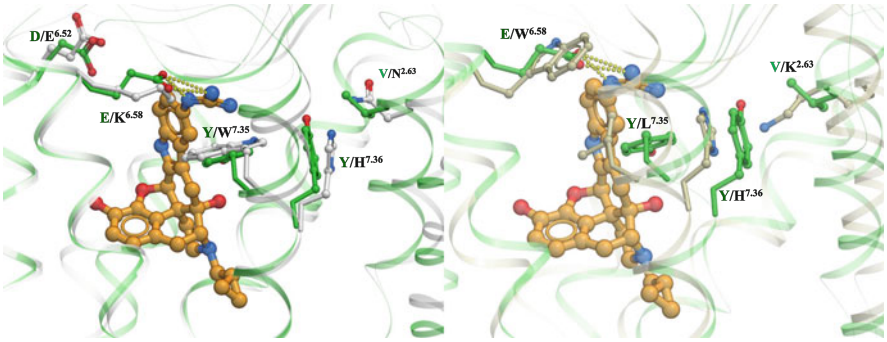

Fig. 4 Determinants of selectivity among opioid receptors. Binding mode of KOR selective 5′-GNTI ligand (orange) in KOR orthosteric pocket (green) with non-conserved residues shown in sticks representation and overlaid on (**a**) MOR (white), (**b**) DOR (gray)

selective KOR ligands (Fig. 4). "Message-Address" concept for KOR selectivity was originally introduced for peptide ligands (Lipkowski et al. 1986) and the non-conserved "address" residues were identified in 6.58 and 7.35 Ballesteros-Weinstein positions. Thus, KOR has acidic E297$^{6.58}$ and aromatic Y312$^{7.35}$ side chains, capable of forming hydrogen bonds and providing hydrophobic interactions. Docking studies show that several highly selective KOR non-peptide ligands (Vardy et al. 2013) such as 5′-GNTI and norBNI (Vardy et al. 2013) (Larson et al. 2000), harness these particular "address-site" interactions to discriminate among the opioid receptors by forming salt-bridging interactions between basic guanidine and E297$^{6.58}$ side chain, as well as hydrophobic interactions between fused indole ring and Y312$^{7.35}$ residue. In contrast, MOP has basic K303$^{6.58}$ and hydrophobic W318$^{7.35}$ residues and DOP has hydrophobic W284$^{6.58}$ and L300$^{7.35}$ residues that cannot support these interactions.

A more detailed analysis of the inactive and active-state structures (Che et al. 2018, 2020) shows additional selectivity determinants that can be exploited for selectivity. The region between TM2-TM3 of KOR is particularly hydrophobic due to presence of a non-conserved hydrophobic residue V118$^{2.63}$. This hydrophobic residue replaced by a polar N129$^{2.63}$ in MOR, which anchors a water-mediated network and changes the hydrophobic nature of the TM2-TM3 sub-pocket. Also important is the water network between TM3 and TM5. As discussed above, these waters are conserved in the opioid receptors and interact with the phenolic hydroxyls of the opioid ligands. However, due to some variation in this water network, the substitution of hydroxy to methoxy moieties was found to have a less detrimental effect on KOR binding than on MOR, which can be utilized to gain KOR selectivity (Che et al. 2018; Huang et al. 2021; Li et al. 2017). Finally, sequence alignment of the opioid receptors indicates a subtle difference in ECL2 and extracellular end of TM5. Thus, ECL2 of KOR is three residues longer than other opioid receptors, which impacts the conformation of several side chains in this sub-pocket and may be exploited for selectivity.

8 Applications of KOR Structure to Computer-Assisted Ligand Discovery

8.1 Understanding KOR Dynamics by MD Simulations

Elucidation of protein structures of KOR in JDTic-bound inactive and MP1104-bound active states has been harnessed for the advancement of several structural biology and structure-based drug design projects. These crystal structures represent only a snapshot in the structurally dynamic lifetime of the receptor. Therefore, several molecular dynamics based studies have been conducted to draw further insights into KOR conformational dynamics, possible pathways of KOR allosteric modulation by Na^+ ions (Shang et al. 2014), binding modes to atypical or novel KOR ligands such as salvinorin A (Leonis et al. 2014) or PF-4455242 (Hu et al. 2013), exploration of the role of the water molecules in ligand binding (Goldfeld et al. 2015), the distinct functional role of opioid ligands within opioid receptor sub-family (Yuan et al. 2015a), prediction of low energy ensemble of KOR structures (Li et al. 2015), prediction of receptor dimerization interface (Provasi et al. 2015), modeling G_i-KOR interface by replacement Nb39 antibody followed by long MDs (Mafi et al. 2020), the difference between agonist and antagonist-bound conformational ensemble (An et al. 2019; Cheng et al. 2016).

8.2 Discovery of New KOR Chemotypes by Structure-Based Virtual Screening

Several research groups have employed the inactive and active state KOR structures for in silico virtual ligand screening (VLS) studies to identify novel lead compounds. In one such study published in 2013 (Negri et al. 2013), authors used JDTic-bound inactive state KOR structure for the VLS study and identified only five very weak hits with affinities in 100 to 500 mM range, one of them showing KOR selectivity. This result might reflect the aforementioned observations that JDTic bound inactive state KOR structure reflects dramatic JDTic-specific induced fit effects. A more recent study, therefore, employed a multi-template screening strategy using (1) JDTic-bound crystal structure, (2) ligand-guided optimization starting with the JDTic-bound structure, and (3) ligand-guided optimization of the KOR homology model based on inactive DOP template (Zheng et al. 2017). This multi-template screening approach yielded a 32% hit rate (hits better than 10 uM) in the initial screening, with the best affinities Ki = 200 nM. Moreover, a further round of SAR study for the best six lead-like scaffolds identified 11 more hits, with the best antagonist Ki = 90 nM. Interestingly, one of the hits was identified as a G protein biased agonist with KB = 170 nM in G-protein assays and bias factor = 6 over arrestin recruitment (Zheng et al. 2017).

8.3 Rational Design for KOR Selectivity Over MOR and DOR

The KOR structures have been widely used to characterize binding modes of several known opioid ligands (Vardy and Roth 2013). Although some ambiguities remain for ligands such as salvinorin A and dynorphin peptides, molecular docking studies along with SAR and site-directed mutagenesis studies have been used to define the receptor interaction maps for several ligands, including several novel scaffolds such as HS665 analogs (Guerrieri et al. 2016), fused β-carboline analogs (Batra et al. 2021), quinoxaline derivatives (Tangherlini et al. 2019), cryptotanshinones (De Caro et al. 2020). The rationally designed KOR selective ligands have either harnessed the non-conserved "address" region in the KOR receptor (Yuan et al. 2015b) or reduced susceptibility of KOR binding to the disruption of water-mediated hydrogen bonding compared to MOR (Huang et al. 2021; Li et al. 2017). The availability of the resolved crystal structures of KOR, MOR, and DOR also facilitated the design of dual KOR/MOR activity opioids (Wang et al. 2021; Yuan et al. 2013; Zheng et al. 2019).

8.4 Rational Design for KOR Functional Selectivity

Pathway-selective KOR ligands with reduced arrestin signaling have been proposed to mitigate undesirable effects of opioids, such as dysphoria, hallucinations, and dissociation associated with KOR stimulation. For KOR, some of the promising G-protein biased leads include triazole 1.1 (Zhou et al. 2013), diphenethylamines (Spetea et al. 2017), 6′-GNTI (Rives et al. 2012), and collybolides (Gupta et al. 2016). Recently, our lab has published two distinct strategies to rationally design functionally selective opioid ligands harnessing solved crystal structures, molecular docking, SAR studies, and understating of the molecular mechanisms of GPCR activation (Fig. 5). In the first approach, we identified subpockets of the orthosteric pocket as the key sites for functional selectivity for the empoxymorphinan analogs of MP1104 (Uprety et al. 2021). Thus, interactions of the ligand amidophenyl arm with TM2-TM3 region were shown to maintain balanced G-protein and arrestin signaling, while switching the contacts to TM5-ECL2 region conferred G protein bias. Subsequently, compounds MP1207/MP1208 were designed to interact with the TM5-ECL2 region specifically, and pharmacological characterization of these resulting compounds shows partial G protein agonism with minimal arrestin recruitment at both KOR and MOR. Furthermore, these compounds show potent analgesia in the mice studies, while no conditional place preference/aversion or respiratory depression was observed (Uprety et al. 2021).

The second structure-based strategy explores conserved and functionally important sodium binding pocket in KOR as a site with the potential to control functional selectivity (Fenalti et al. 2014; Zarzycka et al. 2019). We designed bitopic compounds, which simultaneously interact with both the orthosteric pocket and the sodium binding site and thus blocking certain activation-related conformational changes in the sodium pocket. Pharmacological evaluation of such compounds shows disruption of arrestin recruitment while G protein recruitment is maintained

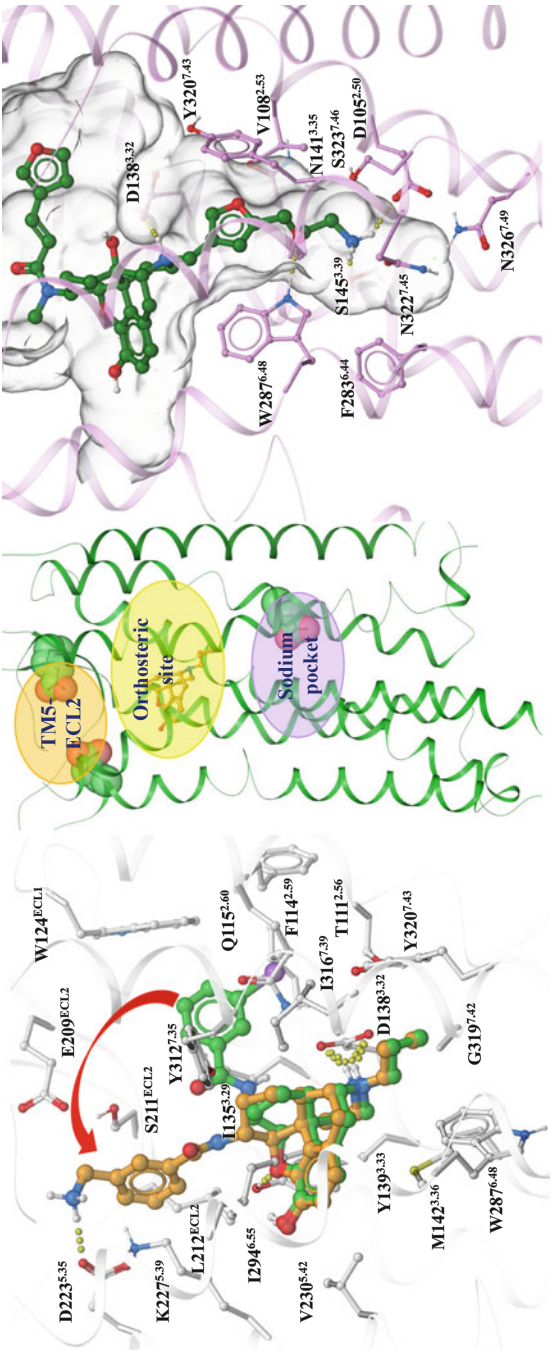

Fig. 5 Rational design of functional selectivity for KOR ligands (**a**) Engagement of TM5-ECL2 sub-pocket (Uprety et al. 2021) (**b**) Schematic indicating positions of two key functional modulation site above and below the orthosteric ligand pocket (**c**) Engagement of allosteric sodium binding pocket (Zarzycka et al. 2019)

(Zarzycka et al. 2019). The decrease in volume of sodium pocket is primarily due to inward movement of TM7 and perhaps this resulting stabilization TM7 produces reduced arrestin recruitment (Latorraca et al. 2017) These examples indicate the utility of structure-based design in discovery of functionally selective compounds that may further our understanding of receptor mechanisms and related physiology.

9 Conclusions and Outlook

The structure elucidation of KOR has brought a paradigm shift in structure-based drug discovery and our understanding of details and dynamics of KOR–ligand interactions. Nevertheless, the three available KOR structures do not fully represent all conformational space and functional states of KOR. Recent advances in the field of structural biology, particularly cryo-EM, provide new opportunities to elaborate KOR conformation co-complexed with a diverse set of ligands and with downstream signaling proteins such as G proteins and arrestins. Such advancements, coupled with the advent of ultra-large virtual small molecule libraries and continued improvements in computational chemistry tools and capacity will present an opportunity to fully harness the KOR structures for structure-based drug discovery programs. Combining structural information with accurate modeling and biochemical validation opens exciting opportunities to optimize known ligands, identify novel scaffolds with biologically distinct pharmacological profiles, as well as target allosteric sites, providing a better molecular understanding of the KOR biology.

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Conflict of Interest The authors declare no conflict of interest.

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Biosensors Monitor Ligand-Selective Effects at Kappa Opioid Receptors

Lucie Oberhauser and Miriam Stoeber

Contents

Abstract

The kappa opioid receptor (KOR) has emerged as a promising therapeutic target for pain and itch treatment. There is growing interest in biased agonists that preferentially activate select signaling pathways downstream of KOR activation on the cellular level due to their therapeutic promise in retaining the analgesic and antipruritic effects and eliminating the sedative and dysphoric effects of KOR signaling on the physiological level. The concept of ligand-selective signaling includes that biased ligands promote KOR to selectively recruit one transducer or regulator protein over another, introducing bias into the signaling cascade at the very receptor-proximal level. Measuring agonist effects directly at the receptor has remained challenging and previous studies have focused on inferring agonist-selective KOR engagement with G protein relative to β-arrestin based on

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downstream signaling readouts. Here we discuss novel strategies to directly assess ligand-selective effects on receptor activation using KOR-interacting biosensors. The conformation-specific cytoplasmic biosensors are disconnected from the endogenous signaling machinery and provide a direct receptor-proxy readout of ligand effects in living cells. Receptor–biosensor interaction is ligand concentration dependent and can be used to determine relative ligand potency and efficacy. In addition, the biosensors reveal the existence of two dimensions of agonist bias in the cellular context: Firstly, agonists can selectively produce discrete protein-engaged KOR states and secondly, agonists can differ in the precise subcellular location at which they activate KOR. We discuss the value and the limitations of using orthogonal receptor-interacting biosensors in the quest to understand functional selectivity amongst KOR agonists in the cellular context.

Keywords

Biosensor · GPCR · Kappa opioid receptor · Ligand bias · Nanobody · Selectivity

1 Introduction: Ligand-Selective Effects at the Kappa Opioid Receptor

Agonists of the KOR comprise various endogenous and exogenous peptide and non-peptide ligands with diverse chemical scaffolds. The binding of agonists induces conformational changes in the receptor that allow binding of active state KOR to transducer and regulatory proteins on the cytosolic side, such as G proteins, GPCR kinases (GRKs), and β-arrestins (Bruchas and Chavkin 2010). Their coupling to KOR elicits transmembrane signal transduction. As a classical member of the GPCR family, KOR signals through allostery, which implies that signal transduction involves a reciprocal, cooperative coupling between the orthosteric agonist-binding site and the intracellular transducer-binding site. Orthosteric ligands can differ considerably in their potency and their efficacy to shift the equilibrium from inactive receptor to active receptor states and produce receptor–transducer coupling. It is also increasingly clear that chemically distinct agonists can introduce allosteric bias into the signaling cascade by promoting KOR to couple to specific downstream pathways (Dunn et al. 2018; Mores et al. 2019; Zhou et al. 2013). The ability of an agonist to activate a signal transduction pathway in vitro is a major determinant of its pharmacological activity and is important for predicting its effects (Smith et al. 2018). Current pharmacological approaches to determine the potency and efficacy of GPCR agonists are based on the measurement of G protein signaling, which can be sampled at multiple levels, e.g. at the level of GTP exchange (GTPγS), at the level of second messenger production (e.g. cAMP), or at the level of gene transcription (Mores et al. 2019). The functional assays of G protein coupling are based on an enzymatic process (nucleotide exchange) and involve different steps of signal amplification, rather than directly measuring protein–protein interaction at the receptor-proxy level. In parallel, pharmacological studies have determined the potency and efficacy of KOR agonists to drive KOR coupling to β-arrestin, another

cellular transducer and regulatory protein. Arrestin coupling has been assayed by measuring β-arrestin recruitment to KOR tagged with reporter proteins, e.g. using bioluminescence resonance energy transfer (BRET), enzyme fragment complementation, or reporter gene expression readouts (Ho et al. 2018). While the assays are based on direct interaction between KOR and β-arrestin, it is now clear that β-arrestin recruitment to GPCRs involves multiple biochemical steps. In particular, full interaction with β-arrestin requires KOR to be phosphorylated by GPCR kinases (GRKs) in the cytoplasmic tail (Chen et al. 2016). Therefore, β-arrestin recruitment measured in such assays reflects a process that is more complex than allosteric coupling by the receptor (Eichel et al. 2018; Nuber et al. 2016). Due to the complexity in G protein- and β-arrestin-based readouts, it remains challenging to reliably determine the degree to which chemically distinct agonists can induce allosteric bias (Conibear and Kelly 2019; Gillis et al. 2020a; Kenakin 2019). An alternative approach to measuring agonist effects at the KOR has recently emerged, which relies on the use of conformation-specific biosensors. Recruitment of biosensors to KOR can serve as direct, unamplified readout for the relative efficacy and potency of distinct agonists. Furthermore, the differential recruitment of different biosensor probes in response to agonists provides new insight into allosteric bias at the receptor-proximal level. In this chapter, we describe the development and the characteristics of nanobody- and mini-G protein-based biosensors, discuss their value in determining ligand effects and ligand bias, and highlight the novel insights into agonist-selective effects at the KOR in living cells.

2 Nanobodies and Mini-G Proteins Act as Conformation-Specific KOR Binders

2.1 Active State Binding Nanobodies Nb39 and Nb33

Current biosensors for the KOR represent repurposed tools that originated from structural and biophysical studies into GPCR function. Over the past decade, tremendous progress in the field of GPCR structural biology has led to the determination of many high-resolution structures of inactive and active state GPCRs by X-ray crystallography or by cryoelectron microscopy (cryo-EM) techniques that reveal the molecular details and dynamics of GPCR signal transduction (Hilger et al. 2018; Mahoney and Sunahara 2016; Nygaard et al. 2013). Since GPCRs, even when bound by ligands, are highly dynamic and sample a continuum of conformational ensembles, researchers developed different tools to stabilize GPCRs in specific conformations. In particular nanobodies, recombinant camelid single-domain antibodies, have been instrumental in obtaining the active state structures of several GPCRs, including the KOR and the mu opioid receptor (MOR) (Che et al. 2018; Huang et al. 2015; Rasmussen et al. 2011a). The nanobodies bind to agonist-bound GPCRs on the cytosolic face and act as G protein mimetics, which means that the nanobodies recapitulate GPCR allostery by increasing the affinity of the agonist at the receptor, similar to G proteins. Pharmacological,

spectroscopic, and structural studies show that conformation-selective nanobodies can stabilize active GPCRs in a confirmation that is strikingly similar to the G protein-coupled state, making them structural surrogates of cellular signaling partners. Nanobodies have unique properties that make them particularly well-suited for GPCR applications. They are stable and small (15 kDa), have a compact shape and three complementarity-determining regions (CDR) that can access small cavities which are inaccessible to conventional antibodies, and often bind conformational epitopes composed of discontinuous amino acid segments in the native protein target (Heukers et al. 2019; Manglik et al. 2017).

The currently available active state-selective nanobodies of KOR were generated by the Steyaert and Kobilka groups in an effort to generate G protein mimetics, originally for the MOR (Huang et al. 2015). For this purpose, lamas were immunized with purified agonist (DALDA)-bound MOR reconstituted into lipid vesicles. Lamas naturally produce heavy chain-only antibodies, which contain variable VHH domains that harbor the full antigen-binding capacity of the antibody and can be cloned and expressed as stable single-domain proteins, resulting in the so-called nanobodies. After immunization, the entire repertoire of variable VHH genes can be cloned and subjected to phage display to select for nanobodies of desired function (Pardon et al. 2014). For the MOR, a family of nanobodies, including Nb39 and Nb33 (Fig. 1a), was identified that binds MOR in an agonist-dependent manner at the intracellular surface and dramatically enhances the affinity of several peptide and non-peptide ligands at MOR (Huang et al. 2015). Subsequently, the crystal structure of active MOR bound to the agonist BU72 and Nb39 was solved, which indeed shows striking similarity to the structure of active MOR in complex with the agonist DAMGO and Gi (Koehl et al. 2018). The Nb39–MOR interface involves residues from ICL2, ICL3 and helix 8 of the MOR, all of which are conserved across the three canonical opioid receptors MOR, delta opioid receptor (DOR), and KOR. This motivated the Roth group to utilize Nb39 as crystallization chaperone for active state KOR (Che et al. 2018). Indeed, Nb39 also binds to active KOR in an agonist- and efficacy-dependent manner as shown by BRET between tagged nanobody and receptor proteins. Nb39 increases agonist affinity at KOR and attenuates the agonist dissociation rate. The crystal structure of active KOR bound to the agonist MP1104 and Nb39 was solved (Fig. 1d). The conformational rearrangements at the intracellular site of active KOR are stabilized by the KOR–Nb39 interactions and just like for MOR, Nb39 binds to a receptor cavity that overlaps with the Gi interaction surface (Che et al. 2018). Consistent with this notion, Gαi1 was found to inhibit the KOR–Nb39 interaction in a dose-dependent manner. Nb33 is another member of the originally detected nanobody family that binds to active state MOR in vitro. Nb33 differs from Nb39 in two amino acids located within CDR1 and CDR2 (Fig. 1a), while all six key residues in the nanobody that engage with active MOR and KOR are conserved. Nb33 has not been as extensively characterized as Nb39 in vitro. However, the sequence homology suggests that Nb33 bears highly similar biochemical and structural features.

Motivated by the fact that nanobodies can be functionally expressed in the cytoplasm of eukaryotic cells and building on the previous success of using an

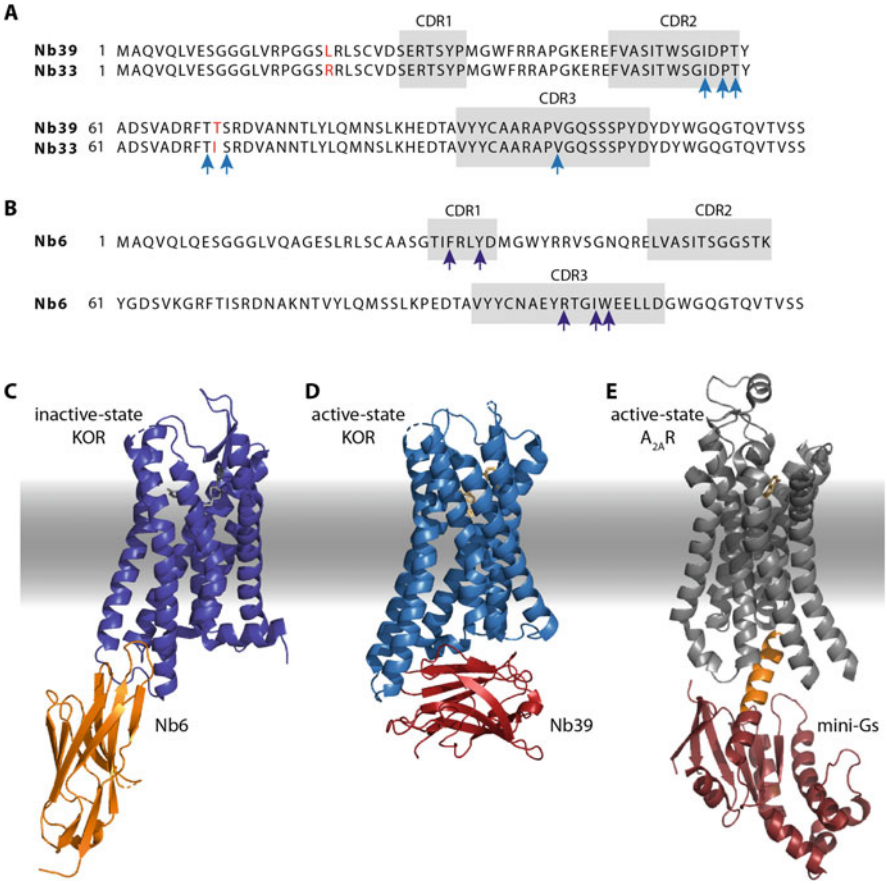

Fig. 1 Nanobodies and mini-G proteins act as conformation-specific KOR binders. (**a**) Sequence alignment of active state-specific nanobodies Nb39 and Nb33. Two amino acids highlighted in red differ between Nb39 and Nb33. The three CDRs of the nanobodies are highlighted and the blue arrows indicate key residues that mediate interactions with active state MOR and KOR (Che et al. 2018; Huang et al. 2015). (**b**) Sequence of inactive state-specific nanobody Nb6 with highlighted CDRs. Arrows point to key residues that interact with inactive state KOR. (**c**) Crystal structure of inactive state KOR (dark blue), bound to antagonist JDTic (gray) and Nb6 (orange), PDB: 6VI4. (**d**) Crystal structure of active state KOR (blue), bound to agonist MP1104 (yellow) and Nb39 (red), PDB: 6B73. (**e**) Crystal structure of active state $A_{2A}R$ (gray), bound to agonist NECA (yellow) and mini-Gs (dark red). PDB: 5G53. The C-terminal region, which differs between the mini-Gs and mini-Gsi probe, is highlighted in orange

active state stabilizing nanobody of the β_2-adrenergic receptor (β_2AR) as sensor for receptor activation in intact cells (Irannejad et al. 2013), we and others have repurposed positive-allosteric Nb39 and Nb33 from crystallization chaperones into biosensors for KOR activation (Che et al. 2018; Stoeber et al. 2018). Of note, Nb39 and Nb33 can also act as biosensors for active state MOR and DOR (Stoeber et al. 2018).

2.2 Inactive State Binding Nanobody Nb6

To generate KOR-specific nanobodies, the Roth group immunized llamas with purified agonist salvinorin A (SalA)-bound KOR and screened the nanobody library by phage display for KOR binders (Che et al. 2018). Selected nanobodies were further tested for agonist- or antagonist-dependency in their binding to KOR, which resulted in the discovery of Nb6, an inactive state-selective KOR nanobody (Fig. 1b, c). Nb6 binds to unliganded and antagonist-bound KOR and dissociates from the receptor upon activation by agonist. Radioligand binding and ligand dissociation studies showed that the presence of Nb6 attenuates agonist affinity and accelerates agonist dissociation, revealing the negative allosteric effect of Nb6 (Che et al. 2020). The crystal structure of inactive KOR bound to antagonist JDTic and Nb6 (Fig. 1c), which is overall strikingly similar to JDTic-only inactive KOR (Wu et al. 2012), revealed that Nb6 binds to KOR at an interface that is distinct from previously reported intracellular nanobody–GPCR interfaces, including the active state Nb39–KOR interface. Interestingly, the CDR3 loop of Nb6 inserts into a cavity between TM5 and TM6 of the KOR, which is different from the intracellular core region that mediates G protein interaction. The anchoring of TM5 and TM6 may prevent TM6 from moving outwards, thereby suppressing a hallmark event in the transition from an inactive to an active receptor conformation. The key residues of the KOR–Nb6 interface are conserved across the canonical opioid receptor family as well as the nociceptin/orphanin FQ peptide receptor. Accordingly, Nb6 binds all inactive state opioid receptors (Che et al. 2020). Since Nb6 and Nb39/Nb33 interact with discrete KOR conformations, Nb6 can serve as a complementary conformation-selective biosensor for the KOR.

2.3 Active State Binding Mini-G Protein Mini-Gsi

Another active state-selective KOR biosensor, which is unrelated to nanobodies in sequence and structure, is based on a minimal G protein (mini-G). The mini-G proteins represent an engineered domain of the G protein α-subunit that mimics heterotrimeric G proteins in inducing pharmacological and structural changes in GPCRs. They have been developed by the Tate group in order to facilitate structure determination of active conformations of GPCRs, similar to the nanobody-based approach (Carpenter and Tate 2016). The crystal structures of the β_2AR bound to heterotrimeric Gs revealed that direct contacts between the G protein and the receptor are almost entirely mediated by the Ras-like GTPase domain of Gαs (Rasmussen et al. 2011b). Therefore, the domain was used as a basis to identify a minimum component of the Gαs subunit that could function as an effective mimetic of the heterotrimeric G protein, while being uncoupled from the βγ subunits (Gβγ), a lipid tether, and receptor binding-driven nucleotide exchange. The first engineered mini-G construct, mini-Gs (22 kDa), was shown to allosterically increase the agonist-binding affinity of Gs-coupled GPCRs to levels comparable to those elicited by heterotrimeric Gs. Furthermore, the crystal structure of the adenosine A_{2A}

receptor in complex with mini-Gs (Fig. 1e) revealed that the mini-G protein indeed recapitulated the native GPCR–G protein interface (Carpenter et al. 2016). Based on the design of mini-Gs, mini-G proteins for all major Gα families (Gαs, Gαi, Gαq, and Gα12) were developed and shown to retain the appropriate GPCR coupling specificity (Nehmé et al. 2017). Developing a mini-G probe based on Gαi1 proved challenging due to stability and coupling issues (Nehmé et al. 2017). However, a successful strategy in developing a mini-Gi probe was to create a chimera wherein specificity-determining residues at the distal C-terminus in mini-Gs were replaced with the corresponding residues from Gαi1. The C-terminal residues form a major determinant of G protein coupling specificity by folding into a helical structure (α5 helix) that occupies the agonist-activated GPCR core (Fig. 1e). The resulting mini-Gsi probe indeed gained coupling to Gi-coupled receptors and lost coupling to Gs-coupled receptors (Nehmé et al. 2017). In addition to their usefulness as surrogates for heterotrimeric G proteins in structural studies, mini-G proteins are currently used extensively as probes to report GPCR activation in living cells, similar to nanobodies (Wan et al. 2018). The mini-Gsi probe has proven to be a robust biosensor for KOR activation (Stoeber et al. 2020). Of note, it can bind to various active state Gi-coupled receptors, while Nb33, Nb39 and Nb6 couple selectively to members of the opioid receptor family.

3 Biosensors Robustly and Rapidly Report on KOR Activation and Deactivation

Measuring the dynamics of ligand-dependent GPCR signaling in living cells is essential for understanding how information is processed and transmitted in the complex cellular environment. In order to study GPCR signal transduction, different biosensors have been developed to monitor ligand binding and ligand-induced conformational changes in GPCRs, G proteins, and β-arrestins (Abreu and Levitz 2020; Haider et al. 2019). The usefulness of a biosensor relies on the recognition of the target under study and the subsequent conversion of recognition into a measurable signal. In addition, desired characteristics of a biosensor include specificity, reversibility, and the ability to report on GPCR signaling without interfering with it. The ability of Nb39, Nb33, Nb6, and mini-Gsi to act as optical biosensors for KOR activation in intact cells with high specificity, sensitivity, and high temporal and spatial resolution has recently been demonstrated (Che et al. 2020; Stoeber et al. 2020). As a straightforward approach, the different nanobodies and mini-G proteins have been fused with fluorescent proteins and ligand-dependent recruitment to fluorescently-labelled KOR has been determined by fluorescence microscopy. Alternatively, BRET measurements were performed in order to monitor close proximity (<10 nm) between the biosensor and KOR. Both assays rely on ligand-dependent biosensor relocalization from the cytosol (unbound) to the membrane (receptor-bound).

Real-time KOR activation and deactivation using Nb39 and Nb6 as biosensors in living cells was first assessed by Che and colleagues (Che et al. 2018, 2020).

Ligand-dependent recruitment of the biosensors to KOR was detected using BRET in HEK293 cells co-expressing KOR C-terminally fused to Renilla Luciferase (RLuc, donor) and Nb39 or Nb6 fused to yellow fluorescent protein (YFP, acceptor). Adding the agonist SalA to cells drove recruitment of Nb39 to active KOR, which was detectable as a pronounced increase in the BRET signal due to close proximity of Rluc and YFP. The signal increase occurred within seconds of agonist addition. Subsequent addition of the antagonist JDTic could rapidly reverse the BRET signal back to baseline, indicative of Nb39 dissociation from inactive KOR. Conversely, adding SalA to cells expressing Nb6-YFP and KOR-Rluc drove dissociation of Nb6 from the receptor, detectable as a strong decrease in the BRET signal. This effect was reversed upon adding the antagonist JDTic reflecting the reassociation of Nb6 with the inactive receptor. The experiments established the usefulness of active state-selective Nb39 and inactive state-selective Nb6 as ligand-dependent and reversible conformational biosensors in living cells. The BRET assay, set up in a 96-well format and based on luminescence- and fluorescence-measurements, further provided an approach to determine ligand concentration-response curves and detect differences in ligand potency and efficacy at the cell population-level. Importantly, the ligand effects on KOR determined by nanobody recruitment corresponded to the known pharmacology of full and partial KOR ligands previously established by classical G protein signaling measurements.

In a parallel approach, we described a total internal reflection fluorescence (TIRF) microscopy-based assay to determine KOR activation and deactivation using Nb33 and mini-Gsi (Stoeber et al. 2020). In TIRF microscopy, the laser illuminates the sample above a critical angle that results in total reflection of the laser beam at the glass-specimen interface, which creates an evanescent excitation field. The evanescent field decays exponentially from the interface and penetrates into the sample medium only to a depth of approximately 100 nm. Thus, TIRF microscopy enables to selectively visualize fluorophores at the plasma membrane and the cytoplasmic zone immediately beneath it (Fig. 2a). We used this approach to follow the ligand-induced relocalization of biosensors from the cytosol to KOR in the plasma membrane in transfected HEK293 cells. To simultaneously image biosensors and receptors in living cells, we fused the biosensors with fluorescent proteins and labelled FLAG-tagged KOR in the cell surface with a fluorescent monoclonal antibody. Binding of mini-Gsi and Nb33 to KOR was monitored upon adding agonists, such as Dynorphin A (DynA), by bath application or perfusion and dissociation measured upon adding the competitive antagonist 5'GNTI in excess. Quantification of the fluorescence intensity throughout time lapse movies acquired at 5 s intervals provided real-time biosensor association traces that showed robust, rapid, and reversible recruitment of Nb33 and mini-Gsi to KOR in response to the full agonists DynA, U50,488, and U69,593. KOR surface levels did not significantly change during the 5–6 min movies, showing that biosensor recruitment could be reliably measured without possible complications of later KOR trafficking (Fig. 2b). Confirming the biosensor specificity, the mini-Gs probe, differing in only nine residues at the distal C-terminus from mini-Gsi, was not recruited in response to KOR activation. The TIRF setup also allowed recording single cell

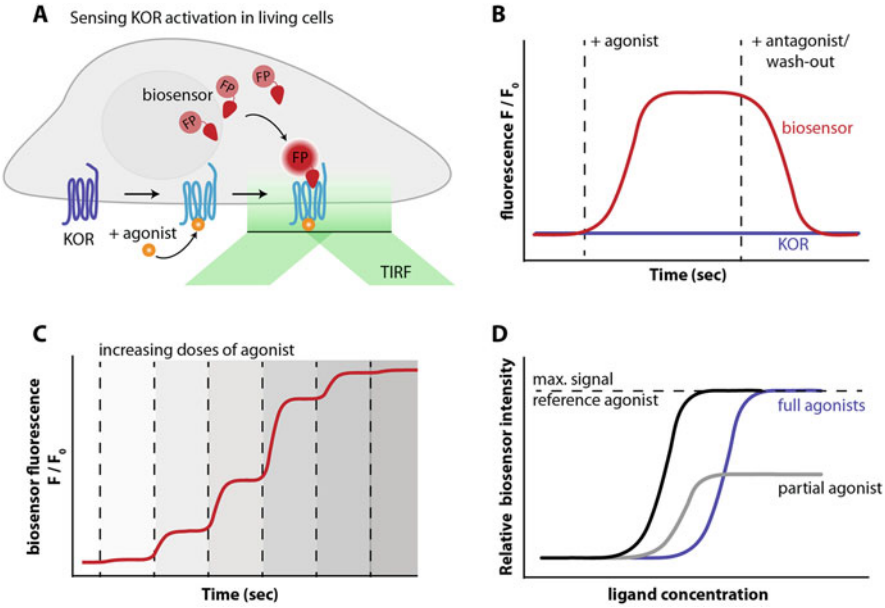

Fig. 2 Biosensors directly detect KOR activation and ligand pharmacology in living cells. (**a**) TIRF assay for measuring biosensor (Nb39, Nb33, or mini-Gsi) recruitment to KOR in the plasma membrane. Biosensor fused to a fluorescent protein (FP, red) re-localizes from the cytosol to active state KOR (blue) in the plasma membrane upon agonist addition. The total internal reflection fluorescence (TIRF) microscopy light beam is indicated. (**b**) Schematic of the fluorescence changes of an active state-specific biosensor (red) and of KOR (blue) detected in the TIRF assay. Agonist and antagonist additions are depicted. (**c**) Schematic of the stepwise increase in biosensor fluorescence intensity during concentration response measurements of single cells using TIRF. The maximum signal of a given ligand is normalized to the intensity generated by an internal reference ligand, which is applied in excess at the beginning (and then washed out) or end of the time series. (**d**) Concentration-dependent recruitment of biosensors to KOR measured by TIRF reveals ligand differences in efficacy and potency

concentration-response curves by following biosensor intensity while increasing agonist concentration in a stepwise manner (Fig. 2c, d). Each dose response was internally normalized to the maximal biosensor intensity measured after adding the reference agonist DynA in excess at the end of each time series. Both Nb33 and mini-Gsi were robustly recruited to KOR in a concentration-dependent manner by chemically diverse full agonists (Stoeber et al. 2020).

Taken together, several KOR-interacting proteins, known to recognize different structural features of the receptor without requiring or engaging other known cellular proteins, can be used as rapid and robust biosensors for KOR activation in living cells. The current assays are based on receptor-proximal biosensor recruitment and present a linear system well-suited to detect differences in relative agonist efficiency and potency.

4 Biosensors Reveal Ligand-Selective Effects at KOR

4.1 Ligand-Selective Recruitment of Distinct Biosensors to KOR

Ligand selectivity or ligand bias is the process whereby chemically distinct agonists can produce different receptor-based effects and represents a well-established pharmacological concept. It is based on the hypothesis that different agonists can drive GPCRs to recruit different cytoplasmic proteins in living cells, introducing allosteric bias into the signaling system at the very receptor-proximal level. To date, most studies on functional selectivity have focused on classifying ligands based on their ability to drive G protein relative to β-arrestin-based pathways and the indirect downstream readouts have required subsequent calculation of bias by operational analysis. We tested whether the GPCR-interacting biosensors could offer a more simple and direct approach for assessing ligand-selective protein recruitment to receptors. While the active state biosensors of KOR have been engineered with the goal to sense and stabilize activation-associated conformational changes in the receptor, it is clear that nanobodies and mini-G proteins present distinct protein folds and that each probe recognizes different structural features of the activated receptor (Fig. 1). Therefore, we reasoned that nanobody-bound KOR and mini-Gsi-bound KOR may represent discrete protein-engaged receptor states that might be selectively produced by diverse agonists (Stoeber et al. 2020). Using mini-Gsi and Nb33 comparatively as KOR-interacting probes, we first noticed that the concentration-response curve for mini-Gsi relative to Nb33 recruitment was consistently left-shifted for all agonists (Fig. 3a, b). The potency shift indicates a difference in allosteric communication of the distinct ligand–KOR–biosensor complexes and further highlights that mini-Gsi and Nb33 interactions with active KOR are not identical. Of note, a similar left shift for mini-Gsi relative to Nb33 was also detected for various agonists at MOR, as determined in BRET and TIRF assays (Gillis et al. 2020b; Stoeber et al. 2020).

We subsequently investigated the effect of the alkaloid agonist etorphine on mini-Gsi and Nb33 recruitment by KOR. Etorphine is an opiate alkaloid drug that efficaciously promotes G protein signaling but drives β-arrestin-mediated KOR internalization poorly and is therefore classified as a G protein-biased agonist by operational criteria (DiMattio et al. 2015; Jordan et al. 2000). In the TIRF-based biosensor recruitment assay, etorphine behaved as a potent but partial agonist for mini-Gsi recruitment, and remarkably, etorphine drove no recruitment of Nb33 even at very high concentrations (Fig. 3c, d). The differential biosensor recruitment indicates that mini-Gsi and Nb33 probes can distinguish receptor-proximal agonist effects in intact cells. In other words, the results provided direct experimental evidence that ligands can impose selectivity on protein recruitment by GPCRs. Differential recruitment of mini-Gsi and Nb33 was not unique to etorphine at KOR but was observed for a range of chemically diverse MOR agonists, including morphine, PZM21, and mitragynine pseudoindoxyl (Gillis et al. 2020b; Stoeber et al. 2020).

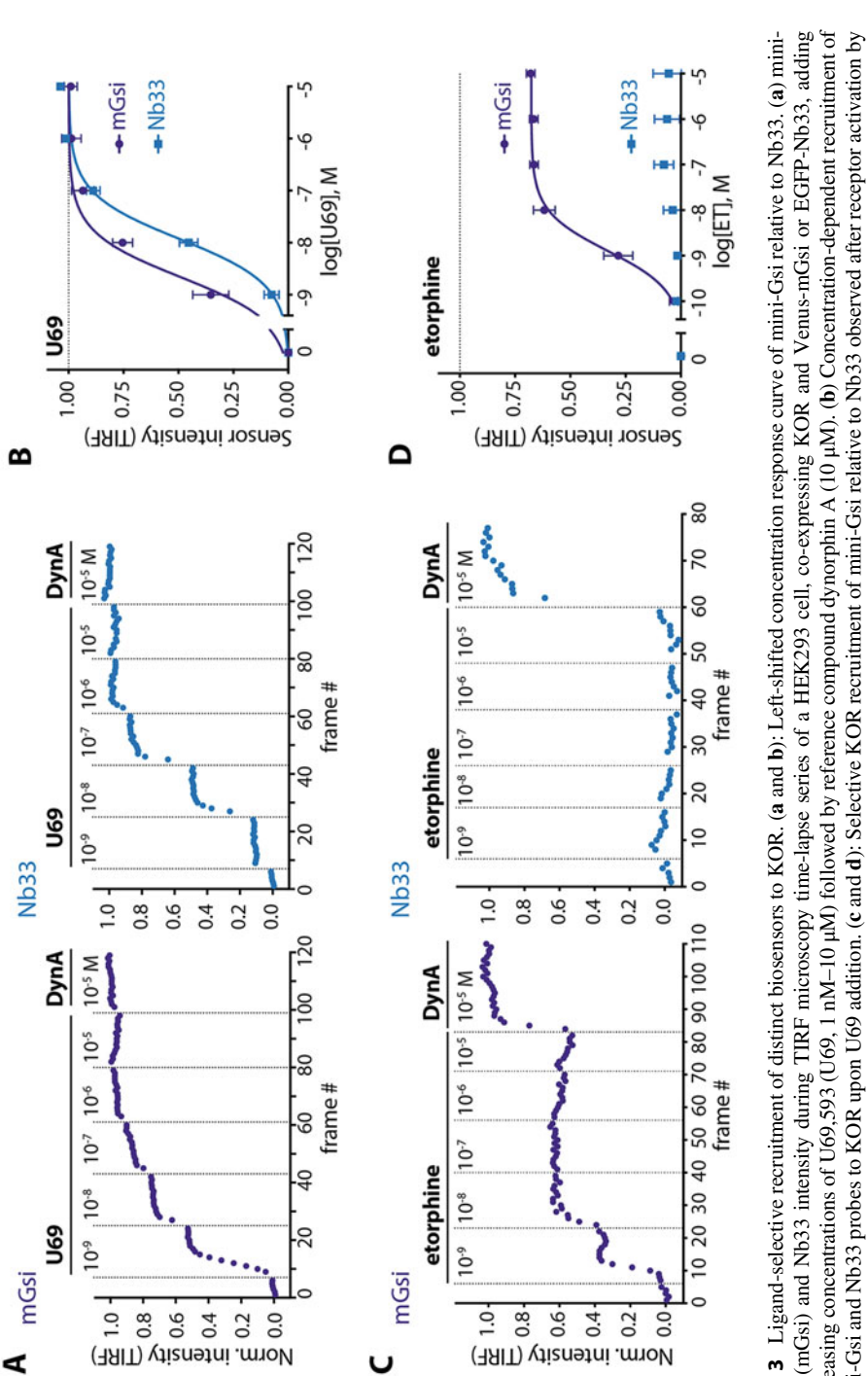

Fig. 3 Ligand-selective recruitment of distinct biosensors to KOR. (**a** and **b**): Left-shifted concentration response curve of mini-Gsi relative to Nb33. (**a**) mini-Gsi (mGsi) and Nb33 intensity during TIRF microscopy time-lapse series of a HEK293 cell, co-expressing KOR and Venus-mGsi or EGFP-Nb33, adding increasing concentrations of U69,593 (U69, 1 nM–10 μM) followed by reference compound dynorphin A (10 μM). (**b**) Concentration-dependent recruitment of mini-Gsi and Nb33 probes to KOR upon U69 addition. (**c** and **d**): Selective KOR recruitment of mini-Gsi relative to Nb33 observed after receptor activation by

While the different biosensors allow to directly measure receptor-proximal protein recruitment in living cells, they present engineered probes that do not directly relate to receptor function. Moving beyond biosensors, we found that agonist-selective protein recruitment to KOR also applies to the physiological relevant GPCR-interacting kinase GRK2. GRK2 drives ligand-dependent KOR phosphorylation in the cytoplasmic tail and it is clear that KOR agonists differ in their ability to stimulate receptor phosphorylation (Chen et al. 2016; Chiu et al. 2017). Using an adapted TIRF protocol that allows to differentiate plasma membrane recruitment from receptor recruitment, we detected that etorphine did not promote GRK2 recruitment to KOR, which was in striking contrast to DynA that strongly drove GRK2–KOR engagement (Stoeber et al. 2020). Ligand-selective GRK2 binding may explain why KOR exhibits agonists-selective phosphorylation and subsequent receptor internalization (Chu et al. 1997; Li et al. 2003). It also suggests that the Nb33 probe can report allosteric effects relevant to GRK engagement.

The findings that conformational biosensors, such as mini-Gsi and Nb33, can be differentially recruited to KOR render them particularly interesting as straightforward tools to probe for ligand bias at KOR. The differential recruitment provides evidence for the hypothesis that agonist bias can manifest in discrete receptor-proximal molecular selection events in the cell. To date, the underlying biophysical differences between the Nb33- and miniG-engaged KOR states remain unresolved. It is possible that the distinct complexes present unique active receptor conformations that are selectively stabilized by agonists. It is also possible that even more diversity in ligand-activated receptor states exists that is not detected by the currently available biosensor probes. Measuring mini-Gsi and Nb33 recruitment comparatively for a larger panel of KOR agonists with diverse pharmacological profiles will deepen our understanding of both agonist bias and the newly available tools to directly assess it.

4.2 Agonist-Selective Activation of KOR at Distinct Cellular Locations

In the past decade, it has become increasingly clear that in addition to receptors at the cell surface, GPCRs in intracellular organelles, such as endosomes, the Golgi apparatus, or the nuclear envelope, can be ligand-activated and mediate physiologically important signaling (Eichel and von Zastrow 2018; Jong et al. 2018). It was also uncovered that ligands differ strikingly in their ability to access GPCRs at different locations in the cell. For example, in order to activate internal β_1-adrenergic

Fig. 3 (continued) etorphine (ET). (**c**) mini-Gsi and Nb33 intensity during TIRF microscopy time-lapse series as in (**a**), adding increasing concentrations of ET (1 nM–10 μM), followed by reference compound DynA (10 μM). (**d**) Concentration-dependent recruitment of mGsi and Nb33 probes to KOR upon ET addition. All data as in Stoeber et al. (2020), published under Creative Commons Attribution Licence CC-BY 4.0

receptors, agonists must be able to pass membranes either by diffusion or by the help of membrane-embedded transporters (Irannejad et al. 2017). Accumulating evidence shows that the location of GPCR signaling can affect both the specificity and the timing of downstream events, demonstrating that cellular location bias in ligand action represents an important new dimension of ligand selectivity (Godbole et al. 2017; Nash et al. 2019; Stoeber et al. 2018; Tsvetanova and von Zastrow 2014).

The use of novel conformation-specific GPCR binders as biosensors in living cells has been instrumental in advancing our understanding of the subcellular organization of GPCR signaling. Recently, we have delineated the spatiotemporal pattern of MOR and DOR activation in living neurons using Nb33 as a biosensor (Fig. 4) (Stoeber et al. 2018). Opioid receptor ligands comprise structurally diverse peptide and non-peptide agonists that differ substantially in their physicochemical properties, which affect membrane permeability. Focusing first on peptide agonists, we found that the endogenous neuropeptides met-enkephalin and β-endorphin drive two spatially and temporally resolved "waves" of MOR and DOR activation, first in the plasma membrane and then in endosomes following agonist-induced internalization of ORs. Extending our studies to clinically relevant opioid drugs, we then found that non-peptide opioid ligands, such as the prototypic alkaloid morphine, drive a discrete and additional wave of MOR and DOR activation in the Golgi apparatus. Golgi-localized OR activation does not require receptor trafficking and is specific to opioid drugs relative to opioid neuropeptides, since drugs can access this internal location due to their ability to diffuse freely across membranes (Fig. 4). Together with studies on other GPCRs, the findings provide a novel cellular framework for understanding how drugs may exert their specific effects (or side-effects) on the cellular level. First inroads into probing the functional significance of internal OR activation show that endosomal ORs contribute a sustained component of adenylyl cyclase inhibition and a subset of ERK and PKC signals on the cellular level and sustained inhibitory actions in sensory neurons on the physiological level (Jimenez-Vargas et al. 2020; Stoeber et al. 2018). The physiological importance of Golgi-localized OR signaling remains to be determined.

Like for the other GPCRs, KOR activation and signaling has generally been assumed to be restricted to the plasma membrane. However, first evidence that KOR ligands strikingly differ in the subcellular location at which they produce receptor activation comes from a recent study that used the biosensor Nb39. Che et al. followed KOR activation in real-time by confocal microscopy and detected a striking difference between the endogenous peptide ligand DynA and the non-peptide hallucinogen SalA (Che et al. 2020). Within seconds, SalA activated the Golgi-localized pool of KOR as detected by recruitment of Nb39 to an internal organelle containing KOR and co-labelling with the Golgi marker GalT. In contrast, DynA drove KOR activation only in the plasma membrane. It is likely that the difference in cellular activation patterns lies in the differential access of ligands to Golgi-localized receptors. SalA may freely diffuse across the membrane, while the peptide DynA penetrates membranes inefficiently. Given the large and increasing number of chemically diverse peptide and non-peptide ligands for KOR, it will be exciting to probe ligand-specific subcellular patterns of KOR activation more

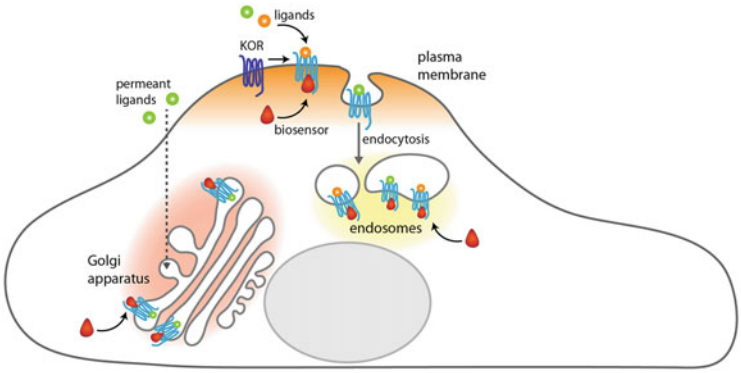

Fig. 4 Location bias of ligand effects: OR activation occurs at distinct subcellular membrane compartments. Peptide ligands (orange circles) first bind and activate ORs at the plasma membrane and initiate a second wave of receptor activation in endosomes following receptor internalization. In addition, membrane-permeant ligands (green-circles) can access receptors that localize inside the cell at steady state and drive OR activation in the Golgi apparatus. Nanobodies allow detecting the spatiotemporal activation profile of distinct agonists

broadly and study the contribution of location-specific activation to the physiological effects of drugs. The KOR-binding nanobodies provide ideal and validated tools for gaining insights into the spatiotemporal organization of KOR signaling and ligand selectivity at the cellular level.

5 Conclusions and Outlook

It is increasingly clear that GPCRs can relay significantly more information about the local chemical environment than the mere binding of an agonist. GPCRs can convey what agonist is binding more specifically by triggering agonist-selective cellular responses. How agonist-selective effects are encoded and transmitted by GPCRs has been studied on many levels and remains an area of intense investigation (Smith et al. 2018). The allosteric complex of ligand, receptor, and transducer mediates communication across the cellular membrane and defines the downstream signaling response. High resolution structural and biophysical studies reveal that distinct agonists can impose bias on the conformational landscape of individual receptors and suggest that unique receptor conformations can couple to distinct cytosolic transducers, leading to a biased cellular response (Weis and Kobilka 2018). The development of orthogonal conformation-specific GPCR binders, including here described nanobodies and mini-G probes for KOR, has opened up new approaches to directly test the hypothesis of ligand-dependent "allosteric processing" at GPCRs.

As G protein mimetics, biosensors can serve as tools in pharmacological assays that measure ligand potency and efficacy. Some advantages over classical G protein assays exist, which include the linear assay system, the receptor-proxy readout, the lack of ceiling effect if sensors are in excess, and their usefulness in in vitro and

cellular assays. Moreover, the distinct protein probes differ in their interactions with receptors and can reveal agonist-selective effects directly at the receptor. The differential recruitment of mini-Gsi and Nb33 to KOR provides direct evidence that different agonists stabilize receptors in distinct conformational ensembles that can interact with selective proteins. Therefore, the probes are promising orthogonal and straightforward screening tools in exploring diversity and selectivity between KOR ligands. Kinetic differences in agonist action at GPCRs can be another contributor to bias and future time-dependent analysis of biosensor recruitment to KOR could provide additional insights into selectivity among agonists (Klein Herenbrink et al. 2016; Livingston et al. 2018). As novel tools, it still remains to be determined what the biophysical basis for the observed selectivity of protein recruitment is and also, if and how the distinct allosteric complexes relate to receptor function.

An emerging additional mechanism that differentiates agonists is the specific cellular location at which they drive GPCR signaling. GPCRs have been recognized for many years to be present at internal membrane locations as well as at the plasma membrane; however, internal receptors have been considered a reserve pool (secretory pathway) or a desensitized pool (endosomal pathway) with no contribution to acute signaling. Assays with high spatiotemporal resolution have revealed that many GPCRs are subject to ligand-dependent activation at internal membrane locations and that ligands differ significantly in their ability to access intracellular GPCR pools. Biosensors provide straightforward inroads into analyzing the ligand-selective subcellular activation pattern of receptor activation. A future challenge is to adapt the biosensor methodology to probe receptor activation in neurons with endogenous receptor expression and to possibly turn the tools into real-time optical sensors for in vivo applications.

Opioid receptors are clinically important targets that provide a striking example for biased agonism. The finding that agonists with different pharmacological profiles can cause different outcomes on the physiological level is the driving force behind the quest to develop novel analgesics with a reduced side-effects profile. However, understanding and quantifying bias in the cellular context remains challenging in part due to the lack in direct comparative transducer-specific assays. The novel orthogonal biosensors for opioid receptor activation can report on various aspects of ligand-selective signaling and are likely to gain traction as valuable tools in cellular pharmacology.

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Does GEC1 Enhance Expression and Forward Trafficking of the Kappa Opioid Receptor (KOR) via Its Ability to Interact with NSF Directly?

Peng Huang, Chunxia Zhao, Chongguang Chen, Sidney W. Whiteheart, and Lee-Yuan Liu-Chen

Contents

Abstract

We reported previously that GEC1 (glandular epithelial cell 1), a member of microtubule-associated proteins (MAPs), interacted directly with the C-tail of KOR (KCT) and tubulin and enhanced cell surface expression of KOR in CHO cells by facilitating its trafficking along the export pathway. Two GEC1 analogs (GABARAP and GATE16) were also shown to increase KOR expression. In addition, to understand the underlying mechanism, we demonstrated that *N*-ethylmaleimide-sensitive factor (NSF), an essential component for membrane fusion, co-immunoprecipitated with GEC1 from brain extracts. In this study, using pull-down techniques, we have found that (1) GEC1 interacts with NSF directly and prefers the ADP-bound NSF to the ATP-bound NSF; (2) D1 and/or D2 domain(s) of NSF interact with GEC1, but the N domain of NSF does not; (3) NSF does not interact with KCT directly, but forms a protein complex with

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KCT via GEC1; (4) NSF and/or α-SNAP do not affect KCT-GEC1 interaction. Thus, GEC1 (vs the α-SNAP/SNAREs complex) binds to NSF in distinctive ways in terms of the ADP- or ATP-bound form and domains of NSF involved. In conclusion, GEC1 may, via its direct interactions with KOR, NSF, and tubulin, enhance trafficking and fusion of KOR-containing vesicles selectively along the export pathway, which leads to increase in surface expression of KOR. GABARAP and GATE16 may enhance KOR expression in a similar way.

Keywords

GABARAP · GATE16 · GEC1 · Kappa opioid receptor · NSF · Tubulin

Abbreviations

AAA	ATPases associated with diverse cellular activities
Atg8	The yeast autophagy protein 8
CHO-FLAG-KOR	CHO cell line stably expressing FLAG-hKOR
DOR	Delta opioid receptor
ER	Endoplasmic reticulum
GABARAP	$GABA_A$ receptor-associated protein
GABARAPL1	GABAA receptor-associated protein like 1
GATE16	Golgi-associated ATPase enhancer of 16 kDa
GEC1	Glandular epithelial cell 1
GOS-28	Golgi-specific v-SNARE of 28 kDa
GST	Glutathione *S*-transferase
hKOR	Human KOR
KCT	KOR C-tail
KOR	Kappa opioid receptor
LC3	Light chain 3 of MAP 1A/1B
MAPs	Microtubule-associated proteins
MOR	Mu opioid receptor
NSF	*N*-ethylmaleimide-sensitive factor
SNARE	Soluble N-ethylmaleimide sensitive factor attachment protein receptor
α-SNAP	Soluble NSF attachment protein

1 Introduction

GEC1 (glandular epithelial cell 1), a 117-amino acid protein, is also named GABAA receptor-associated protein like 1 (GABARAPL1). Its cDNA was first cloned as an early estrogen-regulated mRNA from guinea-pig endometrial glandular epithelial cells by Pellerin et al. (1993). GEC1 is a member of microtubule-associated proteins (MAPs), which also include $GABA_A$ receptor-associated protein (GABARAP), Golgi-associated ATPase enhancer of 16 kDa (GATE16, also named

GABARAPL2), GABARAPL3, light chain 3 (LC3) of MAP 1A/1B, and the yeast autophagy protein 8 (Atg8) (Nemos et al. 2003; Xin et al. 2001). Among these homologues, GEC1 share the highest identity with GABARAPL3 (93%), followed by GABARAP (86%) and GATE16 (61%).

We previously reported that GEC1 interacted with the human KOR (hKOR) C-tail (KCT) directly and enhanced total and cell surface levels of hKOR stably expressed in CHO cells by facilitating its trafficking along the endoplasmic reticulum–Golgi–plasma membrane export pathway without affecting degradation of the receptor (Chen et al. 2006). GABARAP and GATE16 have been also shown to enhance KOR expression (Chen et al. 2009, 2011). Transport of vesicle cargo (e.g., KOR) between intracellular compartments and to the cell surface is thought to be mediated by membrane-bound vesicles and microtubules along the export pathway (Yoon and Munson 2018). Thus, GEC1, GABARAP, and/or GATE16 were proposed to have overlapping functions and share common molecular mechanisms to facilitate trafficking and/or fusion of KOR-containing vesicles in the export pathway and thereby expression of KOR (Chen et al. 2006, 2009, 2011). The underlying mechanisms involve being associated with both microtubule-based cytoskeletal structures and essential membrane fusion machinery.

On the one hand, GEC1, as well as GABARAP, interacts with tubulin and promoted microtubule bundling in vitro (Chen et al. 2006; Mansuy et al. 2004). Deletion of the first 37 amino acids (the tubulin binding domain) at the N-terminal region of GEC1 greatly reduced its influence on KOR expression (Chen et al. 2009). Therefore, GEC1, as well as GABARAP, was proposed to not only interact with tubulin to promote microtubule bundling but also function as a linker between KOR and microtubule-based cytoskeletal structures to enhance the transport of KOR-containing vesicles along microtubules (Chen et al. 2009, 2011). Similarly, the tubulin binding region of GABARAP was important for its effect on clustering of $GABA_A$ receptors (Chen et al. 2000; Wang and Olsen 2000) and enhancing plasma membrane expression of the angiotensin II type 1 receptor (Cook et al. 2008) via the strong association of GABARAP with microtubule-based cytoskeletal structures. Furthermore, GABARAP has already been described to facilitate transport of vesicular structures along microtubules (Labonte et al. 2014; Lystad et al. 2014).

On the other hand, GATE16 and GABARAP were demonstrated to interact directly with NSF (Kittler et al. 2001; Sagiv et al. 2000), a critical component of membrane fusion machinery. NSF belongs to the ATPases associated with diverse cellular activities (AAA) family and catalyzes multiple exocytic/endocytic membrane fusion events [see (Zhao et al. 2007) for a review]. NSF is recruited to membranes via soluble NSF attachment proteins (SNAPs), which bind to integral membrane proteins SNAP receptors (SNAREs). A unitary v-SNARE on a vesicle binds specifically to its cognate t-SNARE complex on the target membrane to form a trans-SNARE complex, which results in the tight association of the vesicle with its target membrane, termed docking. After fusion, highly stable cis-SNARE complexes accumulate in the fused membranes. ATP hydrolysis by NSF dissembles the cis-SNARE complexes, which frees SNAREs for future fusion events (Zhao et al. 2007). The GATE16-NSF and GABARAP-NSF interactions were suggested to

regulate SNARE (soluble N-ethylmaleimide sensitive factor attachment protein receptor) functions, vesicle fusion and thus intracellular membrane transport [see (Elazar et al. 2003; Thielmann et al. 2009) for reviews]. In addition, GEC1 was shown to interact with NSF in rat brain extracts in a co-immunoprecipitation assay (Chen et al. 2006), and purified recombinant GST-GEC1 protein, but not GST, was able to pull down NSF from rat brain extracts (Chen et al. 2006).

The current study is to continue investigating whether GEC1 binds to NSF directly, whether KCT, GEC1, and NSF form a protein complex, and whether NSF and/or α-SNAP affect KCT-GEC1 interaction using purified recombinant proteins in pull-down assays. How GEC1 enhances KOR forward trafficking and expression via its interaction with NSF is also discussed.

2 Experimental Procedures

2.1 Pull-Down Assays Using Purified Recombinant Proteins Expressed in *E. coli*

cDNA Construction and generation of glutathione S-transferase (GST)-tagged Fusion Proteins were as same as described previously (Chen et al. 2006) – Briefly, two GST fusion proteins were used in the studies: GST-GEC and GST-KCT, which were generated in the pGEX-4T-1 bacterial expression system (Amersham Biosciences), expressed in *E. coli* BL21-CodonPlus (DE3)-RP (Stratagene, La Jolla, CA), and adsorbed onto glutathione-Sepharose 4B beads and washed three times with phosphate-buffered saline for pull-down analysis.

cDNA Construction and Purification of His_6/S Tag-GEC1 (Chen et al. 2006) – The full-length cDNA of *GEC1* was cloned into the pET-30a(+) bacterial expression vector, which carries an N-terminal His6 tag and an S-tag. The construct was transformed into *E. coli* BL21(DE3), and the protein was adsorbed onto ProBond™ nickel-chelating resin (Invitrogen). The His6 tag fusion proteins were eluted from the beads with 0.5 M imidazole/500 mM NaCl/50 mM sodium phosphate buffer, pH 8.0, at room temperature for 20 min. Imidazole was removed via multiple rounds of concentration and dilution using Microcon™ centrifugal filter devices (Millipore, Bedford, MA) with TBS-T buffer (25 mM Tris/150 mM NaCl/0.1% Tween 20, pH 7.4).

cDNA Construction and Purification of His_6-α-SNAP and His_6-NSF and domains (D1, D2, and N) (Zhao et al. 2010) – Each cDNA was cloned into pQE9 expression vector, expressed in the *Escherichia coli* strain, Rosetta DE3 pLacI, and purified according to published methods (Zhao et al. 2010).

Interaction of GEC1 and NSF – In buffer BB containing 50 mM HEPES/pH 7.4, 100 mM KCl, 1% Triton X-100 (w/v), 5% glycerol (v/v), 2 mM β-mercaptoethanol and 3% BSA, purified His_6-NSF (3 μg/300 μl, ~100 nM) was incubated with glutathione-Sepharose 4B beads pre-loaded with GST or GST-GEC1 (~5-10 μg/10 μl beads) for 1 h at RT, in the presence or absence of ATP (5 mM), AMP-PNP (5 mM), ADP (5 mM), EDTA (5 mM), and/or $MgCl_2$ (10 mM). The beads were

washed four times with buffer BB without BSA, in the presence or absence of ATP, AMP-PNP, ADP, EDTA, and or $MgCl_2$ accordingly. Then the bound proteins were eluted from the beads by 2× PAGE sample buffer, and resolved by 8% SDS-PAGE, and transferred onto an ImmobilonTM-P PVDF membrane. His_6-NSF was detected by blotting with HisProbe-HRP (Pierce) followed by enhanced chemiluminescence reagents. Images were captured via the Fujifilm LAS1000 plus imaging system, quantified by the affiliated Image Gauge software, and normalized by the signals under the condition (with ATP and Mg^{++}). The same membrane was stained with 0.5% Ponceau S in 5% acetic acid, which shows the molecular weights and amounts of the GST and GST-GEC1.

Interaction of GEC1 or KCT with full-length NSF or its N, D1, or D2 Domains – In buffer BB as mentioned above, purified His_6-NSF full-length or domain(s) (~100 or 200 nM) was incubated with glutathione-Sepharose 4B beads pre-loaded with GST, GST-GEC1, or GST-KCT for 1 h at RT, in the presence of ATP (5 mM) and $MgCl_2$ (10 mM). The beads were washed four times with buffer BB without BSA, in the presence or absence of ATP and $MgCl_2$. Then the bound proteins were detected the same way as mentioned above.

Interactions among GEC1, KCT, NSF, and α-SNAP – The purified His_6-S.tag-GEC1 (1-6 μg/μl, ~100–600 nM) was incubated with glutathione-Sepharose 4B beads pre-loaded with GST or GST-KCT for 1 h at RT, in the presence or absence of His_6-NSF (~100 nM) and/or His_6-α-SNAP (1 μg/300 μl, ~50 nM) (The molar ratio is 1 NSF hexamer: 3 α-SNAP: 6-36 GEC1), in buffer BB with ATP (5 mM) and $MgCl_2$ (10 mM). The bound proteins were washed, eluted from the beads by 2× SDS-PAGE sample buffer, resolved by 15% SDS-PAGE, transferred and detected as described above.

3 Results

Our previous findings indicated that GEC1 interacted with NSF (Chen et al. 2006). Here, recombinant GST-GEC1 and His_6-NSF were generated in *E. coli* and purified. By pull-down assays, a direct interaction between GEC1 and NSF was revealed (Fig. 1). In the buffer, ADP, ATP, or a nonhydrolyzable ATP analog (AMP-PNP) together with Mg^{++} or EDTA was included. As ATP was supposed to be hydrolyzed to ADP by NSF in the presence of Mg^{++}, while this reaction was inhibited by the presence of EDTA, the results demonstrated that GEC1 preferred to bind to the ADP-bound NSF to the ATP-bound NSF (Fig. 1).

Each NSF protomer of the homo-hexamer contains one N-terminal domain (NSF-N), followed by two conserved nucleotide-binding domains (termed NSF-D1 and NSF-D2) (Zhao et al. 2010). NSF-N is required for SNAP-SNARE binding (Zhao et al. 2010). NSF-D1 and NSF-D2 account for the majority of the ATP hydrolysis and oligomerization (hexamerization), respectively (Zhao et al. 2010). It should be noted that (1) recombinant D1 was not able to be expressed by itself, (2) N-D1 and N are monomers, and (3) other D2-containing truncated or

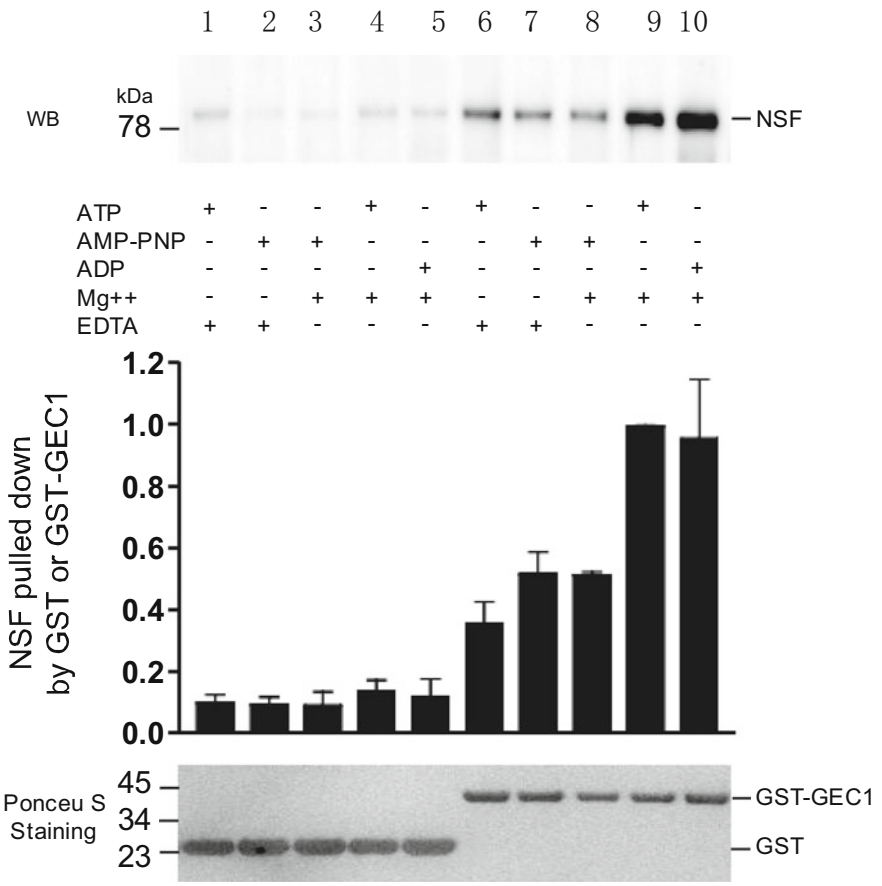

Fig. 1 GEC1 interacts with NSF depending on the form of nucleotide bound to NSF. Pull-down experiments were carried out as described in Experimental Procedures. The purified His_6-NSF (~100 nM) was incubated with glutathione-Sepharose 4B beads pre-loaded with GST and GST-GEC1 for 1 h at RT, in buffer BB with the presence or absence of ATP (5 mM), AMP-PNP (5 mM), ADP (5 mM), EDTA (5 mM), and/or $MgCl_2$ (10 mM). For *upper panel*, the His_6-NSF was detected by blotting with HisProbe-HRP (Pierce) followed by enhanced chemiluminescence reagents. For *middle panel*, the signals from 4–8 times of independent experiments, similar to the representative one shown in the upper panel, were captured via the Fujifilm LAS1000 plus imaging system, quantified by the affiliated Image Gauge software, and normalized by the signals under the condition of lane 9 (with ATP and Mg^{++}). For *lower panel*, the same membrane of upper panel was stained with 0.5% Ponceau S in 5% acetic acid, which shows the molecular weights and amounts of the GST and GST-GEC1

full-length forms of NSF are hexamers. Using pull-down techniques, GEC1 was shown to interact with D1 and/or D2 domain(s), but not N domain of NSF (Fig. 2).

KCT was demonstrated to interact with GEC1 directly (Chen et al. 2006). In current studies, pull-down assays confirmed this previous finding and further

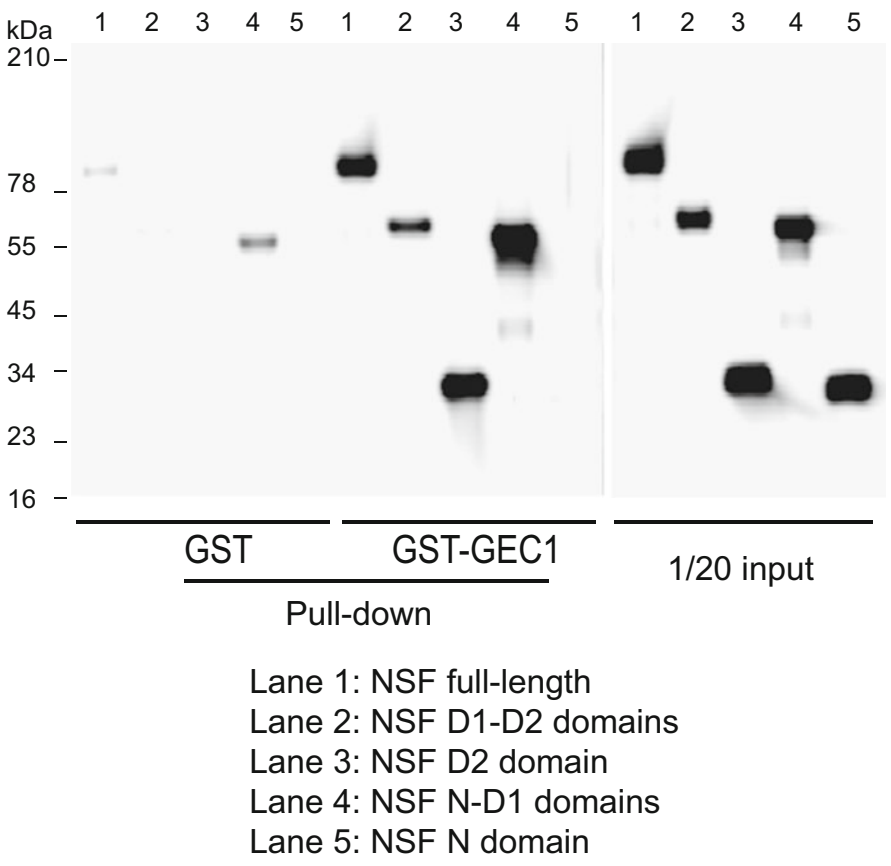

Fig. 2 GEC1 interacts with D1 and/or D2 domain(s) of NSF, but not N domain. Pull-down experiments were carried out as described in Experimental Procedures. Each of the purified His_6-NSF full-length or domains (~200 nM) was incubated with glutathione-Sepharose 4B beads pre-loaded with GST and GST-GEC1 for 1 h at RT, in buffer BB with ATP (5 mM) and $MgCl_2$ (10 mM). His_6-NSF was detected by blotting with HisProbe-HRP (Pierce) followed by enhanced chemiluminescence reagents. The figures represent one of the three experiments performed with similar results

revealed that there was no direct interaction between KCT and NSF (Fig. 3a). However, NSF formed a protein complex with KCT via GEC1 (Fig. 3b).

NSF binds to the SNARE complexes by interacting with an adaptor protein called soluble NSF attachment protein (α-SNAP) (Zhao et al. 2010). α-SNAPs position NSF at the membrane-distal end of the SNARE complex, which stimulates the ATPase activity of NSF and the subsequent hydrolysis-dependent conformational changes drive unwinding of the SNARE complex (Zhao et al. 2010). In current studies, it was tested whether KCT-GEC1 interaction would be dissociated in the presence of NSF and/or α-SNAP. It was observed that NSF alone or with α-SNAP

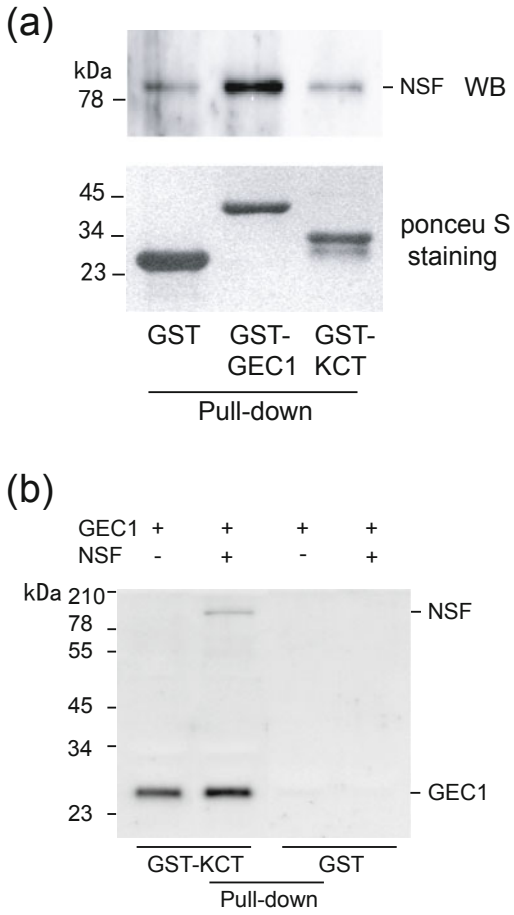

Fig. 3 NSF does not interact with the kappa opioid receptor c-tail (KCT) directly (**a**), but it can form a protein complex with KCT via GEC1 in vitro (**b**). (**a**) The purified His_6-NSF (~100 nM) was incubated with glutathione-Sepharose 4B beads pre-loaded with GST, GST-GEC1, or GST-KCT for 1 h at RT, in buffer BB with ATP (5 mM) and $MgCl_2$ (10 mM). For *upper panel*, the bound proteins were washed, eluted from the beads by 2× SDS-PAGE sample buffer, resolved by 8% SDS-PAGE, transferred onto a PVDF membrane, and detected as described in Fig. 1. For *lower panel*, the same membrane of upper panel was stained with 0.5% Ponceau S in 5% acetic, which shows the molecular weights and amounts of the GST and GST fusion proteins; (**b**) The purified His_6-S.tag-GEC1 (1-6 μg/μl, ~100–600 nM) was incubated with glutathione-Sepharose 4B beads pre-loaded with GST or GST-KCT for 1 h at RT, in the presence or absence of His_6-NSF (~100 nM) (The molar ratio is 1 NSF hexamer: 6-36 GEC1), in buffer BB with ATP (5 mM) and $MgCl_2$ (10 mM). The figures represent one of the 2–4 experiments performed with similar results

did not significantly affect the interaction between KCT and GEC1 in the pull-down assays (Fig. 4). It is interesting that α-SNAP was so sticky and seemed to even bind to GST, and that binding was reduced in the presence of NSF (Fig. 4).

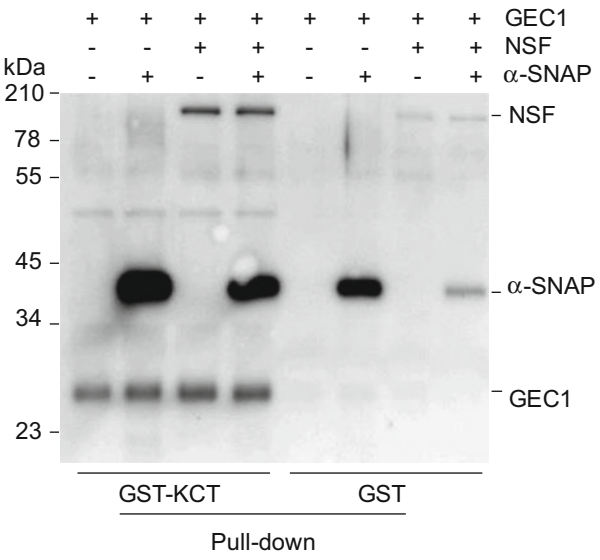

Fig. 4 NSF and/or α-SNAP do not disrupt KCT-GEC1 interaction. The purified His_6-S.tag-GEC1 (~100–600 nM) was incubated with glutathione-Sepharose 4B beads pre-loaded with GST or GST-KCT for 1 h at RT, in the presence or absence of His_6-NSF (~100 nM) and/or His_6-α-SNAP (1 μg/300 μl, ~50 nM) (The molar ratio is 1 NSF hexamer: 3 α-SNAP: 6-36 GEC1), in buffer BB with ATP (5 mM) and $MgCl_2$ (10 mM). The bound proteins were washed, eluted from the beads by 2× SDS-PAGE sample buffer, resolved by 15% SDS-PAGE, transferred, and detected as described in Fig. 1. The figures represent one of the four experiments performed with similar results

4 Discussion

By pull-down techniques, using purified recombinant proteins, we showed that a protein complex was formed among KCT, GEC1, and NSF. The complex was formed by KCT-GEC1 and GEC1-NSF interactions, but no interaction was found between KCT and NSF. The KCT-GEC1 interaction was not affected by NSF in the absence or presence of α-SNAP. Our studies, to the best of our knowledge, are the first to demonstrate a direct interaction between GEC1 and NSF. Therefore, GEC1, GATE16, and GABARAP of the MAPs family have all been demonstrated to interact directly with NSF (Kittler et al. 2001; Sagiv et al. 2000) and enhance KOR expression (Chen et al. 2006, 2009, 2011). Thus, a plausible hypothesis is that three MAPs facilitate anterograde trafficking and thereby expression of KOR via their abilities to interact with tubulin and NSF.

It should be noted that GEC1-NSF binding was better formed when NSF was in the ADP- (vs ATP-) bound form or as a result of ATP hydrolysis. It is unknown whether GATE16-NSF and GABARAP-NSF bindings are better established in a similar way (Kittler et al. 2001; Sagiv et al. 2000). In contrast, NSF-α-SNAP/SNAREs binding was stronger when NSF was in the ATP-bound form (Nagiec et al. 1995). In addition, D1 and/or D2, but not N domains of NSF bound to GEC1 while SNAP-SNARE binding required the NSF-N domain (Zhao et al. 2010). It is

yet to be investigated which domain(s) of NSF are involved in the GATE16-NSF and GABARAP-NSF interactions (Kittler et al. 2001; Sagiv et al. 2000).

The most well-known function of NSF is to unravel cis-SNARE complexes once membrane fusion has occurred, using the hydrolysis of ATP as an energy source. This function allows the dissociated t- and v-SNAREs to be recycled for reuse in further rounds of membrane fusion [see (Yoon and Munson 2018; Zhao et al. 2007) for reviews]. If the interaction of GEC1, GATE16, or GABARAP with NSF plays a role in regulating this SNARE dissembling process which occurs after membrane fusion [i.e., when the step of transport/trafficking of cargo (e.g., KOR) is done], overexpression of any of the three MAPs would enhance vesicle export in a cargo (e.g., KOR)-non-specific manner. However, this inference contrasts with our observations that overexpression of GEC1 enhanced KOR expression, but failed to affect expression of MOR (mu opioid receptor) and DOR (delta opioid receptor) (Chen et al. 2006). This KOR-specific effect of GEC1 was in line with the findings that only the KOR, but not MOR and DOR, interacts with GEC1 (Chen et al. 2006).

Other evidence also indicates that NSF is engaged in cellular functions independent of SNARE complex disassembly [see (Elazar et al. 2003; Thielmann et al. 2009; Zhao et al. 2007) for reviews]. In yeast, for instance, NSF/Sec18p appears to be needed prior to docking for vacuole fusion, possibly for maintaining SNAREs in a primed state (Nichols et al. 1997). In addition, reassembly of Golgi cisternae after mitosis requires ATP-bound NSF, but not ATP hydrolysis (Muller et al. 1999). This process involves GATE16, of which the binding partner is transferred from NSF to GOS-28 (Golgi-specific v-SNARE of 28 kDa), thus preventing interaction with its cognate t-SNARE syntaxin-5 and priming the v-SNARE (Muller et al. 2002). Moreover, NSF-GABARAP complexes were detected in neurons and co-localize within intracellular membrane compartments and may be important for sorting and cycling of the $GABA_A$ receptor between cell surface and intracellular pools (Kittler et al. 2001). GEC1 was localized in the Golgi apparatus and the endoplasmic reticulum (ER) in CHO cells (Chen et al. 2006). It also has widespread tissue distribution (Nemos et al. 2003; Wang et al. 2006; Xin et al. 2001) and, in particular, GEC1 is abundant and widely distributed in the central nervous system (Mansuy-Schlick et al. 2006; Tolle et al. 2008; Wang et al. 2006). Immuno-electro-microscopic studies in the rat brain showed that GEC1 was associated with intracellular compartments (ER, Golgi apparatus, endosomal-like vesicles) and plasma membranes and scattered in cytoplasm in neurons (Wang et al. 2006). Thus, via KCT-GEC1 (or its analogs)-NSF interactions, KCT can serve as a scaffold to bring priming factors (e.g., GEC1, GABARAP, GATE16, and NSF) close to KOR-containing vesicles which facilitate the priming of v-SNARE(s) (e.g., Golgi v-SNARE GOS28 (Muller et al. 2002)) on KOR-vesicles. This step prepares the vesicles ready for docking and fusion to the target membranes, which involves winding v-SNAREs with t-SNAREs on target membranes. This likely leads to more efficient KOR/cargo-specific vesicle fusion within the intracellular compartments (e.g., ER and Golgi apparatus) along the export pathway and thereby enhanced KOR expression eventually.

In addition to aforementioned post-fusion and pre-docking views of NSF functions, the ATPase function of NSF was shown to be involved in highly dynamic reactions that occur after docking and immediately before synaptic vesicles fuse with the presynaptic plasma membrane to release neurotransmitter (Kuner et al. 2008). Although whether GEC1-, GABARAP-, and GATE16-NSF interactions play any roles in these reactions have not been reported, it is intriguing to hypothesize, with this during-docking view, that KCT can recruit factors including GEC1, GABARAP, GATE16, and NSF close to the docking sites, stabilizing the docking and facilitating the ensuing fusion of KOR-containing vesicles. Multiple factors have been recently found to maintain assembled trans-SNARE complexes in the presence of NSF and α-SNAP (Prinslow et al. 2019). It is thus intriguing to study whether GEC1-, GABARAP-, and GATE16-NSF interactions impact on stability of trans-SNARE complexes in the future.

GEC1 and its analogs (GABARAP and GATE16) have been shown to bind to and/or enhance the expression of several membrane-bound proteins. We have defined the residues in both GEC1 and KOR C-tail (KCT) involved in the interaction (Chen et al. 2009). Modeling studies revealed that the interaction was mediated via direct contacts between the kinked hydrophobic fragment FPXXM in KCT and the curved hydrophobic surface in GEC1. Expression of GEC1 also increased cell surface levels of the GluR1 subunit and the prostaglandin EP3.f receptor (Chen et al. 2009), which have FPXXM and FPXM sequences, respectively. GEC1 is required for increased membrane expression of epidermal growth factor receptor during hypoxia (Keulers et al. 2015). In addition, GEC1 has been shown to interact with $GABA_A$ receptor (Mansuy et al. 2004). GABARAP is associated with and increases cell surface expression of many membrane-bound proteins, such as $GABA_A$ receptor (Kanematsu et al. 2007; Kittler et al. 2001; Leil et al. 2004; Wang et al. 1999), KOR (Chen et al. 2009, 2011), Na^+-dependent P_i cotransporter (NaP_i-IIa) (Reining et al. 2009), AT1 angiotensin II receptor (Cook et al. 2008), and TRPV1 (Lainez et al. 2010). In addition, GABARAP also binds transferrin receptor (Green et al. 2002). Recently, all three MAPs were found to directly bind to and essential for plasma membrane localization of HIV-1 Nef, an important pathogenic factor for HIV/AIDS pathogenesis (Boeske et al. 2017).

In summary, GEC1 and its analogs (GABARAP and GATE16) appear to have redundant functions to promote expression of and/or plasma membrane localization of a sub-group of membrane-bound proteins. In GABARAP-null mice, there is no change in $GABA_A$ receptor levels or membrane clustering (O'Sullivan et al. 2005), most likely due to their redundant functions. GEC1, GABARAP, and GATE16 can specifically and, in many cases, directly bind to these membrane-bound proteins (vesicle cargos; e.g., KOR) and NSF at the same time. Such protein complexes allow GEC1, its analogs and NSF to be recruited near cargo-containing vesicles. Therefore, on the one hand, GEC1 and its analogs, via their affinities for tubulin (see Introduction for references), can facilitate transport of these vesicles along microtubules in a cargo-selective manner; on the other hand, GEC1 and its analogs, via their direct interactions with NSF, can promote fusion of the cargo-containing vesicles selectively as priming factors for the v-SNAREs and/or as stabilizing factors

for the trans-SNARE complex, before and/or during vesicle docking, respectively, as discussed above. This may be how GEC1 and its analogs facilitate fusion and anterograde trafficking of vesicles between intracellular compartments in a cargo (e.g., KOR)-selective manner.

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Declarations of Interest: None.

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Kappa Opioid Receptor Mediated Differential Regulation of Serotonin and Dopamine Transporters in Mood and Substance Use Disorders

Durairaj Ragu Varman, Lankupalle D. Jayanthi, and Sammanda Ramamoorthy

Contents

Abstract

Dynorphin (DYN) is an endogenous neurosecretory peptide which exerts its activity by binding to the family of G protein-coupled receptors, namely the kappa opioid receptor (KOR). Opioids are associated with pain, analgesia, and drug abuse, which play a central role in mood disorders with monoamine neurotransmitter interactions. Growing evidence demonstrates the cellular signaling cascades linked to KOR-mediated monoamine transporters regulation in cell models and native brain tissues. This chapter will review DYN/KOR role in mood and addiction in relevance to dopaminergic and serotonergic neurotransmissions.

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Also, we discuss the recent findings on KOR-mediated differential regulation of serotonin and dopamine transporters (SERT and DAT). These findings led to a better understanding of the role of DYN/KOR system in aminergic neurotransmission via its modulatory effect on both amine release and clearance. Detailed knowledge of these processes at the molecular level enables designing novel pharmacological reagents to target transporter motifs to treat mood and addiction and reduce unwanted side effects such as aversion, dysphoria, sedation, and psychomimesis.

Keywords

Addiction · Dopamine transporter · Dynorphin · Kappa opioid receptor · Mood · Serotonin transporter · Signaling

1 Introduction

Dynorphins (DYNs) are endogenous opioid neuropeptides derived from the precursor protein prodynorphin (Chavkin and Goldstein 1981; Kakidani et al. 1982). The DYN peptides are released presynaptically following membrane depolarization and modulate neurotransmitter release (Wagner et al. 1993). Dynorphins are released in response to stressful conditions and drugs of abuse via dual mechanisms involving axonal and somatodendritic release (Bruijnzeel 2009; Bruchas et al. 2010). DYN activates KOR primarily and has a low affinity for MOR or DOR. Other opioid peptides, endorphins, and enkephalins show weak interaction with KOR making KOR a distinct form of the opioid system (Chavkin and Goldstein 1981; Goldstein et al. 1981). Anatomically, DYN/KOR system has a diverse distribution (Drake et al. 1996; Svingos et al. 2001), and KORs are expressed both pre- and post-synaptically on neurons (Arvidsson et al. 1995; Drake et al. 1996; Svingos et al. 2001). Because of their high affinity for DYN and their biological significance in pain, addiction, and mood disorders, DYN/KOR interactions and associated signaling are of great interest to researchers.

2 KOR and the Serotonergic System

2.1 KOR Interaction with Serotonergic System and Its Relevance to Psychiatric Disorders

The serotonin (5-hydroxytryptamine, 5-HT) plays an essential role in the adult central and peripheral nervous system regulating various physiological processes including mood, sleep, aggression, anxiety, sexual drive, appetite, memory, thyroid function, gastrointestinal motility, and vasoconstriction (Fozzard 1989; Jacobs and Azmitia 1992). Dysregulation of 5-HT signaling has been implicated in depression, obsessive-compulsive disorder, anxiety disorders, autism spectrum disorder (Olivier 2015). Activation of KOR signaling produces behavioral effects similar to

neuropsychiatric disorders such as depression, schizophrenia, bipolar, and drug addiction. While KOR agonists produce pro-depressive behaviors, antagonists produce antidepressant effects (Mague et al. 2003; Carlezon et al. 2006). Similarly, KOR activation produces anxiogenic effects and KOR antagonism produces anxiolytic effects (Bruchas et al. 2009). For example, KOR activation decreases social interaction (Robles et al. 2014) while KOR antagonism prevents the deficits resulting from the social-defeat test (Bruchas et al. 2011). Thus, KOR signaling being aversive appears to be necessary to express depressive- and anxiety-like behaviors. KOR antagonism may offer an effective treatment option in treating negative affective states of depression and other psychiatric disorders.

Generally, altered DYN/KOR expressions in limbic brain regions have been implicated in both depression and anxiety disorders in both rodents and humans (Tejeda et al. 2012). A cluster of serotonergic neurons are present in the dorsal raphe nucleus (DRN) and project their axons throughout the brain and these neuronal circuits are involved in mood regulation (Wylie et al. 2010). Functional expression of KOR as a heteroreceptor on serotonergic neurons and neuronal terminals is well documented (Berger et al. 2006). Activation of KOR by endogenous DYN or synthetic KOR agonists decreases 5-HT release (Tao and Auerbach 2002). Studies have shown that KOR agonists produce pro-depressant-like effects (Carlezon et al. 2006). By contrast, antagonists produce antidepressant-like effects in various animal models (Shirayama et al. 2004). Conditioned place aversive (CPA) effects of KOR agonists are observed in experimental animals (Mucha and Herz 1985). Depletion of 5-HT or selective serotonin reuptake inhibitor (SSRI) fluoxetine induces changes in prodynorphin gene expression in a brain-region-specific manner (Di Benedetto et al. 2004). Furthermore, 5-HT plays a role in the chronic effect of KOR agonist or cocaine on DYN mRNA expression (D'Addario et al. 2007). Moreover, depletion of 5-HT affects the ability of KOR agonists to block the behavioral effects of cocaine (Zakharova et al. 2008). Studies have shown that mice lacking DA exhibit intact cocaine-conditioned place preference (CPP) and KOR agonist-induced CPA (Hnasko et al. 2007) suggesting 5-HT as another mediator in these behaviors. KOR expression in serotonergic dorsal raphe neurons of KOR knockout mice reinstates CPA to KOR agonist. In contrast, KOR inhibition in NAc with KOR antagonist blocks CPA induced by KOR agonist (Land et al. 2009). Notably, selective deletion of p38αMAPK in serotonergic neurons obliterates depression-like behaviors and reinstatement of extinguished cocaine CPP (Bruchas et al. 2011). Since 5-HT modulates mesolimbic DA neurotransmission, alterations in synaptic 5-HT can influence psychostimulants effect on dopaminergic neurotransmission and behaviors (Rothman and Baumann 2006; Howell and Cunningham 2015). Interactions of KOR and DA signaling with the serotonergic system may play a role in the behavioral effects of KOR activation (Powell et al. 1994). These findings emphasize the significance of the 5-HT modulatory role in KOR and DA signaling in the CNS.

2.2 KOR Signaling in the Regulation of Serotonin Clearance: Modulation of Serotonin Transporter Function, Trafficking, and Phosphorylation

The presynaptic serotonin transporter (SERT) clears released 5-HT from the extracellular space by Na^+/Cl^- dependent rapid reuptake process (Ramamoorthy et al. 1993; Bengel et al. 1998). SERT function is a key factor in regulating serotonergic neurotransmission (Bengel et al. 1998). Dysregulated SERT functions are linked to the pathologies of several neuropsychiatric disorders, including depression, anxiety, autism, obsessive-compulsive disorder, alcoholism, and drug addiction (Murphy et al. 2004; Hahn and Blakely 2007). SERT blockers such as SSRIs are effective clinical antidepressants and anxiolytics (Kent et al. 1998; Ballenger 1999) and modulate serotoninergic signals in the CNS (Bengel et al. 1998). SERT is also one of the primary targets of psychostimulants, such as 3,4-methylenedioxymethampheamine (MDMA-ecstasy) (Rudnick and Wall 1992), cocaine (Ramamoorthy et al. 1993; Simmler et al. 2017), and bath salts (Eshleman et al. 2019). SERT inhibitor citalopram blocks KOR-agonist-induced cocaine CPP potentiation, and SERT knockout mice fail to produce CPA to KOR agonist (Schindler et al. 2012). These findings suggest that the critical role of SERT function in KOR-mediated behaviors. Extensive studies documented that SERT function and trafficking are dynamically regulated by several cellular protein kinases, including protein kinase B (Akt), calcium calmodulin-dependent kinase II (CaMKII), glycogen synthase kinase-3ß (GSK3ß), protein kinase C (PKC), p38 MAPK, cGMP-dependent protein kinase (PKG), and Src-kinase (Jayanthi et al. 1994, 2005; Miller and Hoffman 1994; Qian et al. 1997; Samuvel et al. 2005; Zhu et al. 2005; Ramamoorthy et al. 2007, 2011; Rajamanickam et al. 2015; Ragu Varman et al. 2020). The autoreceptors and heteroreceptors present on serotonergic neurons modulate intracellular kinase/phosphatase signaling cascades, regulating SERT function, and 5-HT signaling (Ramamoorthy et al. 2011; Bermingham and Blakely 2016). Since KORs are expressed on 5-HT terminals (Berger et al. 2006) and KOR activation triggers multiple intracellular signal pathways, including activating extracellular signal-regulated kinase-½ (ERK1/2), p38 MAPK, PKC, Src, and Akt kinases and increasing intracellular calcium (Bohn et al. 2000; Pan 2003; Bruchas and Chavkin 2010), it is possible that DYN/KOR-linked signals can alter synaptic 5-HT by modulating SERT function. Such regulation may serve as one of the neuronal mechanisms underlying KOR ligand-evoked behaviors. Along these same lines, studies have documented that repeated swim stress and KOR agonists enhance 5-HT clearance, SERT maximal velocity (Vmax) with concomitant increase in surface SERT expression in the ventral striatum via p38 MAPK that is blocked by KOR antagonist, suggesting that KOR signaling regulates SERT (Bruchas et al. 2011; Schindler et al. 2012). The authors hypothesized that stress evoked KOR activation increases SERT function and consequent higher 5-HT clearance in ventral striatum may underlie pro-depressive and pro-addictive behaviors. In contrast, Sundaramurthy et al. (2017) reported that acute treatment with synthetic KOR agonists U69593 and U50488 reduce 5-HT uptake, SERT's Vmax, and surface

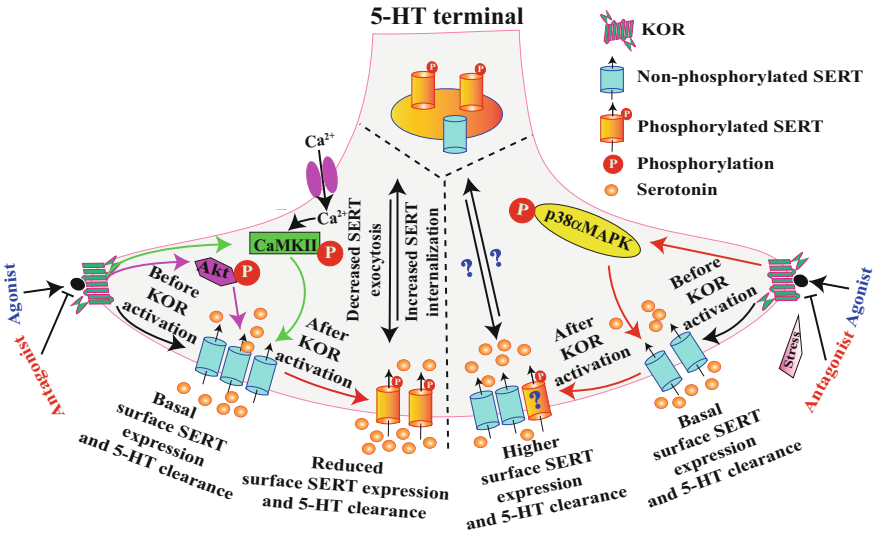

Fig. 1 Simplified diagrammatic depiction of KOR-mediated SERT regulation via Akt and CaMKII or by p38αMAPK dependent pathway. Two depicted mechanisms by which KOR mediates SERT regulation: (1) KOR activation by forced swim stress increases surface SERT expression with a subsequent decrease in extracellular 5-HT levels via p38α MAPK signaling molecule. (2) KOR agonist-induced SERT downregulation reduces surface SERT expression through decreased SERT insertion to the plasma membrane and increased SERT endocytosis and phosphorylation through Akt and CaMKII dependent signaling pathways. Treatment with KOR selective antagonist nor-BNI prevented the KOR-mediated regulation of SERT function

SERT expression while increasing SERT phosphorylation in KOR-SERT coexpressing cell model. The decrease in SERT function and surface SERT expression by U69593 is associated with reduced SERT exocytosis and enhanced SERT endocytosis. Furthermore, KOR-mediated SERT downregulation is blocked by CaMKII and Akt inhibition, but not by p38 MAPK inhibition, suggesting that KOR-mediated regulation of SERT trafficking, phosphorylation are linked to KOR activated CaMKII and Akt pathways. These results show differential regulation of SERT depending on specific kinase pathway activated by KOR (Fig. 1). Some discrepancies in KOR-mediated differential SERT regulation can be attributed to differences in techniques, stress protocols, species or strains, in-vivo versus in-vitro model systems used. Since interactions of DYN/KOR signaling with SERT and the serotonergic system manifest in maladaptive behaviors, understanding the coordination of DYN/KOR signaling with the serotonergic system is essential.

3 KOR and the Dopaminergic System

3.1 KOR Interaction with Dopaminergic System and Its Relevance to Cocaine Use Disorder

Dopamine (DA) signaling in the mesocorticolimbic and nigrostriatal pathways mediates emotion, reward, motivation, learning and memory, and cognitive functions (Volkow et al. 2007; Thomas et al. 2008; Nutt et al. 2015). Perturbed DA neurotransmission is evident in addiction, attention-deficit hyperactive disorder, schizophrenia, and Parkinson's disease (Viggiano et al. 2004; Kurian et al. 2009; Segura-Aguilar et al. 2014; Howes et al. 2015; Nutt et al. 2015). Negative affective states associated with drug withdrawal are the driving force behind drug addiction (Koob and Le Moal 2005), and KORs mediate negative affective states that drive drug addiction (Bruchas et al. 2010; Tejeda et al. 2012). Stress enhances drug-seeking behavior and also increases DYN release (Land et al. 2008). While activation of KOR potentiates stress-induced drug-seeking behaviors (Beardsley et al. 2005), the KOR antagonists block the stress-induced drug seeking (McLaughlin et al. 2003). Since DA dysfunctions contribute to negative affective states and the KOR system regulates mesocorticolimbic DA transmission, dysregulation in KOR-mediated DA modulation is a pivotal contributor to negative affective states that drive drug addiction. Furthermore, upregulation of prodynorphin and KOR is evident in human postmortem brain tissues collected from cocaine users or cocaine-dependent subjects (Frankel et al. 2008) and also in several animal studies (Daunais and McGinty 1995; Fagergren et al. 2003). Upregulation of prodynorphin, the endogenous DYN precursor, and KOR, the DYN receptor, has been implicated in the pathogenesis of depression and mood dysregulation in human cocaine users (Wee and Koob 2010; Butelman et al. 2012; Tejeda et al. 2012; Chavkin and Koob 2016). DYN fibers synapse onto DA terminals (Van Bockstaele et al. 1995) and KORs are present on the same DA terminals (Svingos et al. 1999b), suggesting that the presynaptic DYN/KOR system modulates DA transmission. Indeed, KOR agonists decrease DA release and extracellular DA concentrations via activation of KOR located on DA terminals (Shippenberg et al. 2001). KOR agonists and antagonists affect cocaine self-administration, cocaine-conditioned place preference, and the reinstatement of compulsive cocaine-seeking (Schenk et al. 2000; McLaughlin et al. 2006; Land et al. 2009). KOR activation is also effective in preventing the development of sensitization to the rewarding and psychomotor stimulant effects of cocaine (Shippenberg et al. 1996; McLaughlin et al. 2006). Studies have also shown that specific elimination of KOR in DA neurons reduces anxiety-like behavior, enhances cocaine sensitization, and abolishes KOR agonist-induced reduction in synaptic DA levels and conditioned place aversion (Chefer et al. 2013; Tejeda et al. 2013; Van't Veer et al. 2013). Given the inhibitory effects of KOR agonists on DA transmission, it has been hypothesized that the activity of the DYN/KOR system increases following repeated cocaine use and that this increase opposes alterations in behavior that occur as a consequence of drug use (Shippenberg and Rea 1997; Wee and Koob 2010). It has also been suggested that

upregulation of the DYN/KOR system contributes to the "crash" that occurs following cessation of cocaine use (e.g., depression, anhedonia, and dysphoria) (Shippenberg et al. 2007; Wee and Koob 2010; Tejeda et al. 2012). Therefore, the effects of KOR agonists can be attributed to modulation of DA release (Spanagel et al. 1990; Shippenberg et al. 2001; Chefer et al. 2013). Understanding how the KOR system controls DA transmission may help identify new therapeutic targets for treating negative affective states associated with neuropsychiatric disorders and drug addiction.

3.2 KOR Signaling in the Regulation of Dopamine Clearance: Modulation of Dopamine Transporter Function and Trafficking

The presynaptic dopamine transporter (DAT) is a phosphoprotein that determines the DA signaling by rapid clearance of released DA from extracellular synaptic space. DA transport via DAT is dependent on Na^+/Cl^- reuptake mechanism (Kilty et al. 1991; Giros and Caron 1993). DAT activity plays a critical role in regulating DA homeostasis and behavior (Giros et al. 1996; Gainetdinov and Caron 2003). This possibility is clearly evidenced when the DAT gene is deleted in mice (Giros et al. 1996). DAT is a high-affinity target for several psychostimulants, including cocaine, amphetamine (AMPH), methamphetamine (METH), and therapeutic stimulant methylphenidate. Consequently, these psychostimulants increase extracellular DA and potentiate dopaminergic neurotransmission (Giros and Caron 1993; Giros et al. 1996). Studies from animal models, human postmortem experiments, and brain imaging revealed altered DAT expression by substance use disorder (Volkow et al. 1997; Dubol et al. 2018). Cocaine binding and consequent inhibition of DAT activity are required for the manifestation of behavioral effects of cocaine (Chen et al. 2006; Thomsen et al. 2009). Hence changes in DAT protein levels, DA uptake kinetics, binding affinity of DAT for cocaine, and DAT post-translational modifications are expected to influence drug-induced behaviors. Activation or inhibition of protein kinases such as PKC, ERK1/2, phosphatidylinositol 3-kinase (PI3K), CaMKII, cyclin-dependent kinase-5 (Cdk5), and tyrosine kinase alters DAT activity (Bermingham and Blakely 2016). KOR expression and its colocalization with DAT in striatal dopamine terminals indicate that the KOR-mediated downstream kinase signaling could modulate DAT activity (Svingos et al. 1999b; Bruchas and Chavkin 2010). Consistent with this possibility, KOR agonists regulate DAT-mediated DA uptake in NAc and affect DA neurotransmission via two different mechanisms. Acute KOR agonist treatment increases DA uptake and repeated KOR agonist treatment decreases DA uptake (Thompson et al. 2000). However, a study by Kivell et al. demonstrated that acute treatment with the KOR agonists Sal A, U69593, and U50488 stimulates DAT function, Vmax, and surface expression via ERK1/2-dependent pathway in heterologous system and in striatal tissue (Kivell et al. 2014a). Furthermore, co-immunoprecipitation, BRET, and FRET studies revealed that KOR and DAT exist in a physical complex, and KOR

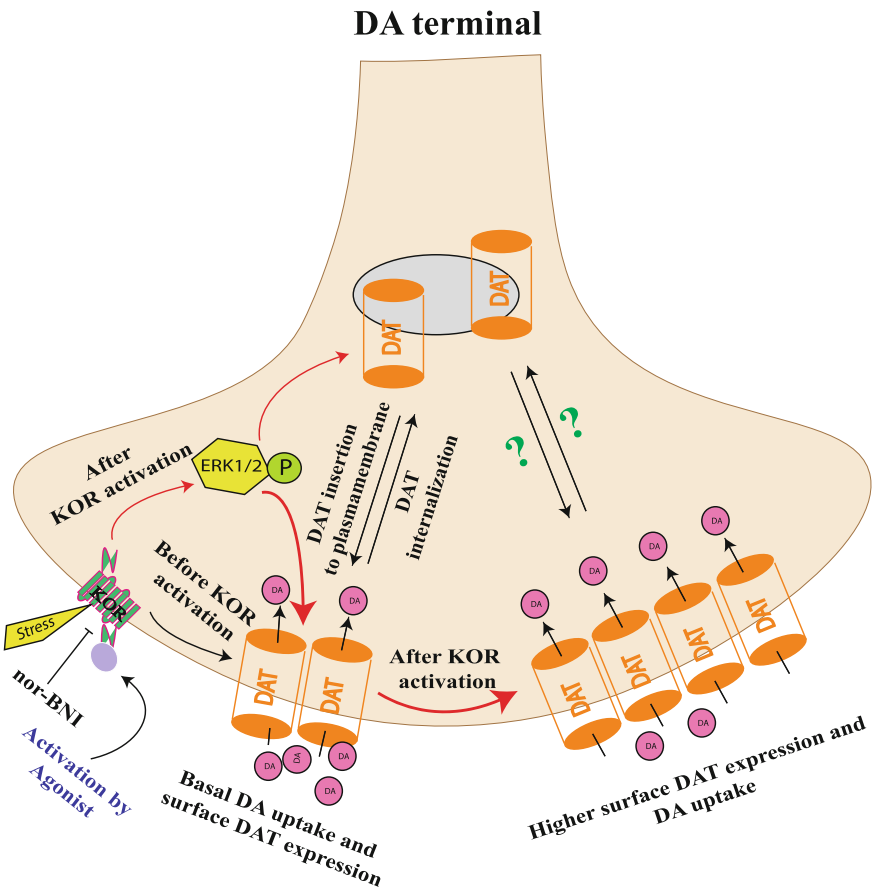

Fig. 2 Simplified diagrammatic depiction of KOR-mediated regulation of DAT via ERK1/2 dependent pathway. Activation of KOR by endogenous agonist DYN or synthetic agonists activates ERK1/2 by phosphorylation (p-ERK1/2). Activated p-ERK1/2 modulates DAT trafficking mechanisms and increases surface resident DAT resulting in enhanced DA-transport. Pre-treatment with KOR selective antagonist nor-BNI prevented the KOR-agonist-induced upregulation of DAT function. Thus DYN/KOR signaling regulates dopaminergic transmission by regulating DA release and clearance

activation enhances DAT-KOR assembling (Kivell et al. 2014a). The interactions of DYN/KOR signaling with dopaminergic signaling may very well modulate synaptic DA both at release and clearance levels (Fig. 2). Recent studies have shined light on the role of DAT phosphorylation and its interactions with KOR system in cocaine-elicited behaviors. For example, enhanced cocaine potency has been reported to occur in parallel with enhanced Thr53 phosphorylation of DAT in females during oestrus cycle (Calipari et al. 2017). DA neuronal activity also modulates DAT-Thr53 phosphorylation, cocaine potency, and cocaine consumption (Brodnik et al. 2020). Very recent study using first knock-in mouse model expressing a

non-phosphorylatable DAT (DAT-Ala53) demonstrated that the potency of cocaine to inhibit DAT and to elicit locomotor activation is reduced in the absence of DAT-Thr53 phosphorylation (Ragu Varman et al. 2021). Furthermore, ERK1/2 inhibitors failed to inhibit striatal DA uptake in DAT-Ala53 mice (Ragu Varman et al. 2021). Given the fact that KOR-mediated DAT regulation is ERK1/2 dependent and KOR activation triggers ERK1/2, it is possible that endogenous DYN/KOR signaling regulates DA dynamics via DAT phosphorylation which in turn affects behavioral response to cocaine.

4 Dynorphin/KOR System as a Therapeutic Target

Dysregulation of the endogenous dynorphin/KOR system in mesocorticolimbic circuitry contributes to the pathogenesis of affective disorders and the depressive symptomology associated with psychostimulant withdrawal (Wee and Koob 2010; Tejeda et al. 2012; Karkhanis et al. 2017). Therefore, the KOR system constitutes a novel target for treating drug addiction, pain, and depression treatments (Prevatt-Smith et al. 2011; Brust et al. 2016; Browne and Lucki 2019; Paton et al. 2020). Several synthetic KOR partial/full agonists are available, and highly selective agonists such as U50488H and U69593 are used widely in experimental studies. *Salvinorin A* (SalA) is a naturally-occurring hallucinogen derived from the *S. divinorum* plant, and it is a fully selective agonist for KOR (Roth et al. 2002). Because of unique structure and receptor selectivity of SalA, SalA and its structural analogs represent novel prospective therapeutic agents for depression and addiction treatments (Prevatt-Smith et al. 2011; Kivell et al. 2014b; White et al. 2015). However, the adverse effects of KOR agonists such as sedation, aversion, dysphoria, depression, and enhanced cocaine reward limit their therapeutic use (Zhang et al. 2005; Carlezon et al. 2006; Tidgewell et al. 2006; Chartoff et al. 2008). There are intensive research efforts going on in identifying KOR ligands that have favorable activity in G-protein signaling over ßarrestin2-signaling. It has been postulated that G-protein pathways downstream of the KOR activation exhibit antinociceptive and antipruritic properties without producing sedation or suppression of spontaneous locomotor activity. In contrast to the G-protein pathways, KOR activated ßarrestin2-p38 MAPK signaling pathway has been associated with dysphoric and pro-addictive effects (Dogra and Yadav 2015; Ehrich et al. 2015; Bohn and Aube 2017; Mores et al. 2019; Paton et al. 2020). Therefore, identifying the neural factors that reduce or eliminate unwanted effects and improve KOR agents' ability to alleviate mood disorders and addiction is critical. In this regard, the mechanisms by which KOR ligands exert their mood-altering effects via their modulation of aminergic neurotransmission are not fully understood. Since KOR is expressed on both presynaptic DA and 5-HT neuronal terminals and regulates aminergic neurotransmission (Svingos et al. 1999a; Berger et al. 2006), studies, including ours, have recently begun to explore the mechanisms of KOR-mediated regulation of SERT and DAT with the aim to identify new therapeutic targets with in the KOR modulated 5-HT and DA signals.

5 Conclusions

Studies show that DYN/KOR system is altered in stress and psychiatric disorders. DYN/KOR signaling decreases amine release and regulates amine transporters. DYN/KOR-mediated amine transporter regulation is still in its early stages. For example, it is important to know whether KOR-mediated amine transporter regulations manifest KOR ligand effects on amine homeostasis and behaviors. It is also important to know how biased KOR ligands affect amine transporters, and which signaling cascades downstream of KOR activation are actually responsible for amine transporter regulations. Equally important is to identify the post-translational mechanisms of transporter proteins involved in the KOR-mediated amine transporter regulations. Future identification of causal molecular link in KOR-mediated amine transporter modulations may offer amine transporter motifs as novel therapeutic targets to fine-tune 5-HT and DA functions. Identification of such motif(s) in the amine transporters forms a basis for developing effective pharmacotherapeutics in the treatment of mood and substance use disorders either alone or in conjunction with existing psychotherapies, and, at the same time, reducing unwanted side effects such as aversion, dysphoria, sedation, and psychomimesis.

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Part III

Preclinical Drug Development

Biased Ligands at the Kappa Opioid Receptor: Fine-Tuning Receptor Pharmacology

Tarsis F. Brust

Contents

Abstract

The kappa opioid receptor (KOR) is a G protein-coupled receptor (GPCR) that can signal through multiple signaling pathways. KOR agonists are known to relieve pain and itch, as well as induce dysphoria, sedation, hallucinations, and diuresis. As is the case with many other GPCRs, specific signaling pathways downstream of the KOR have been linked to certain physiological responses induced by the receptor. Those studies motivated the search and discovery of a number of KOR ligands that preferentially activate one signaling pathway over another. Such compounds are termed functionally selective or biased ligands, and may present a way of inducing desired receptor effects with reduced adverse reactions. In this chapter, I review the molecular intricacies of KOR signaling and discuss the studies that have used biased signaling through the KOR as a way to selectively modulate in vivo physiology.

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Functional selectivity · G protein-coupled receptor · G proteins · Kappa opioid receptor · Ligand bias · Pain · βarrestin

1 Introduction: Biased Signaling and the Kappa Opioid Receptor

The kappa opioid receptor (KOR) is a G protein-coupled receptor (GPCR) which is the main target of the endogenous opioid peptides dynorphins (Chavkin and Goldstein 1981). GPCRs are seven-transmembrane domain proteins that can activate multiple signaling pathways (Hanson and Stevens 2009; Lefkowitz and Shenoy 2005). From a drug discovery perspective, GPCRs (also termed seven-transmembrane domain receptors – 7TMRs) are extremely relevant, given that over one third of all FDA-approved drugs directly target a member of the GPCR protein superfamily (Santos et al. 2017). Moreover, a number of other therapies target GPCRs indirectly. Reuptake inhibitors, for instance, indirectly target GPCRs by increasing the availability of endogenous ligands of the receptors (Price and Brust 2019).

As indicated by the nomenclature, GPCRs activate heterotrimeric G proteins, which are composed of Gα, Gβ, and Gγ subunits (Birnbaumer 2007; Wootten et al. 2018). Activation of G proteins happens once the GPCR is activated, which causes a conformational change in the receptor that is transmitted to the G proteins (Nygaard et al. 2013; Venkatakrishnan et al. 2013). This leads to the replacement of guanosine diphosphate (GDP) by guanosine triphosphate (GTP) in the Gα subunit (Goricanec et al. 2016). Subsequently, Gα and the Gβγ dimer interact with their cellular targets (Rankovic et al. 2016; Birnbaumer 2007). Following G protein activation, the receptor is phosphorylated by G protein-coupled receptor kinases (GRKs) and other kinases that are activated by downstream signaling (such as protein kinase C [PKC] and protein kinase A [PKA]), events that allow for the recruitment of arrestin to the receptor (Komolov and Benovic 2018; Wootten et al. 2018; Yang et al. 2017). Arrestin recruitment to the GPCR causes receptor internalization and can lead to receptor degradation, recycling, or additional signaling events (Peterson and Luttrell 2017; Rankovic et al. 2016).

GPCRs can activate a number of different signaling pathways. Even within the same class of immediate receptor interacting partners there is room for an enormous heterogenicity of signaling responses. For instance, there are sixteen Gα, five Gβ, and thirteen Gγ subunits, in addition to four arrestins (in this chapter we focus on βarrestin2), PDZ-domain-containing scaffolds and numerous A kinase anchoring proteins (AKAPs) that directly interact with GPCRs (Dupre et al. 2009; Khan et al. 2013; Wootten et al. 2018). Notably, decades ago inconsistencies between molecular efficacies and functional physiological responses for GPCR ligands were reported (Jim et al. 1985; Portoghese 1965). And even though these types of inconsistencies can sometimes be explained by pharmacokinetic factors, it was later hypothesized (and since shown a number of times) that it was possible to design GPCR ligands

that would selectively activate certain pathways over others (Kenakin et al. 1991; Rankovic et al. 2016; Roth and Chuang 1987). Those studies formed the basis for what we now know as biased signaling or functional selectivity.

Ligands that upon interaction with a GPCR favor activity in one signaling pathway over another are termed biased or functionally selective (Rankovic et al. 2016; Kenakin 2011). Numerous studies have suggested that activation of certain signaling pathways downstream of a GPCR may be related to desired therapeutic effects, while activation of other signaling pathways may be related to undesired adverse effects (Kenakin 2019). The therapeutic potentials of biased ligands are based on the hypothesis that these compounds can favor GPCR signaling towards a desired pathway over an undesired pathway and, therefore, improve therapeutic efficacy and reduce adverse effects (Kenakin 2018). In fact, a number of studies have suggested that to be indeed the case (Grim et al. 2020; Schmid et al. 2017; DeWire et al. 2013; Brust et al. 2016; Allen et al. 2011; DeWire and Violin 2011; Violin et al. 2010, 2014; Kenakin 2018; Luttrell et al. 2015). However, we are yet to see the full benefits of these compounds reflected clinically.

An important step in the discovery of biased ligands is determining how to identify such compounds. Considerations on this range from selecting the appropriate system (cell line, tissue, etc.) to choosing a method to quantify (or qualify) bias. Several different methods of quantifying ligand bias have been developed, compared, and reviewed in detail (Brust et al. 2015b; Kenakin 2014; Kenakin and Christopoulos 2013a, b; Kenakin et al. 2012; Rajagopal et al. 2011; Rankovic et al. 2016; Stahl et al. 2015; Hoare et al. 2020; Gundry et al. 2017; Zhu et al. 2019). The most commonly used methods of quantifying ligand bias compare the ability of a ligand to activate one signaling pathway over another, to that of a reference compound and result in a bias factor (Kenakin and Christopoulos 2013b; Rankovic et al. 2016). Therefore, ligand bias is relative to the reference compound used for the comparison. Some of the most used methods of quantifying ligand bias use the Black and Leff operational model to calculate transduction coefficients (Black and Leff 1983; Kenakin and Christopoulos 2013b; Kenakin et al. 2012; Rankovic et al. 2016). The transduction coefficients calculate the intrinsic ligand efficacy and the dissociation constant of agonist-receptor-signal transducer complex from functional data to generate bias factors that are independent of signal amplification and receptor reserve (Black and Leff 1983; Kenakin and Christopoulos 2013b). Bias factors can be used in drug discovery efforts to compare the levels of bias of different test ligands. This approach also allows for incorporation of structure activity relationship studies and molecular modeling approaches to pursue potent biased ligands (Lovell et al. 2015; Manglik et al. 2016; McCorvy et al. 2018).

As discussed below, biased signaling at the KOR has been studied in cell models, primary neurons, and animals. The KOR has been pursued as a target in therapies for treating pain and pruritus (itch) (Cowan et al. 2015; Kivell and Prisinzano 2010). However, adverse effects such as dysphoria, sedation, and diuresis have generally precluded the clinical use of these compounds (Brust et al. 2016; Knoll and Carlezon 2010; Mercadante and Arcuri 2004; Pfeiffer et al. 1986). Nalfurafine is the only KOR-selective agonist that is currently in clinical use for the treatment of pruritus

(Kumagai et al. 2010). Notably, compared to opioid analgesics that target the mu opioid receptor for pain relief, KOR agonists are generally devoid of addictive and life-threatening side effects, such as respiratory depression (Bruijnzeel 2009; Brust et al. 2016). In this chapter I review the signaling pathways downstream of the KOR and the available studies on biased signaling at this receptor.

2 Signaling Cascades Downstream of the Kappa Opioid Receptor

2.1 G Proteins

The KOR is coupled to inhibitory $G\alpha_{i/o}$ subunits (Fig. 1) (Meng et al. 1993; Simonin et al. 1995). The most studied effect of these subunits is the inhibition of adenylyl cyclases (Sunahara and Taussig 2002; Syrovatkina et al. 2016), which are enzymes that catalyze the conversion of adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP) (Price and Brust 2019). Therefore, KOR agonists generally lead to a reduction in cellular cAMP production. Even though specific consequences of KOR-induced decrease in cAMP levels have not yet been fully unveiled, certain adenylyl cyclase isoforms have functions that overlap with physiological roles that are attributed to the KOR. For instance, adenylyl cyclase 1 (AC1) has a role in pain perception and adenylyl cyclase 7 (AC7) appears to play a role in anxiety and depressive disorders (Brust et al. 2017; Price and Brust 2019).

A better understanding of how the KOR modulates the activity of different adenylyl cyclase isoforms may enhance our predictions of desired functional

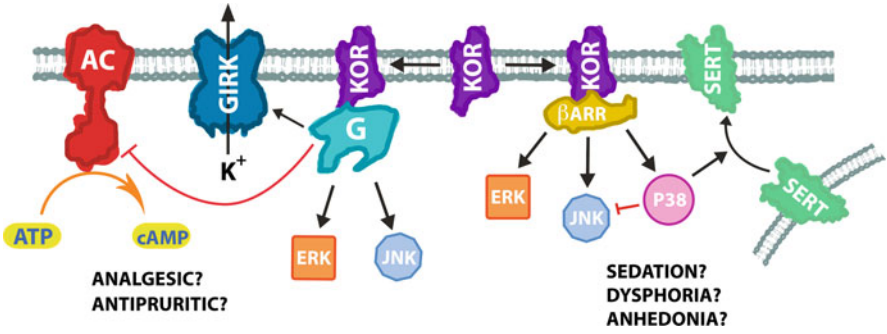

Fig. 1 Summary of the signaling pathways downstream of the KOR. Activation of the KOR leads to signaling events through heterotrimeric G proteins (G) and βarrestins (βARR). The figure depicts the pathways discussed in the chapter: G protein-mediated activation of GIRK, ERK, and JNK, and inhibition of adenylyl cyclases (AC), and βarrestin-mediated activation of ERK, JNK, and p38 MAPK, which inhibits JNK and increases membranous expression of the serotonin transporter (SERT). As noted in the figure, G protein activation downstream of the KOR has been linked to the receptor's analgesic and antipruritic properties, while βarrestin recruitment to the KOR has been related to the sedative, dysphoric, and anhedonic properties of the receptor. Biased ligands are able to favor the activation of certain pathways over others

responses downstream of the KOR. Especially as some adenylyl cyclase isoforms are not inhibited by $G\alpha_{i/o}$ and are conditionally activated by Gβγ subunits (AC2, AC4, and AC7) (Price and Brust 2019). KOR action on these isoforms would be expected to increase cAMP levels. Moreover, AC1, AC3, and AC8 are inhibited by Gβγ subunits, providing an additional layer of biased receptor modulation of tissue-specific cAMP signaling (Price and Brust 2019). The length of KOR activation is also crucial for determining the outcome of KOR modulation of cAMP production. Many studies have shown that through sensitization of adenylyl cyclase (also referred to as cAMP overshoot, heterologous sensitization, superactivation, or supersensitization of adenylyl cyclase), prolonged KOR activation followed by an antagonist can cause a robust increase in cAMP production, a phenomenon that adds a temporal aspect to KOR modulation of the cAMP pathway (Brust et al. 2015a; Nakagawa et al. 1999; Avidor-Reiss et al. 1995; Li et al. 2003; Wang et al. 2003). Therefore, KOR modulation of the cAMP signaling pathway is tissue and time-dependent, resulting in paradoxical inhibitory and excitatory responses.

The process of G protein activation also causes Gβγ subunits to become active. Gβγ subunits can lead to multiple signaling events, including modulation of adenylyl cyclase isoforms, activation of phosphoinositide 3 kinase, phosphorylation of extracellular signal-regulated kinase (ERK), activation of phospholipase C, activation of G protein-coupled inwardly rectifying potassium (GIRK) channels, modulation of calcium channels, and stabilization of microtubules (Dupre et al. 2009; Khan et al. 2013; Wootten et al. 2018). Activation of the KOR has classically been associated with Gβγ-mediated activation of GIRK and inhibition of voltage-gated calcium channels (Ho et al. 2018; Luscher and Slesinger 2010; Ikeda et al. 2002). These mechanisms would cause an overall inhibition of neurotransmission. We have also shown an enhancement of AC2 activity in cell lines that is consistent with the effects of Gβγ subunits (unpublished observations).

2.2 βarrestin2 Activation

Activation of the KOR leads to phosphorylation of the receptor by GRKs, an event that allows arrestin recruitment to the receptor (Bruchas and Chavkin 2010). Arrestins are proteins that were originally thought to serve only to terminate G protein signaling (Shenoy and Lefkowitz 2011). By interacting with the receptor, arrestins can first sterically prevent G proteins from coupling to the receptor (Gurevich and Gurevich 2019). Second, arrestins can internalize the receptor and remove it from the membrane, an event that can prevent additional agonist interactions with the GPCR (Gurevich and Gurevich 2019; Goodman et al. 1996). And third, arrestins can recruit phosphodiesterases and diacylglycerol kinases to the cellular membrane, proteins that act by degrading GPCR second messengers (Nelson et al. 2007; Perry et al. 2002). These actions would justify the name of arrestins and suggest an overall inhibitory role for arrestins on GPCR signaling.

It is now well understood that arrestins interact with a number of proteins that have important cellular signaling functions, such as components of the

mitogen-activated protein kinase (MAPK) cascades, the Src family tyrosine kinases, and Akt (Chavkin et al. 2014; Luttrell et al. 1999; McLennan et al. 2008; Schmid and Bohn 2010). Arrestins appear to act as scaffolding proteins, bringing together several signaling proteins and facilitating their interactions (Gurevich and Gurevich 2019; Lefkowitz and Shenoy 2005). Therefore, in addition to "arresting" GPCR signaling, arrestins can also lead to GPCR-mediated signaling events. Downstream of the KOR, ERK phosphorylation can be induced through G proteins and βarrestin2 in immortalized astrocytes (McLennan et al. 2008). Sequestration of Gβγ subunits with βARKct (C terminus of GRK2), inhibition of G protein signaling with pertussis, and knockdown of βarrestin2 with siRNA, all reduce ERK phosphorylation (McLennan et al. 2008). Moreover, the proportion of G protein-dependent versus βarrestin2-dependent activation of ERK is ligand-dependent, indicating a role for signaling bias (McLennan et al. 2008). Another well-studied signaling pathway downstream of the KOR is the phosphorylation of p38 MAPK. Previous studies have shown that βarrestin2 recruitment to the receptor is required for KOR agonist-induced p38 MAPK phosphorylation (Schattauer et al. 2019; Bruchas et al. 2006). Inhibition of GRK3-mediated phosphorylation of the KOR or knockdown of βarrestin2 prevents p38 MAPK phosphorylation in neurons and astrocytes (Bruchas et al. 2006).

KOR agonists also cause c-Jun N-terminal kinase (JNK) phosphorylation (Jamshidi et al. 2016; Schattauer et al. 2019; Bruchas et al. 2007b). In cell lines, this process occurs in two distinct phases. Early JNK phosphorylation happens in an arrestin-independent fashion and involves G protein-mediated activation of PKC and the small G protein RAC (Schattauer et al. 2019). The late phase JNK phosphorylation happens through βarrestin2 and also involves the activation of RAC in addition to the RHO effector ROCK1 (Schattauer et al. 2019). Whereas the early phase JNK phosphorylation leads to the formation of reactive oxygen species (ROS) through peroxiredoxin 6 (PRDX6), the late phase does not induce significant ROS generation in cell lines (Schattauer et al. 2019). Notably, JNK is inhibited by p38 MAPK and inhibition of KOR-mediated phosphorylation of p38 MAPK causes an increase of KOR agonist-induced ROS generation (Schattauer et al. 2019).

3 Physiological Implications of Signaling Pathways Downstream of the Kappa Opioid Receptor

Studies with genetically modified animals and biased ligands at the KOR have helped to uncover the physiological functions of the different signaling pathways downstream of the receptor. Several studies have correlated the βarrestin2 pathway downstream of the KOR with the aversive and dysphoric properties (Bruchas et al. 2006, 2007a; Brust et al. 2016). This pathway involves the phosphorylation of the KOR by GRK3 followed by the recruitment of βarrestin2 and subsequent phosphorylation of p38 MAPK (Fig. 1). As a way of assessing aversive and dysphoric effects, many of these studies made use of animal models that are commonly used to mirror depressive and aversive states. For instance, repeated swim stress is a method that assesses stress-coping strategies and has been used for identifying compounds that

can treat depression (Commons et al. 2017). In one study, repeated swim stress in mice was correlated with KOR phosphorylation and a KOR-dependent increase of p38 MAPK phosphorylation in GABAergic neurons of the nucleus accumbens of mice by immunohistochemistry and in the striatum by immunoblotting (Bruchas et al. 2007a). Notably, inhibition of p38 MAPK attenuates stress-induced immobility in mice, suggesting a reduction of depressive-like symptoms in the animals (Bruchas et al. 2007a).

The conditioned place aversion (CPA) is another model that is commonly employed to determine the aversive motivational properties of drugs (Cunningham et al. 2006). In this model the animal becomes conditioned to associate the experience elicited by the drug (or other stimulus) with the environment where the experience happened. As the animal is presented with a chance to choose between the drug- or vehicle-associated environment, it will avoid the environment where unpleasant experiences took place (Cunningham et al. 2006). KOR agonists cause aversion in this model and inhibition of p38 MAPK prevents KOR agonist-mediated CPA (Bruchas et al. 2007a). KOR-induced phosphorylation of p38 MAPK is dependent on the expression of GRK3 (Bruchas and Chavkin 2010). Therefore, it is not surprising that in GRK3 knockout mice, KOR agonist-induced CPA and swim stress-induced immobility were significantly decreased (Bruchas et al. 2007a). As KOR-induced phosphorylation of p38 MAPK is dependent on the recruitment of βarrestin2 to the receptor, these results indicate that KOR agonist-induced mouse behaviors that reflect aversion and dysphoria are mediated by the βarrestin2 pathway (Bruchas et al. 2006). However, in stark contrast with these data, KOR agonists still induce CPA in βarrestin2 knockout mice (White et al. 2015).

Dopamine neurons have long been associated with the rewarding and aversive properties of drugs (Volman et al. 2013). KOR activation causes a decrease in dopamine release in the nucleus accumbens, which receives dopaminergic input from the ventral tegmental area (VTA) (Crowley and Kash 2015; Karkhanis et al. 2016; Knoll and Carlezon 2010; Rose et al. 2015; Brust et al. 2016). This phenomenon has traditionally been linked to the aversive and dysphoric properties of the receptor (Knoll and Carlezon 2010). Notably, conditional deletion of the genes for the KOR or p38 MAPK in dopaminergic neurons prevents KOR agonist-induced CPA in mice (Ehrich et al. 2015). In addition, conditional expression of the KOR in VTA dopaminergic neurons of KOR knockout mice rescues KOR agonist-induced CPA, but expression of a mutant form of the KOR (KSA, which is not phosphorylated by GRK3 at serine 369) does not (Ehrich et al. 2015). Interestingly, while genetic or pharmacological inhibition/disruption of p38 MAPK prevents KOR agonist-induced CPA, it does not affect KOR agonist-induced reduction in dopamine release in the nucleus accumbens (Ehrich et al. 2015). These findings suggest that at least part of the aversive properties of KOR agonists may be independent of KOR-mediated inhibition of dopamine release.

The serotonergic system is also closely related with depressive symptoms and is commonly targeted in therapies to treat depressive disorders (Price and Brust 2019). Serotonin and its targets have also been implicated in the dysphoric and aversive effects that result from KOR activation (Schindler et al. 2012; Land et al. 2009). One

study showed that conditional knockout of the KOR in serotonergic neurons blocks KOR agonist-induced CPA in mice (Ehrich et al. 2015). Another study showed that repeated forced swim stress increases membrane expression of the serotonin transporter (SERT) in a GRK3 and p38 MAPK-dependent manner in mice (Schindler et al. 2012). In addition, conditional expression of the KOR in serotonergic neurons of KOR knockout mice rescues KOR agonist-induced CPA (Land et al. 2009). Notably, the KOR antagonist norbinaltorphimine (norBNI) or KOR knockout prevents stress-induced increases in membranous SERT (Schindler et al. 2012). A number of antidepressant agents aim at increasing serotonin concentration at synaptic terminals, many of them act by inhibiting SERT (Price and Brust 2019). Therefore, increased membranous expression of SERT is consistent with depressive and dysphoric symptoms. Furthermore, KOR agonist-induced CPA is inhibited by the selective serotonin reuptake inhibitor (SSRI) citalopram and KOR agonist treatment increases serotonin uptake in whole brain samples from mice (Bruchas et al. 2011).

In contrast to the βarrestin2 pathway, the G protein pathway downstream of the KOR has been associated with antinociceptive and antipruritic properties (Fig. 1). Pertussis toxin causes ADP ribosylation of $G\alpha_{i/o}$ subunits, resulting in an inhibitory effect on G protein signaling (Mangmool and Kurose 2011). In the tail flick assay, which is used as a measure of thermal nociception, intracerebroventricular (ICV) pertussis toxin treatment completely inhibits KOR agonist-induced antinociception in mice (Goicoechea et al. 1999). In rats, intrathecal pertussis toxin treatment also abolishes KOR agonist-induced antinociception in the tail flick assay (Hernandez et al. 1995; Przewlocki et al. 1987). Moreover, KOR agonists retain their antinociceptive properties in βarrestin2 knockout mice, indicating that the βarrestin2 pathway is not necessary for KOR-induced pain relief (White et al. 2015). Notably, intrathecal injections with a cell-permeable cAMP analog (dibutyryl-cAMP) had no effects on KOR agonist-induced antinociception in the tail flick assay (Hernandez et al. 1995), suggesting that $G\alpha_{i/o}$-mediated inhibition of the cAMP pathway is not involved in the antinociceptive properties of KOR agonists. This is consistent with studies showing that while inhibition of certain adenylyl cyclase isoforms relieves inflammatory and neuropathic pain, it has no effects on thermal nociception (Brust et al. 2017; Wang et al. 2011). KOR-mediated inhibition of AC1 has also been recently linked to a mechanism to mask postoperative latent pain sensitization in rodents (Custodio 2019).

G protein activation by the KOR has also been linked to the antipruritic properties. KOR agonists are antipruritic and KOR antagonists cause pruritus (Morgenweck et al. 2015). While βarrestin2 knockout in mice reduced slightly the pruritic effects of KOR antagonists, it did not affect the antipruritic effects of KOR agonists (Morgenweck et al. 2015). These data suggest that βarrestin2 recruitment to the KOR is not required for the antipruritic effects. In addition, the relative potencies of KOR agonists for activating G proteins ($[^{35}S]GTP\gamma S$ binding assay) and for receptor internalization correlate with their antipruritic potencies (Wang et al. 2005). However, as discussed below, the use of biased KOR ligands indicates a G protein-mediated mechanism. KOR agonists also induce diuresis and hallucinations (Mercadante and Arcuri 2004; Pfeiffer et al. 1986). KOR-induced diuresis happens

through the suppression of antidiuretic hormone (ADH or vasopressin) release from the posterior pituitary gland (Kapusta 1995). However, the precise molecular mechanisms underlying KOR-induced diuretic and psychotomimetic effects still remain to be fully detailed.

4 Biased Ligands at the Kappa Opioid Receptor

Several groups have reported biased KOR ligands. Here I will discuss the in vitro and in vivo data available for the compounds that displayed the largest bias factors (Fig. 2) (Mores et al. 2019; Gupta et al. 2016; Schmid et al. 2013; Rives et al. 2012; Zheng et al. 2017; Kivell et al. 2018; Zhou et al. 2013; White et al. 2014; Schattauer et al. 2017; Spetea et al. 2017; Bedini et al. 2020). Important factors to be considered when interpreting the results from those studies are the systems used to report bias, the methods used for quantifying bias, and the pharmacological effects of those compounds in vivo. The last of these factors may also be dependent on the pharmacokinetic properties and the route of administration.

Triazole 1.1 (2-(4-(furan-2-ylmethyl)-5-((4-methyl-3-(trifluoromethyl)benzyl) thio)-4H-1,2,4-triazol-3-yl)pyridine) is a G protein-biased ligand at the KOR that retains potency and efficacy for activating G proteins in the [^{35}S]GTPγS binding assay and is considerably less potent for recruiting βarrestin2 to the receptor in comparison with reference ligands (U50,488 and U69,593) in cell lines (Brust et al. 2016; Zhou et al. 2013). Upon ligand bias quantification, triazole 1.1 had a bias factor of 28 for [^{35}S]GTPγS binding over βarrestin2 recruitment in comparison with U50,488 using the transduction coefficient method (Brust et al. 2016). This bias profile was maintained in transfected primary striatal neurons when comparing [^{35}S] GTPγS binding to KOR internalization, with the apparent difference of triazole 1.1 being a partial agonist for KOR internalization in neurons versus the full efficacy for βarrestin2 recruitment in cell lines (Ho et al. 2018). Triazole 1.1 also presents a significant bias (factor equal to 23) for [^{35}S]GTPγS binding over inhibition of cAMP accumulation in Chinese hamster ovary (CHO) cells (Ho et al. 2018). In addition, the compound displays a bias factor of 26 for GIRK activation over cAMP accumulation in cell lines. These data would suggest a bias among G proteins (Gβγ over Gα), a phenomenon that has been observed for other GPCRs (Brust et al. 2015c; Ho et al. 2018). However, this bias was absent in neuronal cells, further highlighting the importance of choosing an appropriate cell model in studies of functional selectivity (Ho et al. 2018). These results also demonstrate that ligands that appear biased in cell lines may not be biased endogenously.

In vivo, triazole 1.1 reaches the brain of rodents when administered subcutaneously or intraperitoneally (Brust et al. 2016). The compound also displays similar antinociceptive and antipruritic properties to U50,488 in the tail flick assay and to relieve chloroquine phosphate-induced scratching in mice, respectively (Brust et al. 2016). However, in contrast to U50,488, triazole 1.1 does not reduce locomotor activity or decrease dopamine release in the nucleus accumbens of mice. The compound also does not decrease intracranial self-stimulation (ICSS) in rats, a

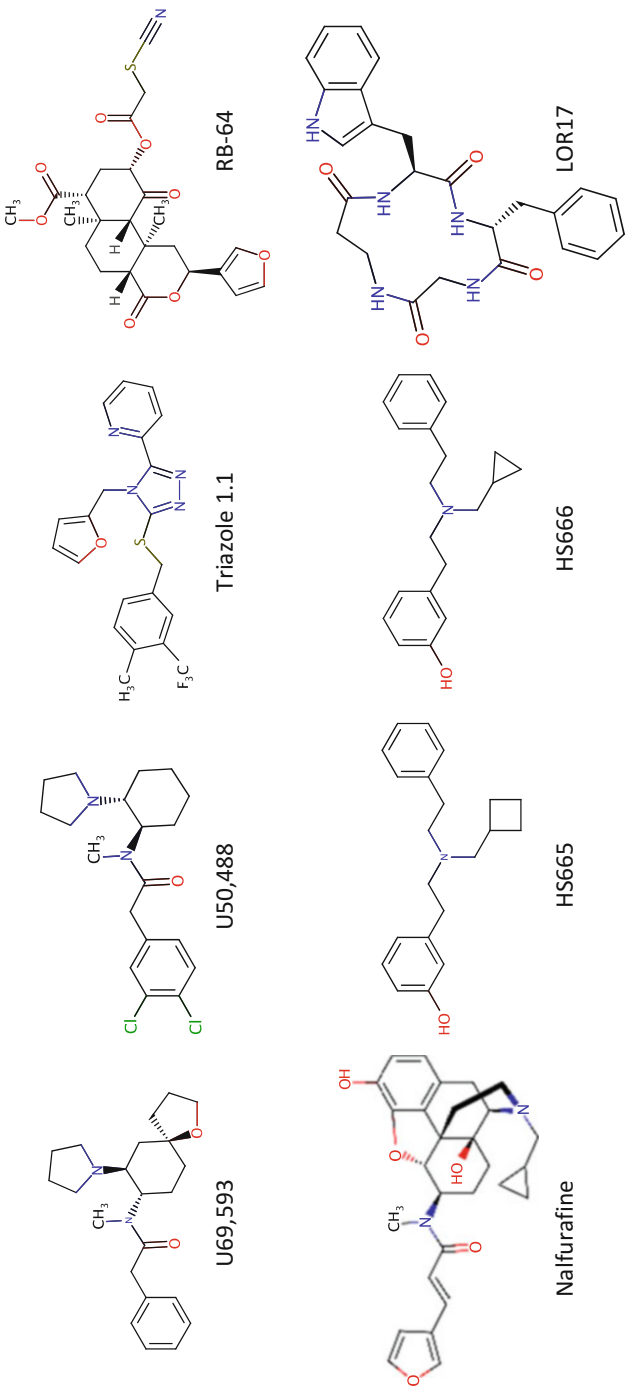

Fig. 2 Chemical structures of reference ligands U50,488 and U69,593 and the biased compounds discussed in the chapter

method that is used to measure anhedonia (Brust et al. 2016). In addition to compounds that induce anhedonia and dysphoric states, acute pain can also disrupt ICSS. Notably, triazole 1.1 is able to recover visceral pain-induced (through intraperitoneal lactic acid injection) decreases in ICSS. The effects of triazole 1.1 were also examined in rhesus monkeys (Huskinson et al. 2020). In that study, a series of behaviors were evaluated in response to the KOR agonists triazole 1.1, U50,488, salvinorin A, and nalfurafine. All compounds decreased scratching, however, in contrast to the other KOR agonists tested, triazole 1.1 did not decrease species-typical activity, increase passive visual (immobility), cause lip droop (muscle relaxation), or induce rest/sleep postures (Huskinson et al. 2020). It should be noted that all compounds were more potent for reducing scratching than for inducing any other behavior studied, indicating some level of separation of desired and undesired effects even for the reference ligands (Huskinson et al. 2020). Moreover, from the KOR agonists tested, triazole 1.1 was the least potent for reducing scratching (Huskinson et al. 2020). Together, these results indicate that at the doses tested, triazole 1.1 retains the antinociceptive and antipruritic properties of KOR agonists, but lacks the sedative and anhedonic characteristics that are commonly associated with these ligands.

RB-64 (22-thiocyanatosalvinorin A) is another G protein-biased ligand at the KOR (White et al. 2014, 2015). Compared to U69,593 and salvinorin A, RB-64 retains potency for G protein activation (luciferase-based cAMP assay) and is less potent for the recruitment of βarrestin2 in cell lines (White et al. 2014). RB-64 presents a bias factor of 35 for cAMP inhibition over βarrestin2 recruitment in comparison to salvinorin A using the transduction coefficient method (White et al. 2014). In mice, RB-64 is antinociceptive in the hotplate assay (used to measure thermal nociception), but the compound also induces CPA. Notably, RB-64 does not impair performance in the rotarod assay nor does it affect novelty-induced locomotor activity in mice. In ICSS studies in mice, RB-64 (1 mg/kg) also caused a small rightward shift in the rate-frequency curve, but did not diminish the maximal response rate compared to vehicle. These data also provide evidence that a G protein-biased ligand at the KOR is antinociceptive and does not induce sedation or as much anhedonia as reference compounds. However, the study also indicates that KOR ligands that are G protein-biased in cell lines still retain the aversive properties of the receptor, a finding that is consistent with the presence of KOR-induced CPA in βarrestin2 knockout mice (White et al. 2015). It is noteworthy that the method used to measure G protein activation for RB-64 was inhibition of cAMP accumulation, which is in contrast to [^{35}S]GTPγS binding that was used for triazole 1.1 (White et al. 2014; Brust et al. 2017). The fact that triazole 1.1 was biased against inhibition of cAMP in cell lines indicates that RB-64 may engage the receptor in a different way and responses that are downstream of the immediate receptor effectors may differ between those two compounds (Ho et al. 2018).

Nalfurafine, the only selective KOR ligand that is clinically used, has also been reported to be biased. The compound is more potent for inducing ERK phosphorylation than for p38 MAPK phosphorylation in cell lines (Schattauer et al. 2017). Nalfurafine has a bias factor equal to 300 for ERK phosphorylation over p38 MAPK phosphorylation in comparison to U50,488 using the transduction coefficient

method (Schattauer et al. 2017). Defining bias using downstream phosphorylation cascades is a valuable strategy that may lead to a more specific correlation with physiological effects. However, as ERK phosphorylation can be mediated both by G protein and arrestin pathways, this bias may not represent a G protein bias (Bruchas and Chavkin 2010; Lovell et al. 2015; McLennan et al. 2008). As the study timepoint for measuring ERK phosphorylation was 5 min, a Gβγ-mediated mechanism would be predicted. However, later studies found that nalfurafine is not biased for G protein ([^{35}S]GTPγS binding) over βarrestin2 recruitment to the KOR in comparison to U50,488, or even that nalfurafine is biased for βarrestin2 recruitment over [^{35}S]GTPγS binding in comparison to U69,593, both using the equiactive comparison (Liu et al. 2019; Dunn et al. 2019). This method of quantifying bias uses ratios of relative efficacies by potencies from standard four-parameter Hill equations to generate a bias factor, which is comparable to the transduction coefficients (Brust et al. 2015b; Ehlert 2008; Griffin et al. 2007). These discrepancies may be reconciled if nalfurafine induces bias among G proteins. In fact, nalfurafine has been shown to be biased for cAMP inhibition over βarrestin2 recruitment in comparison to U50,488 using the equiactive comparison (Kaski et al. 2019). In addition to the different methods used to measure G protein activity ([^{35}S]GTPγS binding vs. cAMP inhibition), these studies also used different cell lines to assess KOR signaling. These cell lines likely present a distinct repertoire of G protein subunit and adenylyl cyclase isoforms, which should also have an impact on the calculated bias factors.

In vivo, nalfurafine is antipruritic and analgesic (formalin-induced inflammatory pain) and (at antipruritic and analgesic doses) does not induce CPA, decrease locomotor activity, or change the baseline threshold of ICSS in mice (Liu et al. 2019). In rhesus monkeys nalfurafine increases passive visual, causes lip droop, and induces rest/sleep postures at doses that are higher than those required for its antipruritic effects (Huskinson et al. 2020). Together, these studies show an interesting correlation of the compound's bias against p38 MAPK phosphorylation and the lower potency for causing aversive effects in animals. It also shows how the activation of downstream signaling pathways may differ from the commonly assessed immediate receptor effectors. The different assay- and cell line-dependent bias factors are also noteworthy, and once more highlight the importance of choosing the appropriate model in studies of functional selectivity. It would be interesting to determine if the bias against p38 MAPK phosphorylation is a feature shared by other biased KOR ligands, as that may be a valuable strategy for screening for aversive properties of KOR ligands.

Additional G protein-biased KOR ligands, HS665 (3-(2-((Cyclobutylmethyl)(phenethyl)amino)ethyl)phenol) and HS666 (3-(2-((cyclopropylmethyl)(phenethyl)-amino)ethyl)phenol) display bias factors equal to 389 and 62, respectively ([^{35}S]GTPγS binding over βarrestin2 recruitment to the KOR in cell lines), in comparison to U69,593 using the transduction coefficients method (Spetea et al. 2017). These compounds have the distinction of being partial agonists. In cell lines, HS665 is a partial agonist for βarrestin2 recruitment and a full agonist for [^{35}S]GTPγS binding and HS666 is a partial agonist in both assays. In mice, these compounds were administered ICV (thus circumventing certain potential pharmacokinetic issues)

and caused antinociception in the tail flick assay, did not induce incoordination (although, data for a positive control was not shown for this assay), and HS665, but not HS666, induced CPA at the dose tested (Spetea et al. 2017). Another study tested HS665 and found that the compound increases serum prolactin levels (a common effect of KOR agonists) and that at 30 mg/kg it causes incoordination in mice (Dunn et al. 2018). As partial agonists also behave as partial antagonists, it would be interesting to determine if these compounds can inhibit KOR agonist-induced adverse effects in vivo (such as sedation, anhedonia, and CPA). Especially as a recent study that examined certain signaling pathways downstream of the KOR made a correlation of KOR ligand efficacy for βarrestin2 recruitment with the efficacy for causing incoordination (Dunn et al. 2019). In this context, KOR ligands that are partial agonists for βarrestin2 recruitment would be desirable, particularly those that retain full efficacy for G protein activation. However, as molecular efficacy is system-dependent, it would be interesting to determine if G protein over βarrestin2 bias factors correlate with improved therapeutic windows as previously reported for ligands of the mu opioid receptor (Schmid et al. 2017).

A recently discovered peptide-derived KOR agonist, LOR17 (c[Phe-Gly-(β-Ala)-D-Trp]) displays a bias factor equal to 853 for inhibition of cAMP over βarrestin2 recruitment in comparison to U50,488 in cell lines using the transduction coefficients method (Bedini et al. 2020). It should be noted that the efficacy of the doses of LOR17 tested (up to 10 μM) for βarrestin2 recruitment to the KOR was very low. Therefore, the extremely high bias factor calculated may not be accurate. Another recently developed method could be used to provide a more accurate bias factor for this compound (Stahl et al. 2019). Alternatively, higher compound doses could be used to provide more accurate measures of transduction ratio and functional affinity for the operational model. Nevertheless, the bias from LOR17 is evident from the concentration response curves (Bedini et al. 2020). The compound also displays differential kinase signaling. LOR17 induces early phase (15 min) ERK phosphorylation, contrasting with U50,488, which induces ERK phosphorylation in early and late phases in cell lines (15 and 60 min) (Bedini et al. 2020). Notably, an increase in ERK phosphorylation at an even earlier time point of 5 min was only observed for U50,488 in HEK293 cells (not in U87-MG cells or human astrocytes and not for LOR17 in any of the cells tested). In contrast to U50,488, LOR17 induces neither p38 MAPK phosphorylation nor astrocyte proliferation. Additionally, LOR17 inhibits both U50,488-induced p38 MAPK phosphorylation and cell proliferation (Bedini et al. 2020). In mice, LOR17 is antinociceptive in the tail flick assay, relieves visceral pain in the writhing assay, and is more effective than U50,488 for reducing thermal hypersensitivity caused by oxaliplatin-induced neuropathy. Contrasting to U50,488, LOR17 did not cause incoordination in the rotarod test or decrease exploratory activity in the hole-board test. The compound also did not reduce locomotor activity or diminish mobility time in the forced swim test (Bedini et al. 2020). As LOR17 inhibits U50,488-mediated p38 MAPK phosphorylation, it would also be of interest to determine if the compound can also reduce KOR agonist-mediated adverse effects, such as CPA, sedation, and anhedonia.

5 Conclusions

Overall, there is good agreement between the studies using genetic manipulations and the studies with biased ligands to suggest that G protein activation over βarrestin recruitment to the KOR may be beneficial for therapies to treat pain and pruritus. However, it is notable that βarrestin2 knockout mice still display CPA to KOR agonists (White et al. 2015). This is in contrast to other studies that showed that knockout of GSK3 (which allows for βarrestin2 recruitment to the KOR) and inhibition (genetic and pharmacological) of p38 MAPK phosphorylation (a signaling event that is downstream of βarrestin2 recruitment to the KOR) prevents KOR agonist-induced CPA (Bruchas et al. 2006, 2007a, 2010). Perhaps those studies could be reconciled if another arrestin (or other proteins) possesses a compensatory function for the lack of βarrestin2 in the βarrestin2 knockout mice. Nevertheless, the G protein-biased RB-64 still induces CPA in mice (White et al. 2015). In contrast, HS666, which is also G protein-biased does not induce CPA in mice (Spetea et al. 2017). However, HS666 has the notable distinction of being a partial agonist. It would be interesting to compare those two compounds (and others) side by side for pathway activation and behavioral tests, as subtle differences in methodological approaches may sometimes lead to different results. Testing additional doses of HS666 would also unveil the complete potential of that compound causing aversion. It should also be noted that certain studies used [^{35}S]GTPγS binding while others used inhibition of cAMP accumulation as an endpoint for G protein activation. However, the two are not interchangeable and bias between the two pathways has been observed (Ho et al. 2018). Moreover, the pharmacokinetic profile of biased ligands should also be considered. As different compounds may have distinct absorption and distribution rates, it would be of interest to test their in vivo pharmacology at time points that reflect their peak plasma and brain concentrations. The different protocols that are used for in vivo studies may result in inappropriate time points for measuring behavior and, therefore, provide inaccurate results. The pharmacokinetic properties may also be affected by ligand binding kinetics. Different binding affinities and off rates are also likely to have an impact on the timing for behavior monitoring as ligands may remain bound to the receptor for different periods of time. This may also influence bias, as the kinetics of G protein and βarrestin recruitment (as well as other signaling pathways) are different (Hoare et al. 2020).

It is also desirable to find compounds that display the reverse type of bias downstream of the KOR (βarrestin2-biased ligands). Those compounds would help to confirm the role of G proteins and βarrestin2 for physiological responses of the receptor. In a similar way to the studies recently done with the mu opioid receptor, it would be interesting to determine if bias factors correlate with therapeutic windows for KOR ligands (Schmid et al. 2017). Future studies should also test the activation of pathways that are downstream of the KOR. Most studies focusing on ligand bias through the KOR were bidimensional and compared G protein activation (using the [^{35}S]GTPγS binding or cAMP inhibition) to βarrestin2 recruitment. However, GPCR signaling is multidimensional and there are numerous signaling

outcomes that may have physiological implications and may not be identified by G protein vs βarrestin2 bias comparisons. Recent studies have started to shift in that direction and present promising avenues for the discovery of new multidimensional bias profiles (Bedini et al. 2020; Ho et al. 2018; Liu et al. 2019). And as discussed above, it will also be important to consider the time variable for many of those signaling pathways.

While promising, it is advisable to be conservative with the expectations regarding the translational potential of these compounds, as this particular area of study is still in its early years. As an example, for other GPCRs contrasting hypotheses and results have been presented regarding the role of bias in improved therapeutic windows and the specific function of signaling proteins (Bohn et al. 1999, 2000; Raehal et al. 2005; Kliewer et al. 2019; Gillis et al. 2020). Nevertheless, the progress described in this chapter is remarkable. It shows how hypotheses generated from signaling studies can guide the pursuit of specific ligands, which present improved pre-clinical outcomes. It is also noteworthy that for the studies discussed, the bias observed in cell models appears to translate to a bias in in vivo pharmacological responses. The use of primary tissue or techniques that measure signaling in vivo is still very attractive and desired. Moreover, ligand binding kinetics and compound pharmacokinetic properties are also expected to play an important role in in vivo experimentation. The discovery of the pharmacological tools described in this chapter is certain to be informative and guide the field. Considering (and pursuing) ligand bias is becoming a norm in GPCR drug discovery and, hopefully, future studies will enlighten us on the clinical potential of these compounds.

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Preclinical Studies on Nalfurafine (TRK-820), a Clinically Used KOR Agonist

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Contents

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Abstract

Nalfurafine has been used clinically in Japan for treatment of itch in kidney dialysis patients and in patients with chronic liver diseases. A one-year post-marketing study showed nalfurafine to be safe and efficacious without producing side effects of typical KOR agonists such as anhedonia and psychotomimesis. In this chapter, we summarize in vitro characterization and in vivo preclinical studies on nalfurafine. In vitro, nalfurafine is a highly potent and moderately selective KOR full agonist; however, whether it is a biased KOR agonist is a matter of debate. In animals, nalfurafine produced anti-pruritic effects in a dose range lower than that caused side effects, including conditioned place aversion (CPA), hypolocomotion, motor incoordination, consistent with the human data. In addition, nalfurafine showed antinociceptive effects in several pain models at doses that did not cause the side effects mentioned above. It appears to be effective against inflammatory pain and mechanical pain, but less so against thermal pain, particularly high-intensity thermal pain. U50,488H and nalfurafine differentially modulated several signaling pathways in a brain region-specific manners. Notably, U50,488H, but not nalfurafine, activated the mTOR pathway, which contributed to U50,488H-induced CPA. Because of its lack of side effects associated with typical KOR agonists, nalfurafine has been investigated as a combination therapy with an MOR ligand for pain treatment and for its effects on opioid use disorder and alcohol use disorder, and results indicate potential usefulness for these indications. Thus, although in vitro data regarding

uniqueness of nalfurafine in terms of signaling at the KOR are somewhat equivocal, in vivo results support the assertion that nalfurafine is an atypical KOR agonist with a significantly improved side-effect profile relative to typical KOR agonists.

Keywords

Abuse deterrence · Alcohol use disorder · Analgesia · Antipruritic effect · Aversion · Opioid use disorder

1 Introduction

Activation of kappa opioid receptor (KOR) produce analgesic and antipruritic effects; however, clinical development of these compounds has been limited by side effects such as psychotomimesis, dysphoria, and sedation (Pande et al. 1996; Pfeiffer et al. 1986; Wadenberg 2003; Walsh et al. 2001). One exception is nalfurafine (TRK-820 in early literature). Nalfurafine (Remitch™), 17-cyclopropylmethyl-3, 14 beta-dihydroxy-4,5 alpha-epoxy-6 beta-[N-methyl-trans-3-(3-furyl) acrylamido]-morphinan, has been used in Japan for treatment of itch in patients undergoing hemodialysis and in those with chronic liver diseases (Kozono et al. 2018; Kumagai et al. 2010, 2012; Nakao and Mochizuki 2009a). Clinical reports have shown that, at therapeutic doses, nalfurafine does not produce dysphoria or psychotomimetic effects, which sets it apart from prototyipcal KOR agonists that have been tested in humans (Kozono et al. 2018; Kumagai et al. 2010, 2012). It should be noted, however, that nalfurafine did not receive approval by the European Medicines Agency because of lack of efficacy.

Nalfurafine was first synthesized by Nagase et al. (1998) and found to be a highly potent and moderately selective KOR full agonist. A unique structural feature of TRK-820 is the 4,5-epoxymorphinan structure with a tyrosine-glycine moiety of endogenous opioid peptides, which is different from other nonpeptide KOR agonists such as U50,488H and salvinorin A.

In this chapter, we summarize preclinical studies on nalfurafine, including in vitro characterization and in vivo pharmacology in laboratory animals, as well as its potential use as an adjunct for pain treatment, and as a deterrent/treatment for drug and alcohol abuse. We also review studies that examine what makes nalfurafine different from other KOR agonists, which may provide some insights into development of KOR agonists with fewer side effects.

In the earlier literature, it was named TRK-820, whereas in the more recent literature, it was called nalfurafine. In this chapter, both terms will be used to be consistent with the original literature.

2 In Vitro Pharmacological Characterization of Nalfurafine

2.1 Bioassays

Nagase et al. (1998) reported that TRK-820 inhibited electrically stimulated contraction of guinea pig ileum (GPI) with an IC_{50} value of 4.8 pM and KOR/MOR selectivity of 279. TRK-820 attenuated mouse vas deferens (MVD) contraction with an IC_{50} value of 36 pM and KOR/MOR selectivity of 104 and KOR/DOR selectivity of 135. TRK-820 was 4,000-fold more potent than morphine in both preparations and 200 and 70 times more potent than U50,488H in the GPI and MVD, respectively.

2.2 Binding Affinity and Selectivity Profile of Nalfurafine at MOR, DOR, KOR and NOR

Several groups determined affinities of nalfurafine for each receptor by competitive inhibition of radioligand binding (Table 1), yielding different results. Its K_i values for the KOR ranged from 0.075 to 3.5 nM, those for the MOR 0.43–53 nM and those for the DOR 51–1,200 nM. Its binding to the NOR was negligible. The differences in its K_i value for a given receptor are likely due to differences in the radioligand, tissues and binding conditions used (Table 1). Its KOR/MOR selectivity ratios

Table 1 K_i values (in nM) of nalfurafine reported in the literature

Reference	MOR	KOR	DOR	NOR	MOR/KOR	DOR/KOR
Seki et al. (1999)[a]	53 ± 12	3.5 ± 0.9	1,200 ± 300	380 ± 50	15.1	343
Wang et al. (2005)[b]	5.2 ± 0.8	0.075 ± 0.007	161 ± 42	N.A.	69.3	214
Nagase and Fujii (2013)[c]	0.431	0.178	51.3	–	2.42	288
Che et al. (2018)[d]	4.20 ± 0.21	0.32 ± 0.02	–	–	13.1	–

N.A. did not bind at 1 μM
– not determined
[a]Inhibition of [^{3}H]bremazocine binding to KOR, MOR and DOR stably expressed in CHO cells, inhibition of [^{3}H]nociceptin binding to NOR stably expressed in CHO cells. Data are expressed as mean ± SEM ($N = 3$)
[b]Inhibition of [^{3}H]diprenorphine binding to KOR and MOR stably expressed in CHO cells, inhibition of [^{3}H]nociceptin binding to NOR stably expressed in CHO cells. Data are expressed as mean ± SEM ($N = 3$)
[c]Inhibition of [^{3}H]U69,593 binding to KOR in guinea pig cerebellum membranes, inhibition of [^{3}H]DAMGO binding to MOR in mouse brain membranes, inhibition of [^{3}H]DPDPE binding to DOR in mouse brain membranes. Data are expressed as mean ($N = 3$)
[d]Inhibition of [^{3}H]diprenorphine binding to KOR and MOR stably expressed in Sf9 cells. Data are expressed as mean ± SEM ($N = 3$)

ranged from 2.4 to 69 and those of KOR/DOR $\geq$ 280. Overall, these data indicate that nalfurafine is moderately selective for the KOR over the MOR and highly selective for the KOR over the DOR.

2.3 No Significant Binding to Targets Other Than Opioid Receptors

Nalfurafine at 10 μM did not bind significantly to 45 pharmacological targets (Nakao and Mochizuki 2009b), including histamine, neurokinin, bombesin, CGRP, somatostatin, ionotropic glutamate, dopamine, adrenergic, muscarinic, adenosine, IL-1β, IL-8, CCR1, CCR2, CCK, $GABA_A$, and VIP receptors and L-type Ca^{++} channel. The only target for which it exhibited a moderate affinity was the M1 muscarinic receptor with a K_i value of 1.7 μM. Nalfurafine did not affect release of several inflammatory mediators (histamine, tumor necrosis factor, interleukin-1β and -6, and prostaglandins D2 and E2), nor did it influence activities of constitutive and inducible nitric oxide synthetase (Nakao and Mochizuki 2009b).

2.4 Efficacies and Potencies of Nalfurafine in Activating MOR, DOR, KOR and NOR: Inhibition of Adenylate Cyclase and Enhancement of [^{35}S]GTPγS Binding

Seki et al. (1999) evaluated agonistic activity of TRK-820 by inhibition of forskolin (10 μM)-stimulated cAMP accumulation in CHO cells stably transfected with cloned MOR, KOR or DOR. The IC_{50} and I_{max} values for MOR, KOR and DOR are shown in Table 2. The data indicate that compared with the reference full agonists, nalfurafine is a potent full agonist at the KOR and high-efficacy partial agonist at the MOR and has a KOR/MOR selectivity factor of 55.

Nalfurafine was also examined for its potency and efficacy in enhancing [^{35}S]GTPγS binding via the MOR, DOR, KOR and NOR. Data are shown in Table 3. Nalfurafine is a high-potency KOR full agonist with an EC_{50} value below 0.1 nM, and has partial agonist activities at the MOR, DOR and NOR with much lower

Table 2 Potency and efficacy of nalfurafine in inhibiting forskolin-stimulated adenylyl cyclase in CHO cells stably transfected with cloned MOR, KOR or DOR

	MOR		KOR		DOR	
	IC_{50} (nM)	I_{max}	IC_{50} (nM)	I_{max}	IC_{50} (nM)	I_{max}
Nalfurafine	8.3 ± 1.4	69 ± 3%[a]	0.15 ± 0.08	81 ± 3%	>1,000	–[b]
Reference full agonist	DAMGO		U69,593		DPDPE	
	5.0 ± 1.1	88 ± 1%	16 ± 6	72 ± 5%,	5.4 ± 1.7	78 ± 3%

Data are expressed as mean ± sem ($N = 3$)
[a]$P < 0.01$ in Student's *t*-test, compared with that of the reference full agonist DAMGO
[b]Did not reach maximum at 10 μM

Table 3 EC_{50} and relative E_{max} of nalfurafine in stimulating [^{35}S]GTPγS binding in membranes of CHO cells stably transfected with cloned MOR, KOR, DOR or NOR

EC_{50} (nM)					E_{max} (% of full agonist)			
	MOR	KOR	DOR	NOR	MOR	KOR	DOR	NOR
Nalfurafine[a]	3.2 ± 1.3	0.025 ± 0.003	289 ± 60	147 ± 30	54 ± 7	93 ± 5	51 ± 6	27 ± 4
Reference full agonist[a]	DAMGO 8.9 ± 2.1[a]	U50,488H 2.2 ± 0.3[a]	DPDPE 3.4 ± 0.7[a]	Nociceptin 1.2 ± 0.2[a]	DAMGO 100[a]	U50,488H 100[a]	DPDPE 100[a]	Nociceptin 100[a]
Nalfurafine[b]	3.11 ± 0.63	0.097 ± 0.018	24.22 ± 2.56	279.7 ± 17.7	73.88 ± 2.93	90.90 ± 3.25	129.1	119.7 ± 2.2
Reference full agonist[b]	DAMGO 6.30 ± 0.43[b]	U50,488H 5.12 ± 0.37[b]	DPDPE 7.06 ± 0.76[b]	Nociceptin 0.046 ± 0.007[b]	DAMGO 99.42 ± 1.07[b]	U50,488H 99.65 ± 1.24[b]	DPDPE 97.67 ± 1.92[b]	Nociceptin 110.8 ± 2.8[b]

Data are expressed as mean ± SEM ($N = 3$)
[a]From Wang et al. (2005)
[b]From Cao et al. (2020)

potency. Its KOR/MOR selectivity is 32 or 128; KOR/DOR or KOR/NOR selectivity factors are much higher. Therefore, nalfurafine is considered as a moderately selective KOR full agonist.

Potency and efficacy of nalfurafine were also examined using several other functional endpoints for G protein- and β-arrestin-mediated signaling, which are discussed in Sect. 2.5.

2.5 Functional Selectivity of Nalfurafine in KOR-Mediated Activation of G Proteins and β-Arrestins

Studies on functional selectivity of nalfurafine for G proteins or β-arrestins have yielded different results, demonstrating nalfurafine to be G protein-biased (Cao et al. 2020; Kaski et al. 2019; Schattauer et al. 2017), un-biased (Liu et al. 2019) and β-arrestin-biased (Dunn et al. 2019). The cell lines, functional endpoints and reference compounds used, and the calculated agonist biases are summarized in Table 4. The discrepancy may be attributed to differences in cell line, end point, assay method for the same end point, receptor expression level and the reference balanced agonist used. Gillis et al. (2020) recently reported that responses having amplification factors and higher receptor reserves tend to enhance potency and efficacy of an agonist, thus confounding agonist bias calculation. The cAMP response has a high signal amplification, whereas [^{35}S]GTPγS binding and β-arrestin recruitment do not, which may contribute to the findings of nalfurafine as G protein-biased agonist in the study of Cao et al. (2020); Kaski et al. (2019), but as a unbiased agonist in Liu et al. (2019).

2.6 KOR Internalization and Down-Regulation

Wang et al. (2005) reported that TRK-820 (1 nM, 30 min, 37°C) caused internalization of FLAG-hKOR stably transfected into HEK 293 cells, similar to U50,488H (1 μM). Following incubation of CHO-FLAG-hKOR cells for 4 h, TRK-820 (1 nM, 37°C) induced down-regulation of the human KOR to the same extent as U50,488H (1 μM).

3 In Vivo Pharmacological Effects of Nalfurafine

Nalfurafine has been demonstrated to have anti-pruritic effects against several pruritogens and in disease models of itch, antinociceptive effects in various pain models, and water diuretic effects. In addition, nalfurafine has been examined for many KOR-mediated side effects, such as hypolocomotion, CPA, and motor incoordination. In general, nalfurafine produces antinociceptive and anti-pruritic effects in dose ranges that are below those causing hypolocomotion, CPA and motor incoordination. This is a unique feature that separates nalfurafine from typical

Table 4 Determination of agonist biases of nalfurafine

References	G protein signaling cells used	β-arrestin signaling	Agonist bias (reference compound)
Schattauer et al. (2017)	ERK1/2 phosphorylation HEK293 cells stably expressing rat KOR-GFP	P38 MAPK phosphorylation HEK293 cells stably expressing rat KOR-GFP	G protein-biased, 7.2 (U50,488H)
Schattauer et al. (2017)	ERK1/2 phosphorylation HEK293 cells stably expressing FLAG-human KOR	P38 MAPK phosphorylation HEK293 cells stably Expressing FLAG-human KOR	G protein-biased, 300 (U50,488H)
Liu et al. (2019)	[^{35}S]GTPγS binding neuro2A cells stably expressing mouse KOR	β-Galactosidase fragment complementation HEK cells stably expressing mouse KOR-donor and β-arrestin1 or 2-acceptor	Unbiased (U50,488H)
Dunn et al. (2019)	[^{35}S]GTPγS binding Human KOR-expressing U2OS cells (PathHunter, DiscoverX)	β-Galactosidase fragment complementation Human KOR-expressing U2OS cells (PathHunter, DiscoverX)	β-Arrestin-biased (U69,593)
Kaski et al. (2019)	GloSensor (cAMP) HEK293T cells transiently transfected with 3 × HA-hKOR and pGloSensor-22F cAMP biosensor	Tango luciferase-based assay HTLA cells transiently transfected with human KOR-V2-TEV-tTA	G protein-biased, 7.7 (U50,488)
Cao et al. (2020)	GloSensor (cAMP) HEK293-T cells stably expressing human KOR were transfected with GloSensor for cAMP	Tango β-arrestin2 recruitment HEK293 cells stably expressing a tTA-dependent luciferase reporter and a β-arrestin2-TEV fusion gene were transfected with the human KOR	G protein-biased, 2.85 (U50,488H)

KOR agonists, such as U50,488H. U50,488H produces the side effects in the dose ranges that induce anti-nociceptive and anti-pruritic effects.

3.1 Antipruritic Effects

In a pioneering study, Cowan and Gmerek (1986) showed that the KOR agonists tifluadom and U50,488 attenuated bombesin-induced scratching and grooming in rats. Subsequent publications confirmed the antipruritic activities of KOR agonists, which ultimately led to the approval of nalfurafine in Japan as an antipruritic drug for treatment of itch in patients undergoing hemodialysis or with chronic liver diseases.

Togashi et al. (2002) were the first to report anti-pruritic activity of nalfurafine in rodents. They demonstrated that in male ICR mice, TRK-820 (p.o., −30 min)

reduced scratching induced by substance P or histamine, with A_{50} values of 19.6 and 7.3 μg/kg, respectively, which was antagonized by pretreatment with norBNI (s.c., overnight), indicating a KOR-mediated effect. No obvious suppression of the spontaneous locomotor activity was observed at the doses used. TRK-820 suppressed running activity at much higher doses with an A_{50} of 102.8 μg/kg.

Subsequently, nalfurafine (s.c) was shown to have inhibitory effects on scratch induced by several pruritogens in a dose-dependent manner in mice, including the malaria drug chloroquine (Inan and Cowan 2004; Snyder et al. 2018), the histamine releasing agent compound 48/80 (Liu et al. 2019; Wang et al. 2005), the KOR antagonist 5'-GNTI (Inan et al. 2009a), the protease-activating receptor 2 agonist SLIGRL, histamine and serotonin (Kardon et al. 2014). The A_{50} values were in the range of 6.6–10 μg/kg and nalfurafine did not affect motor activity up to 30 μg/kg or produce conditioned place aversion (CPA) up to 20 μg/kg in these studies. Snyder et al. (2018) showed that the inhibitory effect of nalfurafine against chloroquine-induced scratching was absent in KOR−/− mice. In addition, Inan et al. (2009a) reported that nalfurafine was also effective as a post treatment in attenuating scratching induced by compound 48/80 and 5'-GNTI. Remarkably, mice treated once/day with 20 μg/kg nalfurafine for 10 days did not develop tolerance to the inhibitory effect of nalfurafine against scratch induced by 5'-GNTI (Inan et al. 2009a). Compound 48/80 and 5'-GNTI enhanced c-fos expression in the dorsal horn of mouse spinal cord, which was inhibited by nalfurafine (Inan et al. 2009a). Umeuchi et al. (2003) found that the anti-scratching activity of TRK-820 (s.c) was antagonized by intracerebroventricular administration of norBNI, indicating an important role of brain KOR in TRK-820 effects. Lastly, nalfurafine (0.01–0.1 mg/kg; i.p.) reduced scratching induced by intradermal injections of serotonin in male Sprague-Dawley rats (Lazenka et al. 2018). However, nalfurafine failed to block itch-induced suppression of responding for intracranial self-stimulation (ICSS) in this same report.

Nalfurafine has also been studied in disease models of itch in rodents. Umeuchi et al. (2005) demonstrated that aged MRL/lpr mice may be used as a model of pruritus related to human autoimmune diseases and found that nalfurafine (p.o.) inhibited the scratching behavior in these mice without causing gross behavioral changes. Inan and Cowan (2006) generated a rat model of pruritus associated with cholestasis by repeated injection with ethynylestradiol and showed that nalfurafine (s.c.) suppressed whole-body scratching with an A_{50} value of 13 μg/kg. Nalfurafine (p.o.) was effective in reducing scratching behaviors in NC/Nga mice, a model of atopic dermatitis (Nakao et al. 2008). Nalfurafine inhibited scratching related to experimentally induced dry skin in mice (Akiyama et al. 2015; Kardon et al. 2014). Topical application of nalfurafine inhibited scratching in an oxazolone-induced murine model of atopic dermatitis (Elliott et al. 2016).

3.2 Anti-nociceptive Effects

KOR agonists have long been established to have antinociceptive effects in laboratory animals (von Voigtlander et al. 1983). Nalfurafine has been found to have antinociceptive effects in rodents and nonhuman primates. Nalfurafine is more active against inflammatory pain and mechanical pain.

Mice Endoh et al. (1999) conducted comprehensive studies in mice. They reported that in male ddY mice TRK-820 given s.c. or p.o. was 351-fold and 796-fold more potent than U50,488H in the acetic acid-induced abdominal constriction test with A_{50} values of 3.3 μg/kg (s.c.) and 32 μg/kg (p.o.), respectively. The effect reached a peak at 30 min and returned to baseline in 120–180 min. Its duration of the antinociceptive effect was longer than that of morphine or other KOR agonists (U50,488H, CI-977, ICI199,441 and PD117302). TRK-820 was also more readily absorbed following oral adminstration than these KOR agonists. The antinociceptive effects produced by TRK-820 in the abdominal constriction test were was antagonized by norBNI, but not by the selective DOR anatagonist naltrindole or a low dose of naloxone that was selective for the MOR, indicating KOR-mediated effect. In addition, in four other antinociceptive assays, 51°C hot plate, thermal tail flick, mechanical tail pressure and tail pinch tests, TRK-820 (s.c.) had A_{50} values of 129, 62, 9 and 35 μg/kg, respectively, and was 68-fold to 328-fold more potent than U50,488H, and 41-fold to 349-fold more potent than morphine in producing antinociception. However, TRK-820 was inactive in inhibiting the high temperature (55°C) hot plate response. These results are consistent with those of Nagase et al. (1998) in a communication report.

Liu et al. (2019) demonstrated that in CD-1 mice nalfurafine (s.c.) produced dose-dependent antinociception in the late phase of the formalin test with an A_{50} value of 8.3 μg/kg, which is 70x more potent than U50,488H.

Snyder et al. (2018) demonstrated following an intraplantar (IPL) injection of capsaicin or acetic acid into a hind paw, mice treated with nalfurafine or the peripherally restricted agonists ICI204,448 or FE200665 showed a significant decrease in the time spent licking the injected hind paw, compared to control mice, indicating antinociceptive effects. In mice, none of the KOR agonists had any effects on paw withdrawal threshold to the application of von Frey filaments (mechanical threshold). In contrast, nalfurafine, but not ICI204,448 or FE200665, significantly increased paw withdrawal latency (PWL) as measured by the Hargreaves assay (thermal threshold). Following acute incision of the hind paw, all the three KOR agonists inhibited mechanical hypersensitivity in the Brennan model, however only nalfurafine, but not the peripherally restricted agonists, inhibited thermal hypersensitivity. Thus, nalfurafine acts both centrally and peripherally to produce antinociceptive effects against thermal and mechanical pain via the KOR.

Kaski et al. (2019) observed that in the tail withdrawal assay (a model for spinal nociception) in C57BL/6 mice, nalfurafine induced significant dose-dependent antinociception at all doses tested (15, 30 and 60 μg/kg). U50,488 also elicited spinal

anti-nociception at 1.25 mg/kg and at 5.00 mg/kg (but not at 2.50 mg/kg). The highest tested dose of nalfurafine (60 μg/kg) was significantly more anti-nociceptive than was the highest dose of U50,488 (5.00 mg/kg).

By single-unit electrophysiological recordings of colonic nociceptive afferents, Snyder et al. (2018) found that inflammatory mediators (histamine, bradykinin, prostaglandin E2, and serotonin) increased the number of action potentials in response to stretch and nalfurafine significantly reduced this sensitization, which was abolished in KOR−/− mice.

Rats Endoh et al. (2000) reported that TRK-820 produced a potent dose-dependent and KOR-mediated antinociceptive effect in the paw pressure test in male Wistar rats with A_{50} values of 64 μg/kg (s.c.) and 75 μg/kg (i.m.). Nalfurafine displayed similar potency in normal rats and adjuvant-induced arthritic rats in the paw pressure test. In the second phase of the formalin test, TRK-820 had a potent antinociceptive effect with an A_{50} value of 9.6 μg/kg (s.c.), similar to mice.

Townsend et al. (2017) reported that nalfurafine (i.v.) produced dose-dependent thermal antinociception using the hot plate test in male Sprague-Dawley rats. More recently, nalfurafine was reported to reverse writhing behavior in rats induced by i.p. administration of lactic acid (Lazenka et al. 2018). However, in this same report, nalfurafine failed to reverse acid-induced suppression of ICSS. Notably, nalfurafine also reduced ICSS in rats in the absence of acid injections, which precludes the ability to make firm conclusions regarding antinociception in this procedure.

Non-human Primates Endoh et al. (2001) demonstrated that in cynomolgus monkeys, TRK-820 (i.m.) produced a potent antinociceptive effect that was 295-fold and 495-fold more potent than morphine in the 50°C and 55°C hot-water tail-withdrawal tests, respectively, and 40-fold more potent than U50,488H and 1,000-fold more potent than pentazocine in the 50°C hot-water test. The duration of antinociceptive effects of TRK-820 (10 and 30 μg/kg, i.m.) was >6 h, much longer than that of U50,488H. The antinociception induced by a lower dose of TRK-820 (10 μg/kg, i.m.) was inhibited by norBNI (10 mg/kg, s.c.), indicating KOR-mediated actions, whereas that produced by a higher dose of TRK-820 (30 μg/kg, i.m.) was not antagonized by norBNI (3.2 and 10 mg/kg, s.c.) or by naloxone (0.1 mg/kg, s.c.), which effectively inhibited the antinociception induced by U50,488H (1.0 mg/kg, i.m.) and morphine (10 mg/kg, i.m.), respectively, suggesting that there may be other targets at 30 μg/kg of nalfurafine.

3.3 Anti-allodynic and Anti-hyperalgesic Effects

Takasaki et al. (2004) used a mouse model of acute herpetic pain in which percutaneous inoculation with herpes simplex virus type-1 induced tactile allodynia and mechanical hyperalgesia in the hind paw on the inoculated side. In female BALB/c mice, TRK-820 (10–100 μg/kg, p.o.) inhibited the allodynia and hyperalgesia in a dose-dependent manner, similar to morphine (5–20 mg/kg) and the KOR agonist

enadoline (1–10 mg/kg). Interestingly, in the effective dose ranges, enadoline greatly reduced spontaneous locomotor activity, but TRK-820 did not. The effects of TRK-820 were blocked by pretreatment with norBNI, but not by a low dose of naltrexone, which antagonized the effects of morphine. Repeated administration of TRK-820 did not attenuate its anti-allodynic and anti-hyperalgesic effects, indicating no tolerance. Intrathecal and intracerebroventricular, but not intraplantar, injections of TRK-820 also inhibited the allodynia and hyperalgesia. Snyder et al. (2018) reported that following surgical incision injury of the hind paw, hypersensitivity to mechanical and thermal stimuli developed. Nalfurafine and peripherally acting KOR agonists ICI204,488 and FE200665 inhibited mechanical hypersensitivity. In contrast, only nalfurafine inhibited thermal hypersensitivity. Thus, the anti-allodynic and anti-hyperalgesic effects of TRK-820 are mediated by the KOR at the spinal and supraspinal levels, an at peripheral sites.

3.4 Inhibition of Neurogenic Inflammation

Snyder et al. (2018) reported that in C57BL/6 mice an IPL injection of capsaicin caused plasma extravasation and an increase in paw temperature, a model of neurogenic inflammation. Nalfurafine or the peripherally restricted KOR agonist ICI204,488 or FE200665 significantly reduced these two reactions. Nalfurafine-induced inhibition of neurogenic inflammation was eliminated by KOR deletion. Similar results were obtained when neurogenic inflammation was induced with a mixture of bradykinin and prostaglandin E2. Thus, activation of KOR in the periphery is sufficient to inhibit neurogenic inflammation.

3.5 Aversive Effects or Lack Thereof

KOR agonists were first shown to produce conditioned place aversion (CPA) in mice and rats in the 1980s (Mucha and Herz 1985; Shippenberg 1986). In both mice and rats, whether nalfurafine causes CPA depends on the dose used. The results in the literature support the notion that at the doses effective in the antinociceptive and anti-scratching tests, nalfurafine did not produce CPA; however, nalfurafine did cause CPA at higher doses. For example, Liu et al. (2019) reported that in CD-1 mice, the A_{50} values of nalfurafine-induced antinociception in the formalin test and in the anti-scratch test were 5.8 and 8.3 μg/kg, respectively, and that nalfurafine (5–20 μg/kg, s.c.) did not cause CPA or CPP.

Mice Tsuji et al. (2001) reported that TRK-820 (3–30 μg/kg, s.c.) did not produce a significant CPP or CPA in mice. Liu et al. (2019) demonstrated that in CD-1 mice, nalfurafine (5–20 μg/kg, s.c.) did not cause CPA or CPP. Zhou and Kreek (2019b) also found that nalfurafine at 10 μg/kg (i.p.) did not produce CPA in C57BL/6 J mice. In addition, Liu et al. (2019) found that nalfurafine (10 μg/kg, s.c.) did not induce CPA in MOR−/− mice, ruling out the possibility that nalfurafine did not

produce CPA because it acted on both the MOR and KOR. Kaski et al. (2019) showed that in C57BL/6 J mice, nalfurafine at 30 μg/kg produced CPA, but not at 15 or 60 μg/kg, whereas U50,488 was aversive at three doses tested (1.25–5.00 mg/kg). The reason for this inverted U-shaped dose-response relationship is not clear.

Rats Mori et al. (2002) reported that in male Fischer 344 rats, TRK-820 at 10, 20, 40 μg/kg did not induce CPA or CPP, but at 80 μg/kg it did cause significant CPA. However, a recent study in male Sprague-Dawley rats reported that 0.18 mg/kg nalfurafine (s.c.) did not produce a significant CPA (Zamarripa et al. 2020b).

3.6 Effects on Locomotor Activity and Motor Coordination

The locomotor-suppressing actions of KOR agonists in rodents have long been recognized (Iwamoto 1981; von Voigtlander et al. 1983). Locomotor activity has commonly been assessed with the rotarod test (Iwamoto 1981), measurement with circular actophotometer cages (von Voigtlander et al. 1983) or rectangular locomotor activity chambers (Liu et al. 2019; White et al. 2015) or the wheel running test (Togashi et al. 2002).

The hypolocomotion induced by KOR agonists generally occurs in the dose ranges that produce antinociceptive and anti-pruritic effects (for example, U50,488H in von Voigtlander et al. 1983; Liu et al. 2019; Kaski et al. 2019, methoxysalvinorin B in Liu et al. 2019). The unique feature of nalfurafine is that in the dose ranges effective for antinociceptive and anti-pruritic effects, nalfurafine either did not cause a hypolocomotor effect (Liu et al. 2019) or produced a small ($\leq$ 25%) but significant effect (Kaski et al. 2019). For example, Liu et al. (2019) reported that the A_{50} values of nalfurafine-induced antinociception in the formalin test and in the anti-scratch test were 5.8 and 8.3 μg/kg, respectively and at 20 μg/kg, it did not affect total distance traveled and slightly impaired rotarod performance. Zhou and Kreek (2019b) also found that nalfurafine at 10 μg/kg (i.p.) had no effect on spontaneous locomotor activity in C57BL/6 J mice.

In some studies, TRK-820 did produce sedation, but at doses which were higher than those producing antinociception (Endoh et al. 1999; Togashi et al. 2002). Endoh et al. (1999) demonstrated that in male ddY mice TRK-820 (s.c.) produced antinociceptive effect in the acetic acid-induced abdominal constriction assay and impaired rotarod performance with A_{50} values of 3.3 μg/kg and 27 μg/kg, respectively. Togashi et al. (2002) reported that in male ICR mice TRK-820 (p.o., at −30 min) inhibited histamine-induced scratching and suppressed running activity with A_{50} values of 7.3 μg/kg and 102.8 μg/kg, respectively. Kaski et al. (2019) found that the inhibitory effect of nalfurafine at 15 and 30 μg/kg on novelty-induced locomotor activity was slight (by $\leq$25%) but significant, whereas at 60 μg/kg, the drug impaired locomotor activity by >50%.

Mori et al. (2002) reported that in a cocaine drug discrimination test using male Fisher 344 rats, 10–40 μg/kg TRK-820 did not affect the response rates, but at 80 μg/kg significantly decreased the response rates.

3.7 Water Diuretic Effects

Inan et al. (2009b) observed that nalfurafine (5–20 μg/kg, s.c.) caused water diuresis in a dose-dependent manner in male rats, similar to U50,488H and methoxymethyl salvinorin B (MOM-SalB). Nalfurafine, like U50,488H and MOM-SalB, increased urine volume and free water clearance and lowered urine osmolality, without excreting sodium ion. The effect of nalfurafine was blocked by the KOR antagonist 5′-guanidinonaltrindole, indicating KOR-mediated effect. Tolerance did not develop following repeated injection of nalfurafine (once/day, 20 μg/kg, s.c.) for 7 days.

3.8 Nalfurafine and U50,488H Induced Different Phosphoproteomic Changes in Mouse Brains

Liu et al. (2019) observed that in CD-1 mice both U50,488H and nalfurafine produced antinociceptive and anti-scratch effects in a dose-dependent manner. However, in the dose ranges effective in both effects, U50,488H, but not nalfurafine, caused CPA, inhibition of novelty-induced hyperlocomotion and motor incoordination. Phosphoproteomics studies on brains of mice revealed that U50,488H and nalfurafine imparted phosphorylation changes to proteins in different cellular compartments and signaling pathways in different brain regions. Notably, U50,488H, but not nalfurafine, activated the mammalian target of rapamycin (mTOR) pathway in the striatum and cortex. Inhibition of the mTOR pathway by rapamycin abolished U50,488H-induced CPA, without affecting analgesic, anti-scratch, and sedative effects and motor incoordination. The results indicate that the mTOR pathway is involved in KOR agonist-induced aversion. Recently, Zhou et al. (2020) reported that excessive alcohol drinking enhanced gene expression of molecules in the mTORC1 pathway in the mouse ventral striatum, and U50,488H (but not nalfurafine)-induced increases in alcohol intake were attenuated by rapamycin pretreatment, suggesting the mTOR pathway is also involved in KOR activation-induced drug taking behavior. In addition, several pathways were differentially regulated (Liu et al. 2019). For example, U50,488H, but not nalfurafine, activated the Wnt signaling pathway in the cortex and hippocampus, but nalfurafine, not U50,488H, activated pathways involved in tight junction and inositol phosphate metabolism in the cortex. How these pathways are involved in the different pharmacological effects of the two agonists remains to be investigated.

3.9 Effect of Nalfurafine on Pharmacological Actions of Morphine

3.9.1 Effects on Morphine-Induced Itch

Morphine-induced itch is a significant clinical issue. In mice, Umeuchi et al. (2003) showed that intracisternal morphine-induced scratching was significantly and dose-dependently inhibited by TRK-820 (s.c.). Nalfurafine is also active via the intrathecal (i.t.) route. Sakakihara et al. (2016) reported that in mice nalfurafine (i.t.)

produced an antipruritic effect against i.t. morphine-induced scratching without affecting sedation scores, and the effect of TRK-820 was blocked by pretreatment with norBNI (i.p.). In rhesus monkeys, TRK-820 (i.v. or p.o.) inhibited systemic skin scratching induced by i.v. or i.t. morphine (Ko and Husbands 2009; Wakasa et al. 2004) without affecting morphine-induced analgesia and respiratory depression.

3.9.2 Effect on Morphine-Induced Antinociception

Endoh et al. (1999) reported that co-administration of TRK-820 (10 or 30 μg/kg, s.c.) with morphine (10 mg/kg) slightly enhanced morphine-induced antinociception in the 55°C hot plate test in male ddY mice. In contrast, pentazocine (3 or 10 mg/kg, s.c.) reduced morphine-induced antinociception.

Co-injection of TRK-820 with morphine (both i.t.) significantly enhanced thermal antinociceptive effects of morphine in mice (Sakakihara et al. 2016).

Kaski et al. (2019) showed that nalfurafine (15 μg/kg, s.c.) co-administered with morphine (5 mg/kg, i.p.) significantly augmented the spinal anti-nociceptive effect of morphine in the 55°C warm water tail withdrawal assay, similar to U50,488H (5 mg/kg, i.p.). In addition, nalfurafine (15, 30, 60 μg/kg, s.c.) significantly increased (~3x) supraspinal anti-nociceptive effect of morphine (5 mg/kg, i.p.) in the 53°C hot plate test (Kaski et al. 2019). A similar augmentation was seen only with 5 mg/kg U50,488H, but not with 1.25 or 2.50 mg/kg.

3.9.3 Effects on Rewarding Properties of Morphine and Oxycodone

Tsuji et al. (2001) found that in ddY mice, an outbred strain, TRK-820 (10 and 30 μg/kg, s.c.) co-injected with morphine (5 mg/kg, s.c.) significantly suppressed morphine-induced CPP, and this effect was antagonized by pretreatment with nor-BNI. TRK-820 alone did not produce CPP or CPA. A similar result was recently reported in male Sprague-Dawley rats with a combination of nalfurafine (0.18 mg/kg; s.c.) and oxycodone (3.2 mg/kg; s.c.) (Zamarripa et al. 2020b). Using C57BL/6 J mice, an inbred strain, Kaski et al. (2019) reported that co-administration of nalfurafine (s.c.) at 15 or 60 μg/kg, but not 30 μg/kg, significantly reduced morphine (5 mg/kg)-elicited CPP. For comparison, U50,488H at 1.25, 2.50 or 5.0 mg/kg inhibited morphine CPP. Nalfurafine alone caused CPA at 30 μg/kg, but not at 15 or 60 μg/kg. The differences between the two studies may be due to genetic backgrounds of the mice. Recently Zhang and Kreek (2020) reported that C57BL/6J mice nalfurafine caused a dose-dependent reduction of oxycodone self-administration and CPP at doses up to 40 μg/kg and the effects of nalfurafine were blocked by pretreatment with nor-BNI.

3.9.4 Effects on Morphine-Induced Tolerance and Dependence

Tsuji et al. (2000b) showed that in mice antinociceptive tolerance to morphine in the 51°C warm plate test following daily injection of morphine (10 mg/kg, s.c.) was suppressed by co-administration of U50,488H (1–10 mg/kg, s.c.) dose-dependently, but not by co-administration of TRK-820 (3–30 μg/kg, s.c.). Tsuji et al. (2000a) also found that co-treatment of mice with nalfurafine (3–30 μg/kg, s.c.) during chronic

treatment with escalating doses of morphine blocked naloxone-precipitated withdrawal syndromes, whereas co-treatment with U50,488H (1–10 mg/kg, s.c.) did not. Thus, co-administration of TRK-820 with morphine prevented naloxone-precipitated withdrawal, but had no effect on morphine tolerance.

3.9.5 Effects on Morphine-Induced Hyperlocomotion

Tsuji et al. (2001) reported that in ddY mice, TRK-820 (10, 30 μg/kg, s.c.) suppressed morphine-induced hyperlocomotion, and this suppression was blocked by nor-BNI. Kaski et al. (2019) found that in C57BL/6J mice nalfurafine (15, 30, 60 μg/kg, s.c.) had similar effects in a dose-dependent manner. In comparison, U50,488 significantly reduced morphine-stimulated locomotor activity only at 5 mg/kg (Kaski et al. 2019).

4 Nalfurafine as an Abuse-Deterring Agent for Prescription Opioid Abuse

4.1 Translational Significance of Contingent vs. Non-contingent Administration of KOR Agonists

As indicated above, treating experimental subjects with KOR agonists has been shown to reduce the reinforcing effects of a number of drugs and nondrug reinforcers in the self-administration design (Cosgrove and Carroll 2002; Mello and Negus 1998; Morani et al. 2009; Simonson et al. 2015). From a translational perspective, studies in which the administration of KOR agonists are not contingent on the behavior of the organism are generally focused on the development of treatments for Substance Use Disorder (SUD) because the administration of the test compound is independent of the seeking and taking of the drug reinforcer (as would be the case with a maintenance therapy). An alternative approach is to study the effects of contingently administered KOR agonists on self-administration of a drug of abuse. Under this experimental arrangement, the animal self-administers a drug reinforcer, and each injection of that drug results in the co-administration of a dose of a KOR agonist. Thus, the effects of the KOR agonist are only experienced when the drug reinforcer is self-administered.

The translational focus of this "yoked" arrangement of drug delivery is the development of abuse-deterrent formulations for therapeutics. That is, self-administering the drugs together models the circumstance in which a pill containing a combined formulation of the therapeutic molecule and the KOR agonist would be taken. Contingent administration of prototypical KOR agonists such as salvinorin A and U69,593 has been demonstrated to reduce self-administration of the MOR agonists, fentanyl and remifentanil, and the stimulant, cocaine, in nonhuman primates (Freeman et al. 2014; Negus et al. 2008), providing evidence that MOR/KOR agonist combinations have less abuse potential than MOR agonists alone.

4.2 Behavioral Pharmacology of Nalfurafine/Oxycodone Combinations

4.2.1 Studies in Rats

The "mild" side-effect profile of nalfurafine increases the feasibility of using a KOR agonist as a combination therapy with a MOR agonist because of its potential to reduce the abuse-related effects of MOR agonists without producing KOR-typical side effects. Townsend et al. (2017) reported that nalfurafine (0.32, 1, or 3.2 μg/kg/injection), when co-administered with a highly-reinforcing dose of oxycodone (0.056 mg/kg/injection; i.v.) in male Sprague-Dawley rats, decreased oxycodone self-administration in a dose-dependent manner under a progressive-ratio schedule of reinforcement. The corresponding oxycodone/nalfurafine ratios were 175:1, 56:1, and 18:1, respectively. At 18:1 ratio, oxycodon no longer had reinforcing effect. In addition, combinations of oxycodone and nalfurafine produced additive thermal antinociception, suggesting that nalfurafine may enable a dose-sparing approach for oxycodone as a combined formulation. Moreover, when a dose of oxycodone that produced significant respiratory depression was compared to an equi-analgesic dose combination of oxycodone and nalfurafine, the drug combination produced no significant respiratory depression. Lastly, Zamarripa et al. (2020b) recently reported that a mixture of nalfurafine and oxycodone blocked acquisition of oxycodone self-administration and oxycodone-induced CPP in male Sprague-Dawley rats. Thus, select combinations of oxycodone and nalfurafine have been shown to produce thermal antinociception equivalent to oxycodone alone without producing abuse-related and respiratory-depressant effects.

4.2.2 Studies in Nonhuman Primates

Zamarripa et al. (2020a) reported that nalfurafine, combined with oxycodone, reduced self-administration of oxycodone under a progressive-ratio schedule of reinforcement in male rhesus monkeys. A notable difference between the self-administration results in rats (Townsend et al. 2017) and rhesus monkeys was the dose ratio of nalfurafine:oxycodone required to reduce self-administration to saline levels. The asymptotic dose of oxycodone under a progressive-ratio schedule of reinforcement is approximately 0.05 mg/kg/inj in both species (Townsend et al. 2017; Zamarripa et al. 2020a). However, the dose of nalfurafine required to reduce self-administration of this asymptotic dose of oxycodone to saline levels is ~3 times higher in rats (0.32, 1, or 3.2 μg/kg/injection) than in monkeys (0.1, 0.18 or 0.32 μg/kg/injection), suggesting that species differences affect the potency of nalfurafine to reduce the abuse-related effects of MOR agonists.

A complementary study was recently conducted to investigate the unconditioned behavioral effects of prototypical and atypical KOR agonists, alone and following oxycodone administration, in male rhesus monkeys (Huskinson et al. 2020). The prototypical KOR agonists, salvinorin A and U50,488H, produced KOR-typical effects including disruption of species-typical activity and "lip droop", a proxy for muscle relaxation. Generally, nalfurafine produced effects that were comparable to the typical KOR agonists at sufficient doses, but the lowest dose required to disrupt

species-typical behaviors in rhesus monkeys was three times higher than the dose required to reduce oxycodone self-administration to saline levels in this species (Zamarripa et al. 2020a) and oxycodone-induced scratching (Huskinson et al. 2020), suggesting that the potency of nalfurafine to produce certain therapeutic effects is greater than its potency to produce side effects. Notably, time course assessments revealed a relatively late onset for some of the behavioral effects of nalfurafine when compared to other KOR agonists. As such, it is important when studying nalfurafine to ensure that adequate time is allowed to capture the potential occurrence of late onset of behavioral effects.

5 Effects of Nalfurafine on Alcohol Drinking

5.1 The Dynorphins/KOR System in Alcohol-Related Behaviors

Dynorphins/KOR activation has been found to be associated with the negative reinforcement aspects of alcohol addictions. It has been demonstrated that selective blockade of KOR attenuates excessive drinking and stress- or cue-induced alcohol-seeking in rodents [see (Anderson and Becker 2017) for a recent review]. These findings provide support for a critical role of the dynorphins/KOR system in alcohol addiction, although the literature is not very consistent. Microdialysis studies have revealed that acute alcohol increases extracellular dynorphin A levels in the CeA and NAc (Lam and Gianoulakis 2011). As the CeA is one of critical brain regions involved in depression- and anxiety-like behaviors (Koob 2021), it is a likely site for potential interaction of alcohol with the dynorphins/KOR system. In fact, in Sardinian alcohol-preferring rats, an increase in prodynorphin mRNA levels is found in the CeA after drinking a large amount of alcohol, suggesting that the dynorphins/KOR system in brain regions related to stress responsivity (e.g., CeA) is activated after excessive alcohol consumption (Zhou et al. 2013). It has been confirmed that there are increases in dynorphin peptide levels and KOR signaling in the CeA in alcohol-dependent Wistar rats after chronic intermittent alcohol vapor exposure (D'Addario et al. 2013; Kissler et al. 2014). Together, the enhanced dynorphins/KOR activity in the CeA may present a homeostatic adaptation of the CNS after chronic alcohol exposure or in the negative affective state during alcohol withdrawal (Haun et al. 2020).

5.2 Development of Nalfurafine as a Potential Therapeutic Agent for Alcoholism

Early work revealed that "classic" KOR agonists decreased alcohol drinking and alcohol reward (Lindholm et al. 2001), but they also produced sedation and dysphoria - side effects that limited their potentials for clinical use as discussed in Introduction. Therefore, the development of novel KOR agonists with reduced side effects may produce useful compounds for the treatment of alcoholism.

As mentioned in the Introduction, nalfurafine is the first KOR agonist approved for clinical use as an anti-pruritus medicine in Japan with few side effects (Kozono et al. 2018). Zhou and Kreek (2019b) thus investigated whether nalfurafine alone or in combination with the MOR antagonist naltrexone (having partial KOR agonist activity) would change excessive alcohol drinking in mice. Both male and female C57BL/6J mice subjected to a chronic intermittent-access drinking paradigm (2-bottle choice, 24-h access every other day) for 3 weeks. On the test day, a single administration of nalfurafine (1–10 μg/kg) decreased excessive alcohol intake and alcohol preference in a dose-dependent manner via a KOR-mediated mechanism. In contrast, nalfurafine does not alter sucrose (caloric reinforcer) or saccharin (non-caloric reinforcer) consumption. Of interest and significance, repeated daily nalfurafine administrations for 10 days decreased alcohol consumption without showing any blunted effects, suggesting tolerance did not develop to nalfurafine. Lack of tolerance to nalfurafine effect is similar to the finding that in humans, tolerance to its antipruritic effects is not observed after treatment of patients for 1 year, and also with no evidence of physical or psychological dependency reported (Kozono et al. 2018). Zhou and Kreek (2019b) demonstrated that nalfurafine at 10 μg/kg did not cause any sedation (spontaneous locomotor activity), anhedonia-like (sucrose preference test), anxiety-like (elevated plus maze test), or dysphoria-like (conditioned place aversion test) behaviors, consistent with the rodent literature (e.g. (Liu et al. 2019)), suggesting that nalfurafine had few side effects at clinically relevant doses. In addition, Zhou and Kreek (2019b) found that combinations of nalfurafine and naltrexone, at doses lower than individual effective doses, profoundly decreased excessive alcohol intake in both sexes of mice. Further, acute administration of nalfurafine alone or in combination with low-dose naltrexone significantly reduced relapse-like drinking in an alcohol deprivation effect model (Zhou and Kreek 2019a). Finally, nalmefene (a clinically utilized KOR partial agonist with MOR antagonism) (Bart et al. 2005) combined with nalfurafine also reduced relapse-like drinking in mice of either sex (Zhou and Kreek 2019a). Together, these in vivo results suggest that nalfurafine alone or combined with naloxone or nalmefene may offer a novel approach to treat alcoholism without the dysphoric properties of classic KOR agonists.

Using different chronic alcohol exposure models, several groups have found that classic KOR agonists increase alcohol intake in mice after excessive alcohol drinking (Rose et al. 2016; Zhou et al. 2020), and trigger alcohol-seeking and relapse-like drinking (Anderson and Becker 2017). Therefore, the data of Zhou and Kreek (2019a, b) that the KOR full agonist nalfurafine decreases, rather than increases, excessive or relapse-like drinking may present a different situation. After chronic excessive alcohol consumption, the endogenous dynorphin peptides (G-protein- and β-arrestin-dependent agonists) and KOR system are activated in several brain regions, including the CeA (Bloodgood et al. 2020; D'Addario et al. 2013; Kissler et al. 2014; Zhou et al. 2013). Activation of mTORC1 or β-arrestin/p38 mitogen-activated protein kinase pathway by stress-related dynorphins/KOR stimulation is associated with aversion, dysphoria, anxiety- and depression-like behaviors (Bruchas et al. 2007; Liu et al. 2018, 2019) that can drive excessive and relapse

drinking (Koob 2021; Zhou et al. 2020). As discussed above, nalfurafine at doses that produced antinociceptive and anti-pruritic effects, caused fewer side effects (sedation, anhedonia, aversion, dysphoria, anxiety- or depression-like behavior) in mice and monkeys (Huskinson et al. 2020; Liu et al. 2019; Zhou and Kreek 2019b). Thus, nalfurafine, a potent KOR agonist, may compete with the released dynorphins to bind the KOR, thereby reducing undesired effects induced by KOR activation, perhaps by reducing mTORC1 or β-arrestin/p38 mitogen-activated protein kinase signaling pathway (Zhou and Kreek 2019a, b; Zhou et al. 2020). This may be responsible, at least in part, for reducing excessive alcohol intake, as nalfurafine may attenuate the dynorphin-induced dysphoria and anxiety- or depression-like behavior after chronic alcohol exposure or during alcohol withdrawal. Taken together, these studies support the notion that nalfurafine may exhibit different molecular, cellular, and behavioral properties than classic KOR agonists and provide support for development of nalfurafine as an anti-addiction medication.

6 Effect of Nalfurafine on CPP and Discriminative Stimulus Effects of Cocaine

Rats: Pretreatment of male Fischer 344 rats with 20 and 40 μg/kg TRK-820, which itself did not produce CPP or CPA, significantly attenuated cocaine-induced CPP and these effects of TRK-820 were reversed by nor-BNI (Hasebe et al. 2004; Mori et al. 2002).

In drug discrimination test in rats, cocaine produced a dose-related increase in cocaine appropriate responses. Pretreatment with 10 and 20 μg/kg TRK-820 significantly shifted the dose-response curve for cocaine to the right without changing the response rate (Mori et al. 2002).

Mice: Pretreatment of C57BL/6 mice with nalfurafine (3 and 10 μg/kg) and U50,488 (3 mg/kg) for 15 min blocked cocaine CPP, but did not cause sedation in the rotarod assay or aversion in a place-conditioning assay (Dunn et al. 2020). Pretreatment of mice with 10 μg/kg nalfurafine and 3 mg/kg U50,488 immediately before test potentiated cocaine self-administration. Further 10 μg/kg nalfurafine also increased progressive ratio break point, indicating enhanced cocaine-seeking behavior (Dunn et al. 2020).

7 Other Effects

TRK-820 attenuated the mecamylamine-precipitated nicotine-withdrawal aversion in a CPP paradigm (Hasebe et al. 2004).

Dunn et al. (2020) reported that serum prolactin levels increased following both nalfurafine (3 and 10 μg/kg) and U50,488 (3 mg/kg).

8 Conclusion

In humans, nalfurafine produces anti-pruritic effects without causing dysphoria or psychotomimesis, side effects associated with typical KOR agonists. Different labs reported different results as to whether in vitro nalfurafine is a biased KOR agonist, depending on the functional end points and assay conditions used. In animal studies, nalfurafine induces anti-scratch effects at doses lower than those producing CPA, hypolocomotion, and motor incoordination, consistent with human data. Thus, it provides a cautionary tale about correlating in vitro biased agonism and in vivo pharmacological effects. Because of its unique side effect profile, nalfurafine may be useful for other clinical uses. Nalfurafine produces antinociceptive effects in experimental animals; however, whether KOR agonists are sufficiently efficacious analgesics in humans is a matter of debate. Nalfurafine may be useful as an adjunct for prescription MOR agonists, which at certain ratios of the two drugs, increases analgesic effects while reducing abuse liability. In addition, nalfurafine may be useful for treatment of opioid and alcohol abuse disorders. Besides being a clinically useful drug, nalfurafine is a very unique experimental tool for elucidating signaling properties underlying KOR-mediated side effects.

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Kappa Opioid Receptor Ligands and Pharmacology: Diphenethylamines, a Class of Structurally Distinct, Selective Kappa Opioid Ligands

Mariana Spetea and Helmut Schmidhammer

Contents

Abstract

The kappa opioid receptor (KOR), a G protein-coupled receptor, and its endogenous ligands, the dynorphins, are prominent members of the opioid neuromodulatory system. The endogenous kappa opioid system is expressed in the central and peripheral nervous systems, and has a key role in modulating pain in central and peripheral neuronal circuits and a wide array of physiological functions and neuropsychiatric behaviors (e.g., stress, reward, emotion, motivation, cognition, epileptic seizures, itch, and diuresis). We review the latest advances in pharmacology of the KOR, chemical developments on KOR ligands with advances and challenges, and therapeutic and potential applications of KOR ligands. Diverse discovery strategies of KOR ligands targeting natural, naturally derived, and synthetic compounds with different scaffolds, as small molecules or

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peptides, with short or long-acting pharmacokinetics, and central or peripheral site of action, are discussed. These research efforts led to ligands with distinct pharmacological properties, as agonists, partial agonists, biased agonists, and antagonists. Differential modulation of KOR signaling represents a promising strategy for developing pharmacotherapies for several human diseases, either by activating (treatment of pain, pruritus, and epilepsy) or blocking (treatment of depression, anxiety, and addiction) the receptor. We focus on the recent chemical and pharmacological advances on diphenethylamines, a new class of structurally distinct, selective KOR ligands. Design strategies and investigations to define structure–activity relationships together with in vivo pharmacology of diphenethylamines as agonists, biased agonists, and antagonists and their potential use as therapeutics are discussed.

Keywords

Agonist · Antagonist · Biased agonist · Diphenethylamines · Kappa opioid receptor · Natural ligands · Partial agonist · Structure–activity relationships · Synthetic ligands

Abbreviations

[^{35}S]GTPγS	Guanosine-5'-O-(3-[^{35}S]thio)-triphosphate
BBB	Blood-brain barrier
Bn	Benzyl
CBM	Cyclobutylmethyl
CHM	Cyclohexylmethyl
CHO	Chinese hamster ovary
CNS	Central nervous system
CPA	Conditioned place aversion
CPeM	Cyclopentylmethyl
CPM	Cyclopropylmethyl
DOR	Delta opioid receptor
EC_{50}	50% effective concentration
ED_{50}	50% effective dose
E_{max}	% maximal stimulation
ERK1/2	Extracellular regulated kinase 1/2
GNTI	Guanidinonaltrindole
GPCR	G protein-coupled receptor
hKOR	Human kappa opioid receptor
i.c.	Intracisternal
i.c.v.	Intracerebroventricular
i.p.	Intraperitoneal
i.t.	Intrathecal
i.v.	Intravenous
K_i	Inhibition constant

KO	Knockout
KOR	Kappa opioid receptor
MAPK	Mitogen-activated protein kinases
MOR	mu opioid receptor
mTOR	Mammalian target of rapamycin
nor-BNI	Nor-binaltorphimine
s.c.	Subcutaneous
SAR	Structure-activity relationship

1 Introduction

The central role of the opioid system, particularly the mu opioid receptor (MOR), in pain relief, reward, and addiction has been the driving force behind many years of research seeking for alternatives to morphine that would produce powerful analgesia, but without its addictive and other undesirable side effects. Misuse and overdose of opioids is an ongoing and rapidly emerging public health crisis, due to the dramatic rise in opioid-related overdose deaths and diagnoses of opioid-use disorder associated with prescription opioids (Stevens 2020; Volkow et al. 2019). This has become a serious health problem in the twenty-first century that needs to be addressed by innovative scientific solutions. Although a lot of research has been conducted on the MOR to find safer medications, as morphine and other opioids primarily act at this receptor, it is the κappa opioid receptor (KOR) that may have the potential, since it does not produce the rewarding effects or the respiratory depression that is the main cause of opioid overdose deaths. There is a significant attention in targeting the KOR in the pursuit of new therapies for the treatment of many human diseases, where the endogenous kappa opioid system (KOR and dynorphins) has a key function. Activation of the KOR is seen as a promising strategy for developing pharmacotherapies for pain, pruritus, and epilepsy, while KOR antagonism is associated with beneficial effects for the treatment of mood disorders (depression and anxiety) and addictive disorders (Fig. 1). Furthermore, the KOR evolves as an important substrate of comorbidity for many neuropsychiatric conditions, including

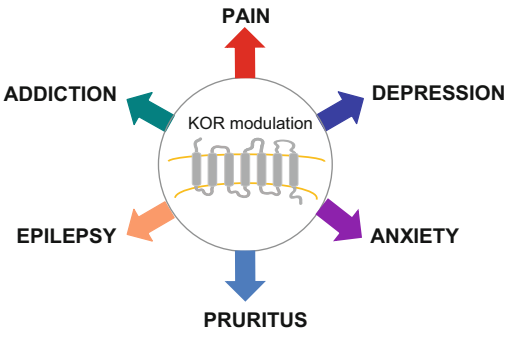


Fig. 1 Differential modulation of KOR signaling, as a strategy for developing pharmacotherapies for several human diseases, either by activating (treatment of pain, pruritus, and epilepsy) or blocking (treatment of depression, anxiety, and addiction) the receptor

chronic pain and affective disorders (depression and anxiety), or addictive and depressive disorders (Cahill et al. 2014; Lalanne et al. 2014; Liu et al. 2019b).

However, the KOR is not devoid of unwanted side effects, with receptor activation producing dysphoria, psychotomimesis, sedation, and anxiety in humans (Pande et al. 1996; Pfeiffer et al. 1986; Ranganathan et al. 2012), and aversion, anhedonia- and anxiety-like effects in animals (Mucha and Herz 1985; Shippenberg and Herz 1986; Lemos and Chavkin 2011; Albert-Vartanian et al. 2016). Cellular and molecular mechanisms of KOR function are extensively investigated to understand receptor signaling pathways leading to therapeutic/beneficial effects (i.e., analgesia, antipruritic, antidepressant and anti-addictive effects, and anticonvulsant/anti-seizure efficacy) and detrimental side effects (see Bruchas and Chavkin 2010; Burtscher and Schwarzer 2017; Bohn and Aubé 2017; Crowley and Kash 2015; Darcq and Kieffer 2018 for reviews). One of the most promising concepts that emerged in the last decade is that of "functional selectivity" or "biased agonism" at the G protein-coupled receptors (GPCR) (Rajagopal et al. 2010; Rankovic et al. 2016). This concept was introduced as a means to separate desirable and adverse responses, where the in vivo relevance of this phenomenon has attained much attention in recent years. Biased agonism describes the condition wherein a ligand stabilizes different conformations of the GPCR, with the ligand selectively engaging G protein or β-arrestin signaling pathways. Accumulated evidence has shown that β-arrestin2-mediated signaling contributes to the undesirable side effects from KOR stimulation (see Bruchas and Roth 2016; Bohn and Aubé 2017; Mores et al. 2019; Zhou and Bohn 2014 for reviews). Observations that these independent signaling mechanisms can be pharmacologically separated by using G protein-biased KOR agonists open a promising avenue for designing KOR-targeted therapeutics with potentially reduced adverse effects. This chapter provides a review on the recent developments of novel KOR ligands, with focus on chemical and pharmacological advances on selective KOR ligands from the class of diphenethylamines and their pharmacotherapeutic potential.

2 KOR Ligands and Their Pharmacology

2.1 Natural Ligands

The natural ligands for the KOR are peptides, the dynorphins, first isolated from the porcine pituitary and named after the Greek word dynamis (power) (Goldstein et al. 1979). Although dynorphin A(1–17) is considered the endogenous KOR ligand, a number of smaller, biologically active dynorphins were identified, including dynorphin A(1–13), dynorphin A(1–11), dynorphin A(1–8), and dynorphin B (1–13). Since dynorphin A and its fragments are rapidly metabolized by various peptidases, and display only limited selectivity for KOR vs. MOR and delta opioid receptor (DOR), a variety of analogues were synthesized and pharmacologically

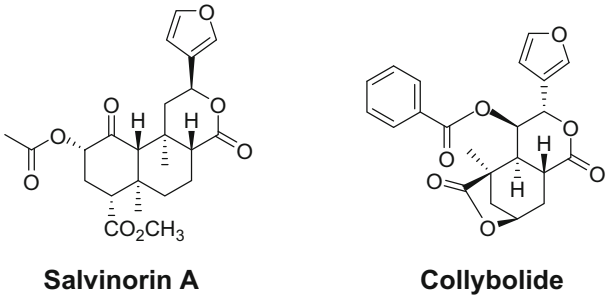


Fig. 2 Structures of naturally occurring KOR agonists, salvinorin A and collybolide

characterized, such as peptides with amino acid substitutions, deletions or additions of natural or unnatural amino acids, or cyclic analogues (see Aldrich and McLaughlin 2012; Carroll and Carlezon Jr 2013; Hall et al. 2016; Hauser et al. 2001 for reviews). Dynorphin A and dynorphin B have been the subject of many structure–activity relationships (SAR) studies with analogues reported as potent agonists or antagonists in vitro and in vivo. SAR studies established that substitution of Tyr^1 and Phe^4 with Ala in dynorphin A and dynorphin B resulted in a decreased biological activity (Joshi et al. 2017; Kawasaki et al. 1993), and residues Arg^7, Lys^{11}, and Lys^{13} in dynorphin A are key for binding and function to the KOR (Naqvi et al. 1998). Deletion of Arg^7 in dynorphin A led to selective KOR antagonists (Ramos-Colon et al. 2016). A comprehensive review on SAR studies on dynorphin A and dynorphin B and related peptides is beyond the scope of this chapter, but some synthetic dynorphin analogues are discussed in Sect. 2.2.

Salvinorin A (Fig. 2), the active component of the hallucinogenic Mexican mint *Salvia divinorum,* was identified as a selective KOR full agonist (Roth et al. 2002). Salvinorin A was the first naturally occurring non-nitrogenous KOR agonist to be discovered, having high nanomolar affinity and selectivity for the KOR (Roth et al. 2002; Wang et al. 2005) with a balanced (unbiased) KOR agonist profile (White et al. 2015). Its pharmacology was extensively explored (see Butelman and Kreek 2015; Coffeen and Pellicer 2019 for reviews). Salvinorin A was found to be effective in inhibiting pain in animals (Ansonoff et al. 2006; McCurdy et al. 2006; Wang et al. 2005; White et al. 2015). However, it had aversive, sedative, anhedonia-like and locomotor-decreasing effects and depression-like behavior in rodents and non-human primates (Butelman and Kreek 2015; Fantegrossi et al. 2005; Kivell et al. 2018; White et al. 2015; Zhang et al. 2005). Salvinorin A has hallucinogenic effects in humans, producing depersonalization and derealization, cognitive alterations and neuroendocrine effects (Butelman and Kreek 2015; Ranganathan et al. 2012). Its effects have an extremely rapid onset (2 min), with a short duration of action (Butelman et al. 2009) attributed to the quick metabolism to the metabolite salvinorin B (Chavkin et al. 2004; Schmidt et al. 2005). Because of this poor pharmacokinetic profile and side effects, salvinorin A cannot be developed as a therapeutic agent. Research efforts have concentrated on structural modifications of the salvinorin A scaffold through synthetical methods resulting in new neoclerodane

diterpenes that have delivered significant SAR information (see Lovell et al. 2011; Prisinzano 2013; Roach and Shenvi 2018 for reviews). In Sect. 2.2, we introduce two synthetic salvinorin A derivatives and their pharmacological actions.

Another recently discovered natural KOR ligand is collybolide (Fig. 2), a non-nitrogenous sesquiterpene, first extracted from the fungus *Collybia maculata* (Bui et al. 1974). Collybolide shares a similar furyl-δ-lactone core to salvinorin A, and is a selective KOR agonist exhibiting dissimilar signaling properties (Gupta et al. 2016). In vitro, collybolide had a high affinity (K_i = 0.9 nM) to the human KOR and was a potent KOR partial agonist in the [^{35}S]GTPγS binding assay (EC_{50} of 2 nM and E_{max} of 124% of basal). Furthermore, collybolide was more potent in activating the mitogen-activated protein kinases (MAPK) than salvinorin A, while having a KOR biased agonistic activity. In mice, intraperitoneal (i.p.) injection of collybolide produced antinociception in the tail-flick assay and reduced chloroquine-induced itch. Collybolide was not sedative at a dose that produced antinociception, but it caused aversion in the conditioned place aversion (CPA) paradigm (Gupta et al. 2016).

2.2 Synthetic Ligands

The main classes of chemically distinct KOR agonists include benzomorphans, arylacetamides, neoclerodane diterpenes, morphinans, triazoles, diphenethylamines, and peptides (see Albert-Vartanian et al. 2016; Aldrich and McLaughlin 2012; Bohn and Aubé 2017; Beck et al. 2019a; Béguin and Cohen 2009; Bruchas and Roth 2016; Faouzi et al. 2020; Fürst and Hosztafi 2008; Mores et al. 2019; Prisinzano 2013; Spetea et al. 2013; Turnaturi et al. 2019 for reviews). Representative examples of therapeutics and potential therapeutics and research probes among KOR agonists are discussed. We also present chemical and pharmacological strategies used to reduce the side effects of KOR agonists, including the development of G protein-biased agonists and peripherally restricted KOR agonists.

The first KOR-preferring benzomorphan agonist, ketocyclazocine (after which the KOR type was named (Martin et al. 1976)) (Fig. 3) produces analgesia with marked sedation and ataxia in animals (Albert-Vartanian et al. 2016).

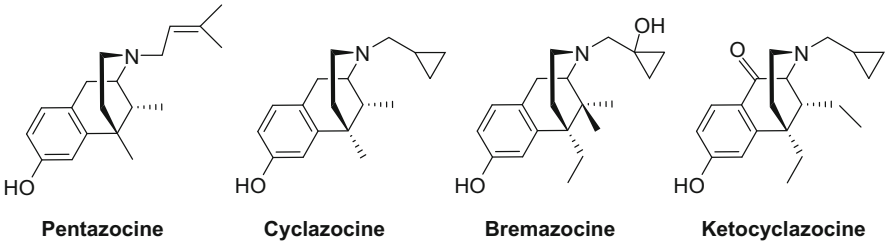


Fig. 3 Structures of KOR agonists from the class of benzomorphans, pentazocine, cyclazocine, bremazocine, and ketocyclazocine

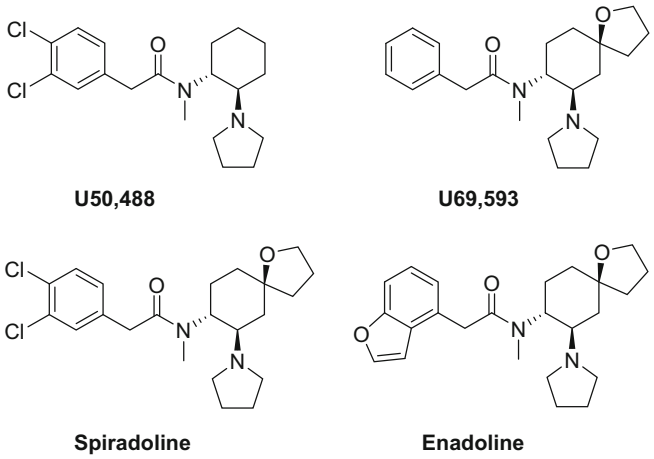


Fig. 4 Structures of KOR agonists from the class of arylacetamides, U50,488, U69,593, spiradoline and enadoline

Benzomorphans, such as pentazocine, cyclazocine, and bremazocine (Fig. 3), were studied for almost 50 years, and have mixed KOR agonist/MOR antagonist activities (see Turnaturi et al. 2018 for a review). Their clinical development was precluded because of psychotomimetic, dysphoric, and diuretic effects (Dortch-Carnes and Potter 2005). Only pentazocine, a relatively specific KOR partial agonist with low affinity and antagonism at MOR, is still used for obstetrical pain (Egede et al. 2017) and acute pancreatitis pain (Basurto et al. 2013), because of its minimal effects to produce respiratory depression (Fürst and Hosztafi 2008). It was thought that the undesirable effects of this class were attributable to their lack of KOR selectivity. However, recent studies showed that pentazocine was significantly more potent at activating p38 MAPK mediated by the human KOR than the rat KOR, and it was very potent in inducing β-arrestin2-dependent activation of extracellular regulated kinase (ERK1/2) (Schattauer et al. 2017).

The arylacetamides U50,488 (Von Voigtlander and Lewis 1982) and U69,593 (Lathi et al. 1985) (Fig. 4) were the first agonists to bind selectively to the KOR. U50,488 had analgesic, antipruritic, antitussive, diuretic, anti-arthritic, and anticonvulsant/anti-seizure effects in animals (Bileviciute-Ljungar et al. 2006; Morgenweck et al. 2015; Von Voigtlander and Lewis 1982; Wang et al. 2005; Zangrandi et al. 2016). While U69,593 produces antinociception, it has minimal effect on gastrointestinal motility (La Regina et al. 1988; White et al. 2015). U50,488 and U69,593 caused aversion, sedation, locomotor impairment, and anhedonia-like effects in animals (Bedini et al. 2020; Brust et al. 2016; Liu et al. 2019a; Morgenweck et al. 2015; Spetea et al. 2017; White et al. 2015). They are unbiased KOR agonists, acting as equipotent and fully efficacious agonists for both G protein and β-arrestin2 signaling pathways (see Mores et al. 2019 for a review). These selective KOR agonists are also valuable ligands in defining the pharmacology of KOR in vitro and in vivo. U50,488 was the first ligand used in vivo to establish that

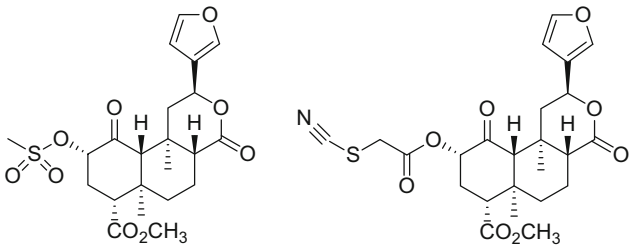


Fig. 5 Structures of KOR agonists from the class of neoclerodane diterpenes, mesyl Sal B and RB-64

KOR-induced aversion requires p38 activation, linked to β-arrestin2 recruitment (Bruchas et al. 2006). Recent phosphoproteomics and behavioral studies showed that phosphosites belonging to the mammalian target of rapamycin (mTOR) pathway were significantly regulated by U50,488, and inhibition of the mTOR abolished U50,488-induced aversion in the CPA test, without affecting analgesic, anti-scratch, and sedative effects, and motor incoordination, thus linking the mTOR transduction pathway to this undesirable side effect (Liu et al. 2018, 2019a).

Chemical work focused on modification of U50,488 and U69,593 led to spiradoline and enadoline (Fig. 4) (Barber and Gottschlich 1997). These compounds were orally active and able to cross the blood–brain barrier (BBB). Tested in humans, they produced pain relief comparable to morphine with less respiratory depression and constipation, but they caused increased urine output and dose-related CNS effects (i.e., dizziness, hallucination-like and dysphoria events, abnormal thinking), thus precluding their further development for clinical use (see Albert-Vartanian et al. 2016 for a review).

Developing salvinorin A analogues as potential therapeutics with improved pharmacokinetics and fewer side effects was highly desirable. Mesyl Sal B (salvinorin B mesylate) (Harding et al. 2005) and RB-64 (22-thiocyanatosalvinorin A) (Yan et al. 2009) (Fig. 5) are semi-synthetic derivatives of salvinorin A and G protein-biased KOR agonists (see Faouzi et al. 2020 for a review). Mesyl Sal B was not as potent as salvinorin A at reducing pain in the warm-water tail-withdrawal and formalin tests in mice after i.p. administration, but it reduced cocaine-induced hyperlocomotor activity and cocaine seeking behavior in rats (Kivell et al. 2018; Simonson et al. 2015). Mesyl Sal B did not produce aversion, anhedonia-like and anxiety-like behaviors, and did not cause motor incoordination, although it produced pro-depressive effects in rodents. RB-64 was identified as a G protein-biased KOR agonist in vitro, with full efficacy in both G protein activation and β-arrestin2 recruitment assays (White et al. 2014, 2015). Compared with unbiased agonists, RB-64 induced considerably less KOR internalization. In vivo, RB-64 was as effective as salvinorin A and U69,593 in eliciting antinociception in the hot-plate test in mice after subcutaneous (s.c.) administration with a longer lasting effect. Antinociceptive effects of RB-64, like the unbiased KOR agonists U69,593 and

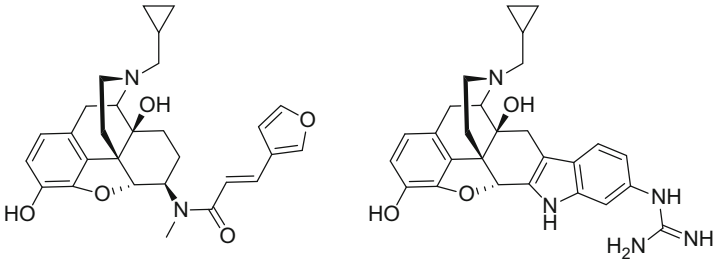
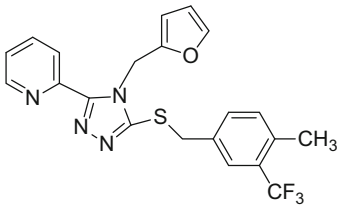


Fig. 6 Structures of KOR agonists from the class of morphinans, nalfurafine, and 6'-GNTI

salvinorin A, were retained in β-arrestin2-knockout (β-arrestin2-KO) mice (White et al. 2015). RB-64 had no effect on anhedonia and motor activity, while it still induced aversion in wild-type and β-arrestin2-KO mice.

Nalfurafine (TRK-820, Fig. 6) is an orally active, centrally penetrating KOR agonist (Nagase et al. 1998). It is the first and currently the only KOR agonist approved for clinical use as antipruritic drug (see Cowan et al. 2015; Shigeki 2015 for reviews). At the therapeutic doses, dysphoria was not reported and hallucinations occurred at a low rate (Kozono et al. 2018). Nalfurafine was initially developed as an analgesic for post-operative pain. Although it showed analgesic efficacy, the safety margin was found to be insufficient for clinical use (see Nagase and Fuji 2011 for a review). In rodents and non-human primates, nalfurafine produces antinociceptive, anti-scratch, sedative, and diuretic effects (Endoh et al. 1999, 2001; Liu et al. 2019a; Wang et al. 2005). Evidence of less analgesic tolerance and sedation in animals was reported for nalfurafine relative to other KOR agonists (Suzuki et al. 2004), and no evidence of rewarding or reinforcing effects or physical dependence (Nakao et al. 2016). These behavioral effects were absent in KOR-KO mice or were blocked by the KOR antagonist, nor-binaltorphimine (nor-BNI) (Inan et al. 2009; Liu et al. 2019a; Schattauer et al. 2017). In vitro, nalfurafine was reported as a G protein-biased KOR agonist with low potency for induction of p38 phosphorylation, as a measure for β-arrestin2 mediated signaling (Schattauer et al. 2017). This mechanism was hypothesized to account for the lack of aversive effects to nalfurafine in humans and minimal aversive responses in animals. Recently, phosphoproteomics investigated mechanisms underlying KOR-mediated effects of nalfurafine in mice, where a large-scale mass spectrometry-based analysis on mouse brains showed that nalfurafine perturbed phosphoproteomes differently from U50,488 in a brain-region specific manner (Liu et al. 2019a).

Another representative example of a KOR agonist from the class of morphinans is 6′-guanidinonaltrindole (6'-GNTI, Fig. 6) (Sharma et al. 2001). It was the first described G protein-biased KOR agonist (Rives et al. 2012), originally reported as a potent KOR partial agonist (Sharma et al. 2001). 6'-GNTI was shown to partially activate G protein-mediated signaling pathways without inducing β-arrestin2 recruitment in cellular systems and striatal neurons (Rives et al. 2012; Schmid et al. 2013).



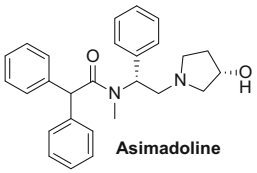
Triazole 1.1

Fig. 7 Structure of triazole 1.1

Consistent with its very low ability to recruit β-arrestin2, 6'-GNTI did not promote subsequent receptor internalization, it blocked U69,593-induced internalization of the KOR, and it induced the phosphorylation of ERK1/2. A recent study reported on 6'-GNTI anticonvulsant/anti-seizure efficacy in mice after central (intracisternal, i.c.) administration (Zangrandi et al. 2016). In the same study, 6'-GNTI was not significantly aversive in the CPA paradigm nor did it cause locomotor impairment. It produced analgesia only when administered intrathecally (i.t.), but not intracerebroventricularly (i.c.v.) (Waldhoer et al. 2005). The high hydrophilic character of the molecule and its reduced ability to enter the CNS limited 6'-GNTI suitability for clinical development.

Triazole 1.1 (Fig. 7) is the outcome of a high-throughput screening campaign utilizing PathHunter KOR β-arrestin2 cells to identify novel KOR agonists and antagonists (Frankowski et al. 2012). This compound was found to be preferentially biased toward G protein signaling with minimal effects on β-arrestin2 recruitment and downstream ERK1/2 activation, as well as to be brain penetrant after systemic application, and capable of inducing antinociception and anti-scratching effects in mice (see Bohn and Aubé 2017 for a review). A more detailed analysis of behavioral effects of triazole 1.1 revealed that this biased KOR agonist did not reduce ambulatory locomotion at antinociceptive doses or reduce dopamine release in the nucleus accumbens, which could indicate that triazole 1.1 will not produce dysphoria/aversion (Brust et al. 2016).

Several derivatives of dynorphin A were examined for analgesic activity following systemic administration (see Aldrich and McLaughlin 2012; Carroll and Carlezon Jr 2013; Hall et al. 2016; Hauser et al. 2001; Hruby and Agnes 1999; Schiller 1991 for reviews). Dynorphin A(1–8) analog E-2078 (MeTyr-Gyl-Gly-Phe-Leu-*N*MeArg-LeuNHEt) was extensively studied in rodents (Nakazawa et al. 1991; Salas et al. 1992), non-human primates (Butelman et al. 1999), and humans (Fujimoto and Momose 1995). After intramuscular administration in humans, E-2078 exhibited analgesic activity comparable to pentazocine. While this peptide crossed the BBB in monkeys, it produced some of the effects exhibited by non-peptide KOR agonists (Butelman et al. 1999). Peptide ligands unrelated to dynorphin A that display agonist activity at the KOR were designed, including the recently reported cyclic pentapeptide LOR17 (c[Phe-Gyl-Gly-(β-Ala)-D-Trp]) (De Marco et al. 2016). LOR17 was reported as a G protein-biased KOR agonist (Bedini et al. 2020) that inhibited adenylyl cyclase and activated early-phase ERK1/2 phosphorylation. Conversely to the unbiased U50,488, LOR17 neither induced p38 MAPK phosphorylation nor increased KOR-dependent, p38 MAPK-mediated



CR665 D-Phe-D-Phe-D-Nle-D-Arg-NH-4-Picolyl
CR845 D-Phe-D-Phe-D-Leu-D-Lys-[γ-(4-N-piperidinyl)amino carboxylic acid
JT09 D-Phe-D-Phe-D-Nle-D-Lys-4-Picolyl

Fig. 8 Examples of peripherally restricted KOR agonists, small molecules (asimadoline), and peptides (CR665, CR845 and JT09)

cell proliferation in astrocytes. In vivo, while this cyclic peptide was effective in inhibiting pain after s.c. administration in mice, it did not alter motor coordination, locomotor and exploratory activities nor induced pro-depressant-like behavior (Bedini et al. 2020).

The KOR is expressed in peripheral sensory neurons (Ji et al. 1995; Snyder et al. 2018; Stein et al. 1989) and activation of peripheral KORs reduces response to painful stimuli and suppresses neurological inflammation (Albert-Vartanian et al. 2016; Jamshidi et al. 2015; Snyder et al. 2018; Stein 2016). Targeting peripheral KORs is seen as a promising strategy to avoid CNS-mediated side effects (see Albert-Vartanian et al. 2016; DeHaven-Hudkins and Dolle 2004; Kivell and Prisinzano 2010 for reviews). The goal of achieving analgesia while avoiding CNS penetration has focused on both small molecules and peptides. An example of such a peripherally acting KOR agonist is asimadoline (EMD 61753; Fig. 8) (Barber et al. 1994). Asimadoline produced antinociception in a variety of inflammatory pain models and anti-itch effects, both attributed to actions on KORs on the endings of sensory nerve fibers in rodents. It was inactive in the absence of inflammation and its activity was minimal in models of central activation. Asimadoline showed high plasma protein binding, which also helped to decrease passage into the brain (see Cowan et al. 2015; DeHaven-Hudkins and Dolle 2004 for reviews). Asimadoline was the first peripherally selective KOR agonists to enter clinical trials with hopes that it might treat peripheral pain. Oral administration to patients who underwent arthroscopic knee surgery caused a hyperalgesic response and diuresis at analgesic doses. Unfortunately, asimadoline did not achieve clinically relevant efficacy at doses that lacked CNS adverse effects (Vanderah et al. 2008).

More recent peripherally restricted KOR agonists include peptides CR665 (FE-200665) (Binder et al. 2001), CR845 (difelikefalin) (Cara Therapeutics 2020), and JT09 (Beck et al. 2019b) (Fig. 8). They contain D-amino acids (Beck et al. 2019b; Wisniewski et al. 2000) and have higher peripheral selectivity than asimadoline (Vanderah et al. 2008). They have high potency and efficacy in experimental pain models (see Albert-Vartanian et al. 2016; Aldrich and McLaughlin 2012; DeHaven-Hudkins and Dolle 2004 for reviews). CR665 was active in animal models after s.c., intraplantar (i.pl. in the inflamed paw), and i.t. administration (Binder et al. 2001). Clinical studies with CR665 after intravenous (i.v.) administration established CR665 to have comparable analgesic efficacy to oxycodone against visceral pain, but a paradoxical hyperalgesia action on skin-pinch pain (Arendt-Nielsen et al. 2009). There were few side effects with CR655, including an increase in urine output, pruritus, and facial paresthesia. Because CR665 was not orally active

and induced hyperalgesia, its clinical development was discontinued (Albert-Vartanian et al. 2016).

CR845 (Fig. 8) is a peripherally specific, highly selective KOR agonist. It produced antinociception in animals after systemic (s.c., i.p.) administration, and inhibited scratching behavior induced in mice by pruritogens, without affecting gastrointestinal motility (see Albert-Vartanian et al. 2016; Cowan et al. 2015 for reviews). CR845 demonstrated good oral efficacy in rat models of visceral pain, but not in the hot-plate test (53°C) as a model of nociceptive pain (Hughes Jr et al. 2013). It has shown antipruritic and analgesic properties, along with being safe and well tolerated in several clinical studies for pruritus (i.v. formulation for uremic pruritus in hemodialysis patients) and acute post-operative pain (i.v. formulation in laparoscopic hysterectomy, ventral hernia, and bunionectomy) (Cara Therapeutics 2020; Fishbane et al. 2020). An oral formulation of CR845 is being evaluated in a Phase 2 study in chronic kidney disease-associated pruritus Stage 3–5 patients, as well as chronic liver disease-associated pruritus in patients with primary biliary cholangitis and patients with atopic dermatitis. Additionally, studies for abuse liability in humans have demonstrated that CR845 is unlikely to be recreationally abused or lead to physical dependence. In a human abuse liability trial, i.v. CR845 demonstrated statistically significant reductions in "drug liking," "feeling high," "overall liking," and "take drug again" scores in comparison with i.v. pentazocine (Cara Therapeutics 2020).

JT09 (Fig. 8) is a peptide analogue of CR665 (Beck et al. 2019b), reported as an orally active peptide, peripherally acting KOR agonist and as efficacious as morphine in alleviating peripheral pain, with less propensity for CNS-mediated side effects in animals (see Beck and Dix 2019 for a review).

Initially, KOR antagonists were used as pharmacological tools for studying the in vitro and in vivo actions of KOR activation. The fact that the endogenous kappa opioid system influences behaviors that reflect motivational and emotional states in animal models of depression, anxiety, and addiction (Cahill et al. 2014; Crowley and Kash 2015; Darcq and Kieffer 2018; Lalanne et al. 2014) has drawn the interest in developing selective KOR antagonists as potential pharmacotherapies for treating mood and addictive disorders (see Aldrich and McLaughlin 2012; Béguin and Cohen 2009; Carlezon Jr and Krystal 2016; Carroll and Carlezon Jr 2013; Helal et al. 2017; Jacobson et al. 2020; Urbano et al. 2014 for reviews). Representative examples of therapeutics and potential therapeutics and research tools among KOR antagonists, small molecules, and peptides are discussed.

Nor-BNI (Fig. 9) was the first potent and selective KOR antagonist design based on the "message-address" concept (Portoghese et al. 1987). Portoghese et al. suggested that the *N*-17′ of nor-BNI mimics Arg^{7} of dynorphin which may be responsible for KOR selectivity. Using a 3D representation of nor-BNI, they designed a series of compounds that contain the selective DOR antagonist naltrindole on which they attached substituents that can be protonated at physiological pH. The most promising compound in this series was 5′-guanidinonaltrindole (5'-GNTI, Fig. 10), which was found to be more selective and more potent as KOR antagonist than nor-BNI (see Béguin and Cohen 2009 for a review). Molecular docking studies based on the inactive crystal structure of the KOR have revealed that the *N*-17 of 5'-GNTI interacts with Asp^{138} and the *N*-17′ confers selectivity for the KOR by ionic interaction with

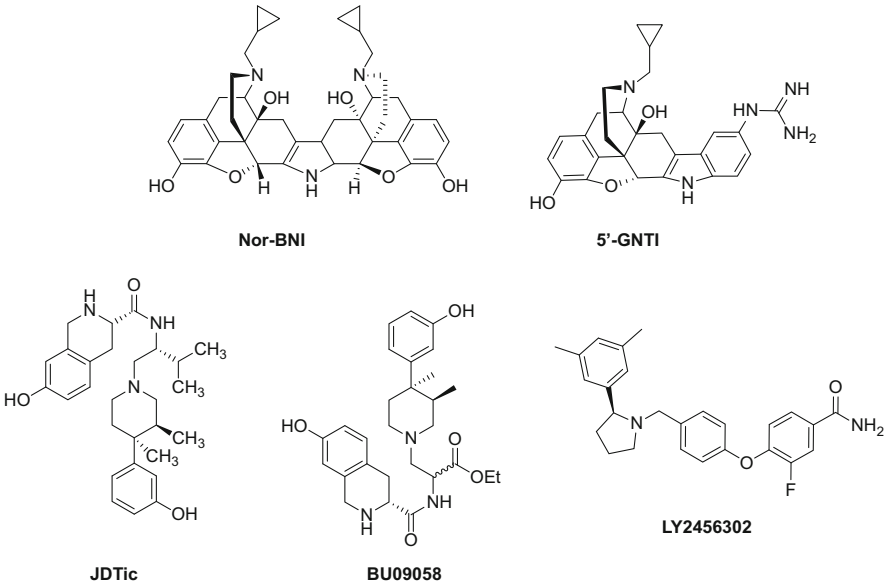


Fig. 9 Representative long-acting (nor-BNI, 5'-GNTI, and JDTic) and short-acting (BU09058 and LY2456302) KOR antagonists

Glu^{297} located near the extracellular end of the sixth transmembrane domain (Wu et al. 2012). JDTic (Fig. 9) is structurally different from nor-BNI and 5'-GNTI (Thomas et al. 2001), and was used for the elucidation of the crystal structure of the inactive conformation of the KOR (Wu et al. 2012), and structural analysis of conformational changes related to KOR activation and inactivation (Che et al. 2020). JDTic was the first KOR antagonist tested in humans. During Phase 1 human clinical trials for the treatment of cocaine abuse, development of JDTic was terminated because of adverse effects and specifically non-sustained ventricular tachycardia. Additionally, JDTic showed an unfavorable brain-to-plasma concentration ratio, indicating poor CNS penetration (Bailey and Husbands 2018; Chavkin and Martinez 2015).

The three KOR antagonists, nor-BNI, 5'-GNTI, and JDTic, share an unusual pharmacokinetic property in that there is a slow onset of antagonist activity (typically peaking at 24 h) and an exceedingly long duration of action in vivo (weeks after a single administration) (Carroll et al. 2004; Endoh et al. 1992; Negus et al. 2002). Behavioral studies showed that these KOR antagonists produce antidepressant- and anxiolytic-like effects in animals, as well as their possible application in the treatment of drug addiction (see Carroll and Carlezon Jr 2013; Carlezon Jr and Krystal 2016; Urbano et al. 2014 for reviews).

Concerns about the feasibility of developing KOR antagonists for the clinics have centered around the long duration of action and CNS penetration, leading to the development of a number of short-acting KOR antagonists, including BU09059 and LY2456302 (Fig. 9). BU09059 is a structural analogue of JDTic (Casal-Dominguez et al. 2014). In addition to being short-acting, BU09059 is more selective for the

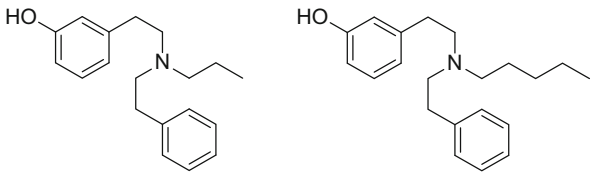
KOR than JDTic. The pyrrolidine derivative LY2456302 (also referred to as CERC-501, JNJ-67953964, or Aticaprant) (Rorick-Kehn et al. 2014) is a recently developed short-acting KOR antagonist for the treatment of depression and substance use disorder (see Browne and Lucki 2019 for a review). It was proposed that ligand-directed signaling might account for the duration of activity of KOR antagonists, which was demonstrated to correlate with activation of c-Jun N-terminal kinase (JNK) (Jamshidi et al. 2016; Melief et al. 2011). The long-acting KOR antagonists, such as nor-BNI and JDTic, activate JNK, whereas shorter acting KOR antagonists do not. Additionally, there were differences in BBB permeability and bioavailability, with JDTic having poor brain penetration, and short-acting antagonists such as LY24563021 showing relatively rapid absorption (Naganawa et al. 2016). Irrespective of the duration of activity, the long-lasting blockade of the KOR was not necessary to block stress-induced or pro-depressant response.

Early attempts to design selective KOR antagonists by modification of dynorphin A had limited success with analogues exhibiting weak antagonist activity, residual agonist activity, and/or low selectivity for KOR vs. MOR, but in recent years antagonist analogues with improved pharmacological profiles were reported (see Aldrich and McLaughlin 2012; Carroll and Carlezon Jr 2013; Remesic et al. 2016 for reviews). Arodyn (Ac-Phe-Phe-Phe-Arg-Leu-Arg-Arg-ala-Arg-Pro-LysNH_2) (Bennett et al. 2002), a linear analogue of dynorphin A, is a selective KOR antagonist with relatively long activity in vivo (days) (Carey et al. 2007). The cyclic dynorphin A analogue, zyklophin (*N*-benzylTyr^1,*cyclo*(D-Asp^5,Dap^8)-dynorphin A-(1–11)NH_2) (Patkar et al. 2005) is a selective KOR antagonist with short duration of action (Aldrich et al. 2009). Zyklophin exhibited enhanced metabolic stability compared to arodyn and antagonized central and peripheral KORs after s.c. administration. It induced scratching behavior when injected s.c. to mice (DiMattio et al. 2014). Both arodyn and zyklophin blocked drug-seeking behavior in a model of stress-induced reinstatement of cocaine abuse, suggesting that such KOR peptide antagonists could have potential therapeutic applications in the treatment of drug abuse.

The KOR is among the few GPCRs of which the crystal structures were determined both in inactive (Wu et al. 2012) and active conformations (Che et al. 2018). The active KOR structure gives valuable hints for the structural basis of biased agonism (Che et al. 2018) and allosteric modulation (Che et al. 2020). Moreover, the structures of the KOR have provided an unprecedented opportunity for computational drug discovery. Structure-based virtual screening enabled discovery of potent KOR agonists (Negri et al. 2013), KOR antagonists and new G protein-biased agonist scaffolds (White et al. 2014; Zheng et al. 2017). Future drug development is expected to significantly benefit from the KOR structures and availability of modern computational methods, with references to recent reviews (Ferré et al. 2019; Filizola 2019; Manglik 2020).

2.3 Diphenethylamines, a New Class of Selective KOR Ligands

The synthesis of the first diphenethylamine derivative, RU 24213 (**1**) (Fig. 10), was reported in 1978, originally developed as a potential anti-Parkinson's drug (Nedelec et al. 1978). RU 24213 (**1**) was described as a selective dopamine D_2 receptor agonist



1 (RU 24213)
KOR affinity IC_{50} = 57 nM
KOR selectivity vs. MOR = 25
KOR antagonist

2
KOR affinity IC_{50} = 57 nM
KOR selectivity vs. MOR = 15
KOR antagonist

Fig. 10 Structures of diphenethylamines RU 24213 (**1**) and **2** and their KOR activities. (Data from Cosquer et al. 1992 and Fortin et al. 1991)

(Euvrard et al. 1980), also found to bind with moderate affinity and selectivity to KOR and to have a KOR antagonist activity in vitro (Fortin et al. 1991). The *n*-pentyl analogue **2** (Fig. 10) exhibited moderate affinity to the KOR, and showed KOR antagonist activity in vivo by antagonizing U50,488-induced antinociception and diuresis in rats after s.c. administration (Cosquer et al. 1992). These simple structures were used as leads for the design of small molecules targeting the KOR and featuring a diphenethylamine scaffold. In this section, we discuss their SARs and in vitro and in vivo pharmacological activities.

2.3.1 Agonists and Partial Agonists

The first strategy for designing new diphenethylamine derivatives involved extension of the *n*-alkyl substituent at the nitrogen, with replacement of *n*-propyl group in RU 24213 (**1**) by an *n*-butyl group (**3**), and *n*-pentyl group in **2** by an *n*-hexyl group (**4**) (Fig. 11) (Spetea et al. 2012). In vitro binding studies demonstrated that replacing the *n*-propyl group by an *n*-butyl substituent at the nitrogen (**1** vs. **3**) resulted in similar KOR affinity and potency, but lower KOR selectivity for **3** to the human KOR expressed in Chinese hamster ovary cells (CHO-hKOR) (Table 1). Extension of the N-substituent in **2** by one methylene group resulting in the *n*-hexyl analogue **4** further decreased KOR affinity and selectivity. Diphenethylamines RU 24213 (**1**) and **2** were reported previously to have moderate affinity and selectivity to the KOR in the rat brain (Cosquer et al. 1992; Fortin et al. 1991), while a higher affinity was found to the human KOR in CHO cells (Table 1) (Spetea et al. 2012). Earlier in vitro biochemical studies measuring [^{3}H]diprenorphine binding to rat brain membranes in the presence and absence of NaCl and guanosine triphosphate (GTP) established diphenethylamines RU 24213 (**1**) and **2** as KOR antagonists, based on the decrease in ligand affinity to the receptor in the presence of NaCl/GTP (Cosquer et al. 1992; Fortin et al. 1991). In the [^{35}S]GTPγS binding assay using CHO-hKOR cells, compounds **1** and **2** had moderate potency and low efficacy, being KOR partial agonists (Spetea et al. 2012). Extension of the *n*-alkyl substituent at the nitrogen in diphenethylamines **1** and **2** retained KOR partial agonism with moderate potency of derivatives **3** and **4**, respectively (Table 1).

	R^1	R^2	R^3	R^4	R^5
3	*n*-butyl	H	OH	H	H
4	*n*-hexyl	H	OH	H	H
5 (HS665)	CBM	H	OH	H	H
6 (HS666)	CPM	H	OH	H	H
7	CPeM	H	OH	H	H
8	CHM	H	OH	H	H
9	Bn	H	OH	H	H
10	CBM	H	OH	OH	H
11	CPM	H	OH	OH	H
12	CPeM	H	OH	OH	H
13	CHM	H	OH	OH	H
14	CBM	H	OH	H	OH
15	CHM	H	OH	H	OH
16	CBM	F	OH	H	H
17	CHM	F	OH	H	H
18	CBM	F	OH	OH	H
19	CBM	F	OH	H	OH

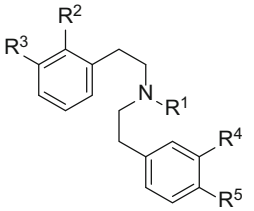


Fig. 11 Design strategy of new diphenethylamines (**3**–**19**), as KOR full or partial agonists. Bn, benzyl; CBM, cyclobutylmethyl; CHM, cyclohexylmethyl; CPeM, cyclopentylmethyl; CPM, cyclopropylmethyl

The next design strategy of new diphenethylamine derivatives examined the character of the N-substituent (alkyl vs. cycloalkylmethyl and arylakyl) on KOR activities and emerged SAR (Fig. 11) (Erli et al. 2017; Schmidhammer et al. 2019; Spetea et al. 2012). An *N*-cyclopropylmethyl (*N*-CPM in derivative **6**, HS666) and particularly an *N*-cyclobutylmethyl (*N*-CBM in derivative **5**, HS665) substitution was more favorable for the interaction with the KOR than *n*-alkyl groups in analogues **1**–**4** by increasing KOR affinity, selectivity, agonist potency, and efficacy, with HS665 (**5**) being a KOR full agonist (Table 1) (Spetea et al. 2012). Besides the high affinity and selectivity of HS665 (**5**) to the human KOR stably expressed in CHO cells, direct in vitro binding studies with the tritium-labeled HS665 ([^{3}H] HS665) established its high selectivity to the native neuronal KOR in the guinea-pig brain (Guerrieri et al. 2015). Moreover, [^{3}H]HS665 was employed as a research tool to establish in vitro opioid activity profile of new ligands (Dumitrascuta et al. 2017; Erdei et al. 2018; Martin et al. 2018; Szűcs et al. 2020).

In silico studies using the crystal structure of the inactive human KOR) (Wu et al. 2012) and an active-like structure of the KOR attained by molecular dynamics (MD) simulations explored binding modes of the 3-monohydroxy substituted diphenethylamines, **1**–**6** at the KOR (Guerrieri et al. 2016). The size of the N-substituent hosted by the hydrophobic pocket formed by the residues Val^{108}, Ile^{316}, and Tyr^{320} influenced ligand binding to the KOR. The *N*-CBM (in **5**) and *N*-CPM (in **6**) groups had the optimal size. As to the linear substituent at the nitrogen, increasing the chain length from *n*-propyl to *n*-hexyl caused a decrease in KOR affinity (Table 1). Whereas KOR affinities of the *n*-propyl, *n*-butyl, and *n*-pentyl N-substituted derivatives were in the same range, the presence of the *n*-hexyl chain at the nitrogen in compound **4** produced a larger decrease in KOR affinity, signifying that five carbon atoms in linear alkyl substituents was the critical size for

Table 1 Binding affinities and agonist activities to the KOR of diphenethylamines **1**–**19** (Data from Erli et al. 2017; Spetea et al. 2012)

	KOR affinity[a]	KOR selectivity[a]		KOR activity[b]	
	K_i (nM)	MOR/KOR	DOR/KOR	EC_{50} (nM)	% stim.
1	8.13	73	457	49.1	21.2
2	12.6	26	104	86.4	36.2
3	10.9	38	223	46.2	45.5
4	141	5.6	25	647	24.0
5 (HS665)	0.49	1,106	>20,000	3.62	90.0
6 (HS666)	5.90	140	>1,700	35.0	53.4
7	0.017	16,118	133,471	3.87	82.8
8	0.061	8,803	35,066	0.23	61.9
9	0.71	652	2,623	4.65	79.5
10	0.38	605	8,789	4.44	71.1
11	4.62	137	617	20.6	51.3
12	0.31	1,884	8,952	13.7	80.4
13	0.14	1,193	10,229	17.6	91.1
14	3.43	5	125	22.2	76.4
15	1.85	126	885	22.2	84.3
16	0.072	5,529	>138,000	6.90	66.1
17	0.040	21,275	>250,000	2.77	88.9
18	0.12	4,642	>83,000	1.49	57.5
19	3.37	155	389	36.7	69.1

[a]Determined in competition binding assays using membranes of CHO cells stably expressing the human opioid receptors

[b]Determined in the [^{35}S]GTPγS binding assay using membranes of CHO-hKOR cells. For compound structures, refer to Figs. 10 and 11

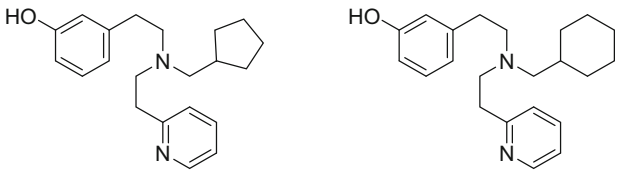
interaction with KOR (Guerrieri et al. 2016). Moreover, the hydrogen bond formed by the phenolic 3-OH group of *N*-CBM substituted HS665 (**5**) with His^{291} was essential for binding affinity and agonist activity to the KOR. Docking of diphenethylamine HS665 (**5**) to the human KOR also revealed similar binding orientation and ligand–receptor interactions to the endogenous KOR agonist dynorphin (first four amino acids, Tyr-Gly-Gly-Phe) (Guerrieri et al. 2016).

Interesting SAR observations were made in the 3-monohydroxy diphenethylamines series (compounds **5**–**9**, Fig. 11), where the presence of more bulkier N-substituents, such as *N*-cyclopentylmethyl (*N*-CPeM, in **7**) and *N*-cyclohexylmethyl (*N*-CHM, in **8**), led to the largest increase in KOR affinity in the picomolar range and excellent KOR selectivity, that was much enhanced when compared to *N*-CBM substituted HS665 (**5**) and *N*-CPM substituted HS666 (**6**) (Table 1) (Erli et al. 2017). Introduction of an *N*-benzyl group also resulted in very high KOR affinity and selectivity of diphenethylamine **9**, although less prominent than *N*-CBM (**5**), *N*-CPeM (**7**), and *N*-CHM (**8**) substitutions. In the [^{35S}]GTPγS binding assay using CHO-hKOR cell membranes, derivatives **5** and **7** were potent

and full agonists to the KOR, while analogue **8** had a profile of a potent KOR partial agonist (Table 1).

Within the 3,3′-dihydroxy diphenethylamines series (compounds **10–13**, Fig. 11), an *N*-CBM (**10**), *N*-CPeM (**12**), and *N*-CHM (**13**) substitution afforded derivatives with very high KOR affinity and selectivity (Table 1) (Erli et al. 2017). Introduction of an additional hydroxyl group at position 3′ into *N*-CBM substituted HS665 (**5**) did not affect KOR affinity, selectivity, and potency of the resulting in 3,3′-dihydroxy substituted analogue **10**, but changed the full agonist profile of derivative **5** into a potent KOR partial agonist **10**. Similar observation on the lack of alterations in KOR activity was made for the 3-hydroxy, *N*-CPM substituted HS666 (**6**) and its 3,3′-dihydroxy analogue **11**, with no change in KOR partial agonism. However, the 3,3′-dihydroxy, *N*-CHM substituted **13** showed lower KOR affinity, selectivity, and potency than its analogue **8**, as well as a shift from a partial agonist of **8** to a full agonist activity of **13**. A larger decrease in KOR affinity, selectivity, and potency was shown for 3,3′-dihydroxy, *N*-CPeM derivative **12** when compared to its analogue **7**, with both diphenethylamines being KOR full agonists (Table 1) (Erli et al. 2017). Additional SAR studies reported on the consequence of shifting the 3-′-hydroxyl group to position 4′ with a decrease in both binding affinity and selectivity to the KOR, specifically for *N*-CBM substituted **14** and *N*-CHM substituted **15** when compared to their 3,3′-dihydroxy analogues **10** and **13,** respectively (Table 1). Regarding functional activities to the KOR, the 3'-OH to 4'-OH switch did not change KOR partial agonist activity (**10** vs. **14**) or full agonist activity (**13** vs. **15**) (Table 1).

The inclusion of fluorine in drug candidates is nowadays driven by a deeper understanding of this element and its effects on biological activity. The strong electronegativity, small size, and large dipole moment of a C-F bond, all subtend versatility in drug design (Johnson et al. 2020). To examine the influence of the presence of fluorine in position 2 in diphenethylamines on KOR activity and physicochemical properties, different 2-fluoro substituted derivatives were designed (compounds **16–19**, Fig. 11) (Erli et al. 2017). The *N*-CBM (**16**) and *N*-CHM (**17**) analogues with a single 3-hydroxyl group showed very high KOR affinities in the picomolar range and an extraordinary KOR selectivity. Compound **17** was the most selective KOR ligand in the series, and a very potent full agonist (Table 1) (Erli et al. 2017). It was demonstrated that in the case of an *N*-CBM substitution, introduction of a 2-fluoro substituent (**16**) enhanced both KOR affinity and selectivity remarkably in comparison with its counterpart HS665 (**5**), but converted a full agonist (**5**) into a potent KOR partial agonist (**16**). A substantial increase in KOR selectivity was also established for the 2-fluorinated *N*-CHM substituted **17** compared to analogue **8**, with both displaying very good KOR affinity (in the picomolar range), with **17** also being a potent full agonist. Introduction of a fluoro substituent in position 2 into the 3,3′-dihydroxy *N*-CBM derivative **10** enhanced KOR affinity and potency, paralleled by a considerable increase in KOR selectivity for **18**, without modifying KOR partial agonist activity (Table 1). While the 3,4′-dihydroxy *N*-CBM derivative **19** showed similar KOR affinity to **14**, the presence of a 2-fluoro substituent in **19** increased KOR selectivity, and left KOR partial agonist profile unchanged. Based on



20
EC_{50} = 8.2 nM, E_{max} = 86.7%

21
EC_{50} = 11.8 nM, E_{max} = 81.6%

Fig. 12 Structurally related diphenethylamines **20** and **21** and their KOR agonist activities to the human KOR in the [^{35}S]GTPγS binding assay. (Data from Dunn et al. 2019)

the calculated partition coefficients (*c*logP) and distribution coefficients at pH 7.4 (*c*log$D_{7.4}$) (MarvinSketch 17.10, Chem Axon), fluorinated compounds **16**, **17**, **18**, and **19** had similar values to analogues HS665 (**5**), **8**, **10**, and **14,** respectively and a good capability to enter the CNS (Erli et al. 2017). Thus, a fluorine substitution in position 2 in this class of diphenethylamines was very advantageous concerning the KOR activity profile in vitro.

The *N*-CPeM substituted **20** and *N*-CHM substituted **21** (Fig. 12) (Dunn et al. 2019) are structurally related to diphenethylamines **1**–**19** (Figs. 10 and 11), but their KOR binding affinities and selectivities were not reported. Both compounds **20** and **21** were described as KOR agonists in the [^{35}S]GTPγS binding assay using membranes of U2OS cells stably expressing the human KOR (Dunn et al. 2019), although being less potent agonists than analogues **7** and **8**, respectively (Table 1).

2.3.2 Biased Agonists

In vitro functional activity studies on reported diphenethylamine KOR agonists, **3**, **5**–**7**, **20,** and **21**, as G protein-biased KOR agonists with variable degree of bias (Table 2) (Dunn et al. 2018, 2019; Spetea et al. 2017). These studies compared ligand potency and efficacy across two functional assays measuring G protein coupling (the [^{35}S]GTPγS binding assay) and β-arrestin2 recruitment (PathHunter β-arrestin2 assay) to the human KOR (Table 2). The *N*-*n*-butyl substituted **3** was a KOR agonist in the G protein activation assay, with no inducible stimulation of β-arrestin2 signaling. Diphenethylamines HS665 (**5**) and **7** and structurally related **20** and **21** showed weak partial agonism for the β-arrestin2 recruitment, while they were very potent and fully efficacious in promoting KOR-dependent G protein activation. The *N*-CPM substituted derivative HS666 (**6**) was a KOR partial agonist in the G protein coupling assay with very little to no measurable β-arrestin2 recruitment (Dunn et al. 2019; Spetea et al. 2017).

2.3.3 Antagonists

Within the series of diphenethylamines, several molecules with KOR antagonism were reported (Erli et al. 2017; Guerrieri et al. 2016). The first diphenethylamine displaying KOR antagonism was the *N*-phenylethyl substituted **22** (Fig. 13) (Guerrieri et al. 2016). This derivative exhibited no appreciable agonist activity to

Table 2 Ligand bias comparing G protein and β-arrestin2 signaling to the KOR of diphenethylamines **3**, **5–7**, **20** and **21**

	G protein activation[a]		β-arrestin2 recruitment[b]			
	EC_{50} (nM)	E_{max} (%)	EC_{50} (nM)	E_{max} (%)	ΔLogRA (Bias factor)[c]	Reference
3	14 14.3	94 75.3	–[d] –[d]		1.8 (59) 1.2	e, f
5 (HS665)	4.98 1.8 1.5	88 110 84.9	463 380 676.2	55 30 49.4	– (389) 1.6 (42) 0.6	e, f, g
6 (HS666)	35.7 22.7	50 75.3	449 –[d]	24	– (62) 0.2	f, g
7	0.64 0.5	100 79.0	720 261.9	55 72.6	1.3 (18) 0.2	e, f
20	8.2	86.7	3,956	61.2	0.4	f
21	11.8	81.6	2,082	57.7	0.1	f

[a]Determined in the [^{35}S]GTPγS binding assay to the human KOR
[b]Determined in the PathHunter β-arrestin2 recruitment assay to the human KOR
[c]Positive ΔLogRA values indicate bias for G protein over β-arrestin-2 signaling
[d]Denotes no stimulation/no fit. For compound structures, refer to Figs. 11 and 12
[e]Dunn et al. (2018)
[f]Dunn et al. (2019)
[g]Spetea et al. (2017)

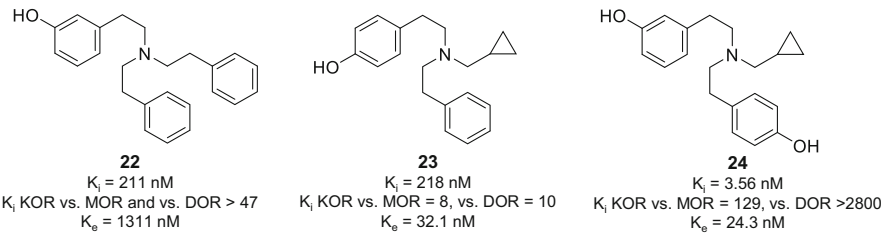


Fig. 13 Structures of diphenethylamines **22**–**24** as KOR antagonists, and their in vitro antagonist activity to the human KOR determined in the [^{35}S]GTPγS binding assay. (Data from Erli et al. 2017 and Guerrieri et al. 2016)

the KOR in vitro, and antagonized U69,593-induced [^{35}S]GTPγS binding with relatively low potency. Radioligand binding studies with CHO cells expressing the human KOR demonstrated that the introduction of a phenylethyl group at the nitrogen (**22**) also led to a reduction in KOR affinity when compared to other diphenethylamines, with completely loss of binding to MOR and DOR (Table 1).

An interesting SAR observation was made by shifting the 3-hydroxyl group in *N*-CPM substituted **6** to position 4 in analogue **23** by converting a KOR partial agonist HS666 (**6**) into a KOR antagonist (**23**) (Fig. 13), that had also higher KOR antagonist potency than **22** in vitro. The in vitro pharmacological profiles of diphenethylamines

22 and **23** were supported by molecular docking studies to the structure of the human KOR (Guerrieri et al. 2016). The *N*-phenylethyl group in **22** was relatively bulky to be hosted by the hydrophobic pocket formed by the residues Val^{108}, Ile^{316}, and Tyr^{320}, which resulted in a different orientation of the phenolic moiety compared to the full agonist HS665 (**5**), and making this compound a weak KOR antagonist. Diphenethylamine **23** having a phenolic 4-hydroxy group did not form the hydrogen bond with His^{291}, an important residue for KOR affinity and agonist activity (Guerrieri et al. 2016). Notable was the observation on the alteration of the functional activity in vitro of HS666 (**6**) from a KOR partial agonist to an antagonist **24** upon introduction of an additional hydroxyl group in position 4′ (Table 1 and Fig. 13). Another remarkable finding was that an additional hydroxyl group at position 3′ into *N*-CPM substituted **23** retained the high KOR antagonist potency in vitro, but also increased KOR affinity and selectivity of analogue **24** (Fig. 13) (Erli et al. 2017; Guerrieri et al. 2016). The KOR antagonist activity of 3,4′-dihydroxy *N*-CPM derivative **24** was also demonstrated in vivo, as this compound blocked U50,488-induced antinociception in the acetic acid-induced writhing assay in mice after s.c. administration (Erli et al. 2017).

2.3.4 In Vivo Pharmacology

In vivo pharmacological studies on the diphenethylamine class of KOR full/partial agonists have established them as effective antinociceptives with reduced liability for KOR-mediated adverse effects (Erli et al. 2017; Spetea et al. 2017). In a mouse model of acute thermal nociception, the 55°C warm-water tail-withdrawal assay, the KOR full agonist HS665 (**5**) and the partial agonist HS666 (**6**) administered centrally (i.c.v.) produced effective antinociception in C57BL/6J wild-type mice, that were absent in KOR-KO mice (Spetea et al. 2017). The onset of the antinociceptive response was rapid after i.c.v. administration. In the tail-withdrawal assay, HS665 (**5**) was two-fold more potent than U50,488, whereas HS666 (**6**) had similar antinociceptive potency to U50,488, and was only slightly less (< two-fold) potent in inducing antinociception than HS665 (**5**) (Table 3). Both HS665 (**5**) and HS666 (**6**) showed antinociceptive efficacy after s.c. administration, by inhibiting the acetic acid-induced writhing response in CD1 mice, which was blocked by the KOR antagonist nor-BNI (Erli et al. 2017; Spetea et al. 2012). In this mouse model of visceral pain, HS665 (**5**) had a potency equivalent to that of U50,488, while HS666 (**6**) was two-fold less active (Table 3).

Significant inhibition of the writhing response with a KOR-specific effect was demonstrated for other diphenethylamines **7**–**19** after s.c. administration in CD1 mice (Table 3) (Erli et al. 2017). In the writhing assay, both full and partial agonists effectively produced antinociception, with interesting SAR outcomes. The *N*-CPeM substituted **7** was the most active in inducing an antinociceptive effect, with increased potency than U50,488, HS665 (**5**) and HS666 (**6**). The KOR partial agonist, *N*-CHM substituted **8** was also very efficacious as an antinociceptive, with two-fold less potency than its full agonist analogue **7**, but with an activity near to that of *N*-Bn substituted **9**. A reduction in the in vivo agonist potency (two-fold) was shown by the 3,3′-dihydroxy *N*-CPeM derivative **12** compared to

Table 3 Antinociceptive activities of diphenethylamines **5–19** in mice (Data from Erli et al. 2017, Spetea et al. 2012 and Spetea et al. 2017)

		Antinociception	
	In vitro agonism	Writhing assay, ED_{50} (mg/kg, s.c.)	Tail-withdrawal assay, ED_{50} (nmol, i.c.v.)
U50,488	A	1.54	7.21
5 (HS665)	A	1.91	3.74
6 (HS666)	PA	3.23	6.02
7	A	0.49	
8	PA	1.01	
9	A	1.21	
10	PA	1.71	
11	PA	4.73	
12	A	1.19	
13	A	0.95	
14	PA	1.73	
15	A	1.90	
16	PA	2.64	
17	A	1.33	
18	PA	2.25	
19	PA	2.14	

A full agonist, *PA* partial agonist. For compound structures, refer to Fig. 11.

its 3-monohydroxy substituted analogue **7**, a SAR observation that correlated with the decrease in the in vitro KOR agonist potency of **12**. The selective KOR full agonists **13** and **17**, with an *N*-CHM substituent, were equipotent to the partial agonist **8**. The 2-fluorinated *N*-CBM substituted **16** was slightly less active than its analogue HS665 (**5**) due to the reduced in vitro agonist potency and efficacy of compound **16** (Table 1 and Table 3) (Erli et al. 2017). Exchanging the *N*-CBM substituent in 2-fluorinated derivative **16** by the *N*-CHM substituent in **17** not only altered the in vitro agonist activity by converting a KOR partial agonist **16** into a full agonist **17** (Table 1), but also increased in vivo agonism of **17**. The 2-fluorinated, *N*-CHM substituted **17** was more effective in inhibition of the writhing response than its analogue **16** (Table 3). It was also established that 2-fluorinated analogues **18** and **19** had comparable antinociceptive activity to the *N*-CBM derivatives **10** and **14**, respectively, all having a KOR partial agonist profile in vitro (Erli et al. 2017).

Behavioral effects with diphenethylamine KOR full/partial agonists (Table 1) and G protein-biased KOR agonists (Table 2) on the motor activity in the mouse rotarod test were reported (Dunn et al. 2018, 2019; Erli et al. 2017; Spetea et al. 2017). Neither the KOR full agonist HS665 (**5**) nor the partial agonist HS666 (**6**), administered centrally i.c.v., had any effect on motor coordination in C57BL/6J mice at antinociceptive doses effective in the 55°C warm-water tail-withdrawal assay (Spetea et al. 2017). The lack of motor impairment in the rotarod test was also confirmed when HS665 (**5**), HS666 (**6**), and the *N*-CPeM substituted analogue

7 were given s.c. to CD1 mice at threefold to fivefold of the antinociceptive ED_{50} dose in the writhing assay (Table 3) (Erli et al. 2017). Only when injected i.p. HS665 (**5**) and diphenethylamine **7** at a dose of 30 mg/kg (16- and 61-fold of the antinociceptive ED_{50} doses in the writhing assay, Table 3) produced a modest (submaximal) motor incoordination in C57BL/6 mice (Dunn et al. 2018, 2019). Other diphenethylamines, the *N*-CHM substituted **8** and the 2-fluorinated derivatives **16** and **17** (Fig. 11) also produced no significant changes in the rotarod performance in CD1 mice at doses equivalent to a fivefold the effective antinociceptive ED_{50} dose in the writhing assay (Table 3) (Erli et al. 2017). In rotarod studies, diphenethylamine **3**, with an *n*-butyl group at the nitrogen (Fig. 11), administered i.p. to C57BL/6 mice in a dose of 30 mg/kg, did not cause locomotor incoordination (Dunn et al. 2018, 2019). Structurally related diphenethylamines **20** and **21** (Fig. 12) at doses of 30 mg/kg i.p. produced modest motor impairment, however without reaching the level of impairment produced by U50,488 in C57BL/6 mice (Dunn et al. 2019). None of derivatives **3**, **20**, and **21** were evaluated for antinociceptive activity, therefore no correlation between the effective antinociceptive dose and doses inducing motor incoordination could be made. Diphenethylamines **3**, HS665 (**5**), HS666 (**6**), **7**, **20**, and **21** were established as G protein-biased KOR agonists (Table 2), having low to no β-arrestin2 recruitment efficacy and potency in vitro, that appear to correlate with the reported KOR-specific effect on the locomotor activity.

Activation of central KORs causes dysphoria in humans, an undesirable side effect reported in early human studies (Pande et al. 1996; Pfeiffer et al. 1986). In animals, conventional KOR agonists such as U50,488, U69,593 and salvinorin A produce aversive effects in the CPA test in rodents (Liu et al. 2018, 2019a; Spetea et al. 2017; White et al. 2015; Zangrandi et al. 2016). KOR-mediated aversive properties were described to be associated with the recruitment of β-arrestin2 to the receptor, and G protein-biased KOR agonists may deliver the desired analgesia and be devoid of dysphoric effects. In vivo activation of central KORs by HS665 (**5**) and HS666 (**6**) was evaluated in the CPA test in C57BL/6J mice (Spetea et al. 2017). Unlike the unbiased KOR agonist U50,488, the G protein-biased agonist HS666 (**6**) produced no aversion when given i.c.v. in very high doses, equivalent to a 25-fold the effective antinociceptive ED_{50} dose in the 55°C warm-water tail-withdrawal assay. In the same CPA assay, HS665 (**5**), also established to be a G protein-biased agonist in vitro (Table 2), caused aversive-like effects in C57BL/6J mice when i.c.v. tested in a dose eightfold higher than the effective antinociceptive ED_{50} dose (Spetea et al. 2017). Further studies on the contribution of biased signaling toward the differences in behavioral effects of HS665 (**5**) and HS666 (**6**) will be required. A recent study compared the phosphoproteomes of the mouse striatum (among other brain regions) following central (i.c.) injection of HS665 (**5**) and HS666 (**6**) and other KOR agonists, including U50,488, 6'-GNTI and RB-64 to C57BL/6 mice in doses established to be effective in pharmacological and behavioral studies (Liu et al. 2018). The G protein-biased HS666 (**6**) similarly to 6'-GNTI, as non-aversive KOR agonists, elicited differential dynamic phosphorylation of synaptic proteins as compared to aversive KOR agonists, HS665, RB-64 and U50,488 (Liu et al. 2018). Particularly, the weak β-arrestin2 recruiting KOR agonists HS666 (**6**) and 6'-GNTI

did not activate the mTOR signaling pathway, which was found to be involved in mediating aversion of unbiased KOR agonists, such as U50,488 (Liu et al. 2018, 2019a). The KOR specificity was proven by the absence of significant phosphorylation changes in KOR-KO mouse brain (Liu et al. 2018).

3 Conclusion

The complexity of the kappa opioid system pharmacology is long recognized, but it was only in the past decades that the significance of receptor activation, signaling, particularly biased agonism, and distinct opioid drug action, were understood and systematically analyzed. Cellular and molecular mechanisms behind the KOR specific side effects of dysphoria and psychotomimesis have been intensively studied. Numerous KOR ligands have been designed over the years, with increased efforts in the twenty-first century towards the discovery of innovative and safer ligands targeting the receptor with the prospective to emerge as new therapies for human disorders, including pain, drug addiction, mood disorders, neurological conditions, and pruritus. The recently available structures of the KOR together with efficient computational methods provide significant insights into binding modes of known and new ligands to the receptor, with the prospective of translating the gained knowledge into the discovery of novel bioactive molecules.

In this review, we summarized chemical and pharmacological advances in the pursuit of KOR ligands with different scaffolds and distinct pharmacology. We discussed diverse strategies to bypass KOR associated adverse effects, including the development G protein-biased agonists and peripherally restricted agonists. We particularly emphasized the design strategies and pharmacology of new KOR ligands from the class of diphenethylamines, with diverse activation profiles ranging from potent and selective agonists to G protein-biased agonists and to selective antagonists. The first lead molecules in the series of diphenethylamines included the selective KOR agonist HS665 (**5**) and the partial agonist HS666 (**6**) (Spetea et al. 2012). Extended SARs disclosed that binding affinity, selectivity, and functional activity to the KOR can be altered by simple structural modifications, such as bulkier N-substituents, a 2-fluoro substitution or additional hydroxyl groups at positions 3′ and 4′ (Erli et al. 2017; Guerrieri et al. 2016). For example, *N*-CBM (in HS665 (**5**)) and *N*-CPM substitutions (in HS666 (**6**)) were more advantageous than *n*-alkyl groups leading to an increase in affinity, selectivity, agonist potency, and efficacy to the KOR. The presence of CPeM and CHM groups at the nitrogen led to excellent selectivity for the KOR. Introduction of a 2-fluoro substitution in *N*-CBM and *N*-CHM substituted diphenethylamines caused an additional increase in the KOR selectivity and affinity in the picomolar range. The shift of the 3-hydroxyl group to position 4 and additional hydroxyl groups at positions 3′ or 4′ had different consequences on KOR activity in vitro and in vivo. Furthermore, G protein-biased KOR agonists with various levels of bias were identified among the diphenethylamines. Development of an effective KOR agonist is particularly urged by the need for an efficacious analgesic without the burden of side effects of

currently used MOR agonists, including a lower abuse potential and risk for physical dependence. Diphenethylamines with a KOR agonist profile are effective antinociceptives in mouse models of acute thermal nociception and visceral pain with a KOR-specific mechanism of action. Additional behavioral studies in mice proved them as potential therapeutics with reduced liability for KOR-mediated side effects, including aversion and sedation/motor dysfunction.

Although selective KOR antagonists were initially developed as pharmacological tools for studying the in vitro and in vivo properties of KOR agonists, the currently encouraging results from preclinical and clinical studies, particularly with short-acting antagonists, as potential antidepressants, anxiolytic and anti-addiction medications represent an exciting development in opioid drug discovery. Diphenethylamines displaying antagonist activity to the KOR were identified, with the 3,4′-dihydroxy, *N*-CBM substituted **24**, as a high affinity and selective KOR ligand with in vitro and in vivo antagonism, with further studies remaining to establish their therapeutic value.

New KOR ligands with a diphenethylamine scaffold represent potential drug candidates that merit further investigation as prospective pharmacotherapeutics for the treatment of pain and other human diseases where the KOR/dynorphin system plays a key function in their etiology. Selective KOR antagonists with favorable pharmacokinetics are of significant interest with potential pharmacotherapeutic effects in mood, anxiety, or addictive states.

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Peptide Kappa Opioid Receptor Ligands and Their Potential for Drug Development

Jane V. Aldrich and Jay P. McLaughlin

Contents

Abstract

Ligands for kappa opioid receptors (KOR) have potential uses as non-addictive analgesics and for the treatment of pruritus, mood disorders, and substance abuse. These areas continue to have major unmet medical needs. Significant advances have been made in recent years in the preclinical development of novel opioid peptides, notably ones with structural features that inherently impart stability to proteases. Following a brief discussion of the potential therapeutic applications of

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KOR agonists and antagonists, this review focuses on two series of novel opioid peptides, all-D-amino acid tetrapeptides as peripherally selective KOR agonists for the treatment of pain and pruritus without centrally mediated side effects, and macrocyclic tetrapeptides based on CJ-15,208 that can exhibit different opioid profiles with potential applications such as analgesics and treatments for substance abuse.

Keywords

All D-amino acid tetrapeptides · Analgesia · Blood brain barrier permeability · CJ-15,208 · *cyclo*[Pro-Sar-Phe-D-Phe] · Dynorphin · Kappa opioid receptor · Macrocyclic tetrapeptides · Mood disorders · Oral activity · Peripherally restricted ligand · Pruritus · Respiration · Stress · Substance abuse

1 Introduction

While clinically used mu opioid receptor (MOR) agonists such as morphine produce robust analgesia, they are less effective against chronic pain such as peripheral neuropathy, and worse, also produce liabilities such as respiratory depression and abuse that greatly limit their usefulness (Yaksh and Wallace 2018). To attempt to retain opioid analgesia while limiting side effects, considerable effort has focused on developing ligands for the other opioid receptors (Aldrich and Vigil-Cruz 2003), with a number of selective ligands developed for both δ (DOR) and κ (KOR) opioid receptors (Yaksh and Wallace 2018).

With the significant therapeutic promise of KOR agonists and antagonists, this review will focus on new peptide ligands for KOR with promising results in vivo that suggest their potential for therapeutic development.

1.1 Actions of the Endogenous Kappa Opioid System and Resultant Potential Therapeutic Applications of Ligands for Kappa Opioid Receptors

Kappa opioid receptors are found throughout the brain, with dense expression found in the rat claustrum, olfactory tubercle, lateral nucleus of the amygdala, nucleus accumbens, caudate putamen, the bed nucleus stria terminalis and preoptic area, hypothalamus, thalamus and medial habenula, interpeduncular nucleus, and nucleus tractus solitarius (Mansour et al. 1987, 1994), and to a lesser degree hippocampus (Mansour et al. 1994). These earlier results were confirmed and extended recently with three-dimensional imaging via CLARITY of tdTomato-KOR in a knock-in mouse model (Chen et al. 2020). Like the other opioid receptors, the KOR is a member of the superfamily of seven transmembrane domain G-protein coupled receptors. Agonist-induced activation of KOR leads to conformational changes and the dissociation of G_i and G_o heterotrimeric G-proteins (Law et al. 2000). This results in the hyperpolarization of the neuron, mediated in part by activation of

inwardly rectifying potassium channels (Grudt and Williams 1993) and inhibition of voltage-gated calcium ion channels (Gross et al. 1990). Further actions by the dissociated G_i-alpha subunits decrease adenylyl cyclase activity and cyclic adenosine monophosphate (cAMP) levels (De Montis et al. 1987) and regulate the activity of a number of kinases (Bruchas and Chavkin 2010), notably the c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase 1 and 2 (ERK1/2) (Belcheva et al. 2005; Fukuda et al. 1996). Activation of KOR also results in β-arrestin-dependent signaling (McLennan et al. 2008), including activation of p38 mitogen-activated protein (MAP) kinase (Bruchas et al. 2006).

The dynorphin (Dyn) peptides, dynorphin A, dynorphin B, and α-neoendorphin, are the endogenous ligands for the KOR (Chavkin et al. 1982; Goldstein et al. 1979). A product of the prodynorphin (*pDyn*) gene (Horikawa et al. 1983; Sharifi et al. 1999) and isolated from a larger 32-amino acid peptide dynorphin-32 (also called "big dynorphin") (Merg et al. 2006), the initial dynorphin A product is 17 amino acids long with high affinity for KOR (Goldstein et al. 1981), but it is rapidly processed by endopeptidases (Schwarzer 2009) to shorter versions, with varying agonist potency (Minamino et al. 1980). Dynorphin peptides are expressed in the pituitary (Hollt et al. 1980; Schäfer and Martin 1994) and throughout the brain including in the striatum, hippocampus, amygdala and hypothalamus (Watson et al. 1983), spinal cord (Duan et al. 2014; Sardella et al. 2011), and dorsal root ganglia (Baseer et al. 2012).

The localization of KOR and the sites of production and distribution of dynorphin peptides result in many physiological, pathophysiological, and psychological processes (reviewed in more detail elsewhere), offering opportunities for drug development. For KOR agonists, this includes treatment of pruritus (Inan and Cowan 2020), convulsions (Simonato and Romualdi 1996; Zangrandi and Schwarzer, 2021), respiratory depression (Dosaka-Akita et al. 1993; Haji and Takeda 2001), pain ((Calcagnetti et al. 1988); but see also (Liu et al. 2019) and (Massaly et al. 2019)), and substance abuse (see the reviews in this Handbook by Banks (2020) and Kreek and coworkers (Reed et al. 2020)). KOR agonists may also be useful as water diuretics (Slizgi and Ludens 1986). Moreover, KOR antagonists may have therapeutic value to prevent stress-induced relapse to drug seeking behavior (Wee and Koob 2010) and in migraine (Xie et al. 2017), mood disorders (Knoll and Carlezon 2010; Carlezon and Krystal 2016; Jacobson et al. 2020), and other psychological disturbances such as schizophrenia (Clark and Abi-Dargham 2019).

1.2 Review of Key Non-Peptidic Kappa Opioid Receptor-Selective Ligands

While there are clinically used analgesics that exhibit activity at KOR as well as at MOR (notably buprenorphine as well as the so-called mixed agonists/antagonists nalbuphine, nalorphine, and pentazocine, Fig. 1), other attempts over the past 40 years to develop ligands with activity at KOR as potential therapeutic agents have met with very limited success. KOR-selective benzacetamides were

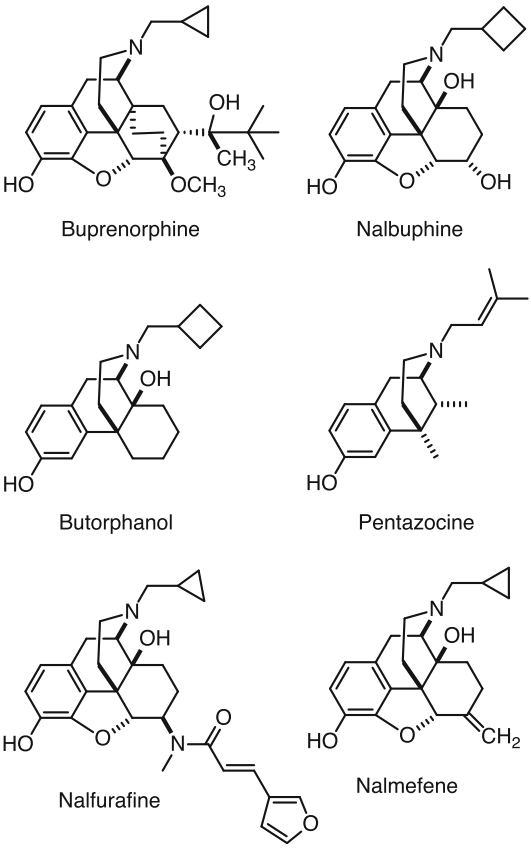


Fig. 1 Clinically used drugs with activity at kappa opioid receptors

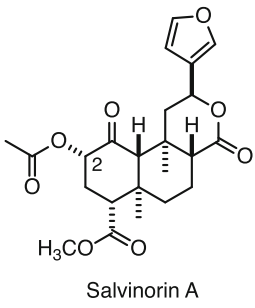
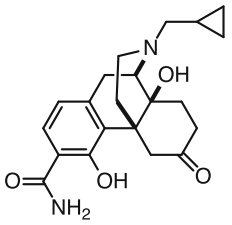


Fig. 2 Structure of the kappa opioid receptor agonist salvinorin A

extensively explored in the 1980s as potential analgesics (Szmuszkovicz 1999, 2000), but centrally acting KOR agonists produced dysphoria and psychotomimesis among other side effects (Pfeiffer et al. 1986; Wadenberg 2003), preventing their development as therapeutic agents. The novel non-nitrogenous natural product salvinorin A (Fig. 2) is a highly selective KOR agonist (Roth et al. 2002) that has served as a lead compound for extensive modification, especially to the C2 ester to



Samidorphan

Fig. 3 Structure of the mu opioid receptor antagonist samidorphan

increase metabolic stability (see Paton et al. (2020) for a recent review); however, it is well known to produce psychoactive effects in humans (Maqueda et al. 2016) and is not used clinically. The only KOR agonist approved to date is nalfurafine (Fig. 1) which is marketed in Japan to treat pruritus associated with kidney dialysis and chronic liver diseases (Miyamoto et al. 2020). While its antipruritic activity appears to be mediated by central KOR (Umeuchi et al. 2003), nalfurafine does not produce aversion characteristic of other KOR agonists (Mori et al. 2002). These actions have been attributed to a bias for G-protein signaling, but evidence from in vitro studies from independent laboratories has demonstrated that nalfurafine may act as a G protein-biased, unbiased, or β-arrestin-biased agonist, leaving this matter a subject of ongoing debate (see Zhou et al. 2021 for a review). The naltrexone derivative nalmefene (Fig. 1) exhibits high affinity and partial agonist activity at KOR along with MOR antagonism (Bart et al. 2005), and is used in the European Union to reduce alcohol consumption in alcohol-dependent individuals. This is consistent with reports on mediation by the KOR/dynorphin system of negative effects in alcohol dependence, and the reduction of alcohol consumption by the KOR antagonist activity of nalmefene in dependent subjects (see Mann et al. (2016) for a review).

Potential therapeutic application of KOR antagonists in humans was initially demonstrated using a combination of buprenorphine (Fig. 1) and the MOR antagonist naltrexone as a "functional KOR antagonist" in open-label studies in opioid-dependent individuals (Gerra et al. 2006; Rothman et al. 2000). More recently a combination of buprenorphine and the MOR antagonist samidorphan (Fig. 3) (ALKS 5461) was developed for adjunct treatment of major depressive disorder, resulting in submission of a New Drug Application (NDA) to the FDA. However, the FDA requested additional clinical data to demonstrate effectiveness in 2019 (https://www.drugs.com/nda/alks_5461_190201.html). The remaining active clinical trial of ALKS 5461 (NC03610048) on clinicaltrials.gov was terminated by Alkermes in May, 2020.

The prototypical small molecule KOR antagonists nor-BNI (nor-binaltorphimine), 5'-GNTI (5'-guanidinonaltrindole), and JDTic (Fig. 4) exhibit exceptionally long durations of KOR antagonist activity, lasting weeks to longer than a month after a single dose (see Carroll and Carlezon (2013)). A Phase 1 clinical trial of JDTic was stopped due to concerns about potentially adverse cardiac effects (Buda et al. 2015). This has prompted exploration of other non-peptide KOR antagonists that exhibit shorter durations of action (see Carroll and Carlezon (2013)

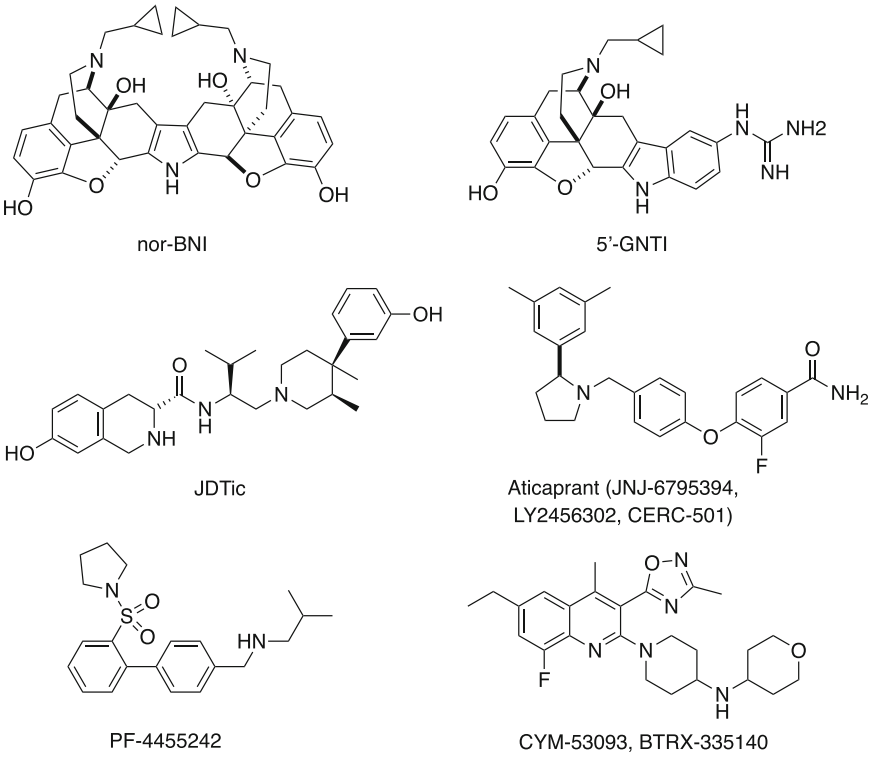


Fig. 4 Small molecule KOR antagonists

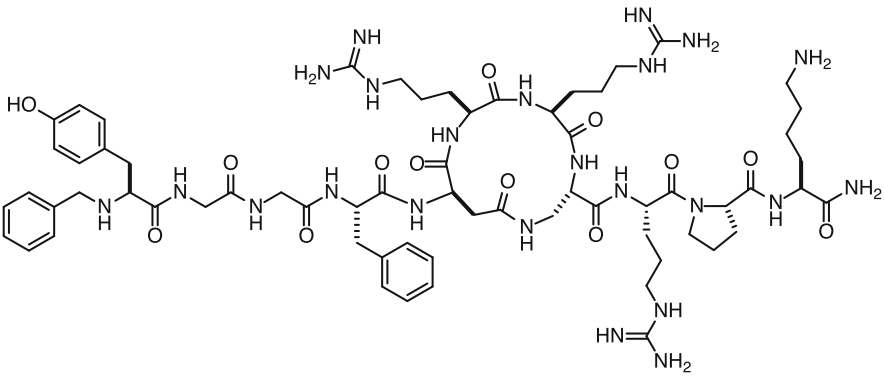
for a review), some of which have advanced into clinical trials (see Helal et al. (2017)) focusing primarily on their potential application in mood disorders. Aticaprant (JNJ-6795394, previously known as LY2456302 and CERC-501, Fig. 4) has been the most extensively studied KOR antagonist monotherapy in humans to date. While a trial did not find positive results in treatment of nicotine use disorder (Jones et al. 2020), promising results were recently reported in a clinical trial (NCT02218736) of this KOR antagonist as a potential treatment for anhedonic behavior (Krystal et al. 2020). The sulfonamide derivative PF-04455242 (Fig. 4) was also advanced into clinical trials, but these studies were terminated due to toxic effects in animals following prolonged exposure (NCT00939887, clinicaltrials.gov). A recently reported KOR-selective antagonist CYM-53093/BTRX-335140 (Guerrero et al. 2019) (Fig. 4) is also currently undergoing a Phase 2a clinical trial (NCT04221230) in patients with major depressive disorder.

H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-OH

Dynorphin A

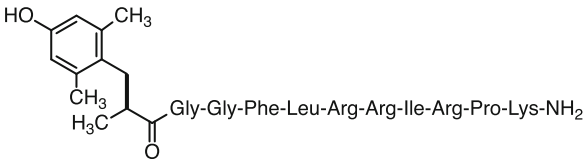
N-MeTyr-Gly-Gly-Phe-Leu-Arg-N-MeArg-D-Leu-NHEt

E2078



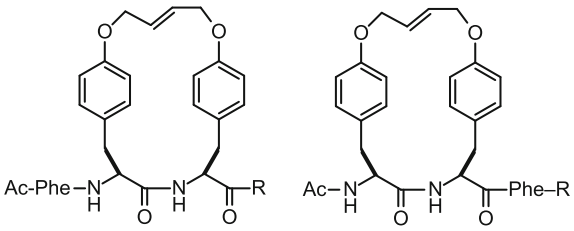
$PhCH_2$-Tyr-Gly-Gly-Phe-D-Asp-Arg-Arg-Dap- Arg-Pro-Lys-NH_2

Zyklophin



Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-NH_2

Dynantin



Cyclic arodyn analogs (R = Arg-Leu-Arg-Arg-Ile-Arg-Pro-Lys-NH_2)

Fig. 5 Dynorphin A and analogs discussed in the text. Dap = 2,3-diaminopropionic acid

2 Peptide Ligands for Kappa Opioid Receptors

2.1 Dynorphin A Analogs

Dynorphin A (Fig. 5) has served as a primary lead peptide for structural modification (see Aldrich and McLaughlin (2009); Aldrich and Vigil-Cruz (2003); Hall et al. (2016) for reviews). However, the metabolic lability of Dyn A complicates utilizing its derivatives systemically (Aldrich and McLaughlin 2009), and multiple modifications are typically needed in these longer peptides to provide sufficient metabolic stability for systemic administration. For example, the Dyn A-(1-8) analog E2078 (NMeTyr^{1},N-MeArg^{7},D-Leu^{8}]Dyn A-(1-8)-NHEt, Fig. 5), which in humans has analgesic effects comparable to pentazocine (Fujimoto and Momose 1995), contains multiple modifications to stabilize it to proteolytic cleavage, but the KOR selectivity of this peptide is low (Aldrich and McLaughlin 2009). In longer Dyn A analogs cyclization along with terminal modifications can impart sufficient metabolic stability for systemic administration, e.g. as in the cyclic KOR antagonist zyklophin (Patkar et al. 2005) (Fig. 3), which after subcutaneous administration antagonizes central KOR to prevent stress-induced relapse of cocaine-seeking behavior (Aldrich et al. 2009). Alternatively, delivery approaches have been explored to achieve sufficient concentrations of peptides in vivo for activity after systemic administration. CJC-1008, a peripherally acting Dyn A-(1-13) derivative that was modified to promote covalent attachment to serum albumin after administration and prolong its duration of action, exhibited activity in patients with postherpetic neuralgia (Wallace et al. 2006). N-Terminal methylated derivatives of Dyn A-(1-13) exhibited antinociception in morphine-tolerant rats following inhalation as well as after intravenous administration (Brugos et al. 2004). Recently the linear KOR selective peptide antagonist dynantin (Fig. 5) was encapsulated in a novel glycoliposome to improve its plasma stability and delivery across the blood-brain barrier (Lewicky et al. 2020).

As an alternative to cyclization via lactam or disulfide bonds, dynorphin A analogs cyclized by ring-closing metathesis (RCM) have also been explored. In addition to dynorphin A-(1-11)NH_2 analogs cyclized via allylglycine residues (Fang et al. 2009), a novel cyclization strategy utilizing Tyr(allyl) was employed to synthesize analogs of the KOR-selective antagonist arodyn (Ac[Phe$^{1-3}$,Arg^{4},D-Ala^{8}]dynorphin A-(1-11)-NH_2) cyclized in the N-terminus (Fig. 5) that retained KOR affinity and selectivity (Fang et al. 2018).

2.2 Tetrapeptide KOR Ligands

Smaller peptides with structural features that inherently impart metabolic stability are appealing leads for the development of systemically activity peptides with activity involving KOR. Novel tetrapeptides have shown promise as leads for systemically active KOR peptides with potential for development as clinical therapeutics.

2.2.1 All-D-Amino Acid Peptides

Exploration of a combinatorial tetrapeptide library for ligands binding to opioid receptors led to the unexpected identification of tetrapeptides containing all D-amino acids with high affinity for KOR (Dooley et al. 1998). Containing only D-amino acids, such peptides should be stable to cleavage by proteases, and thus represent promising peptides for potential development of peptidic therapeutic agents. The peptide D-Phe-D-Phe-D-Nle-D-Arg-NH_2 (ff-nle-r-NH_2, Fig. 6) exhibited exceptional selectivity for KOR in vitro (>30,000- and 68,000-fold over MOR and DOR, respectively), and its activity was primarily restricted to the periphery after

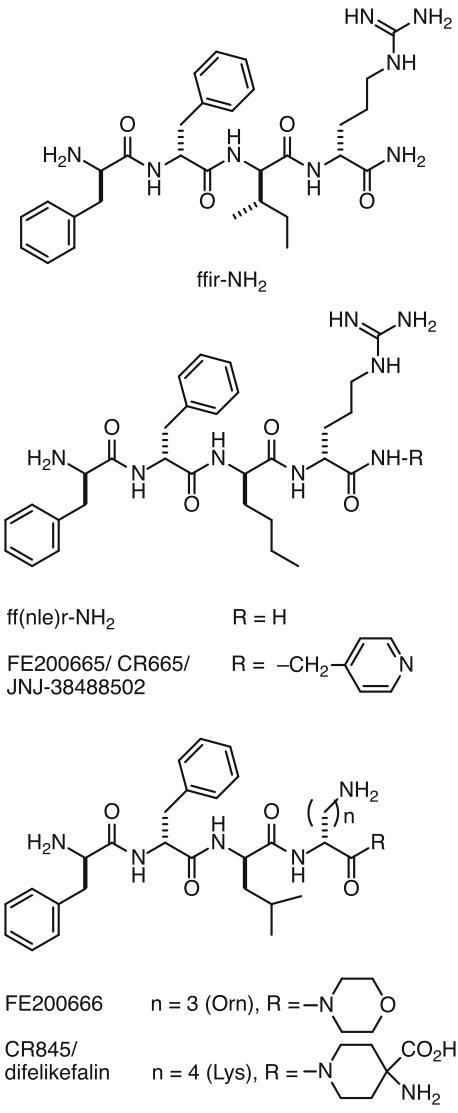


Fig. 6 Peripherally selective all-D-amino acid tetrapeptides

systemic administration (Vanderah et al. 2004). It exhibited significant antinociception in the acetic acid writhing assay that was antagonized by peripheral, but not central, administration of nor-BNI, verifying that the antinociception was mediated by peripheral KOR. It also was active in the formalin assay, but did not produce sedation or motor impairment at a ten-fold higher dose. These results demonstrated that peripheral-restricted KOR agonists offer promise as analgesics while minimizing centrally mediated adverse effects, including dysphoria (Albert-Vartanian et al. 2016).

The C-terminally modified analogs FE200665 and FE200666 (Binder et al. 2001) (Fig. 6) also exhibited high KOR affinity ($K_i < 0.25$ nM) and selectivity over MOR (16,900- and 88,600-fold, respectively) and DOR (84,600- and >1,250,000, respectively) in vitro. These analogs exhibited exceptional selectivity (548- and 182-fold, respectively) between antinociception and centrally mediated side effects (as measured in the rotarod assay) (Vanderah et al. 2008), and FE200665 (subsequently referred to as CR665 or JNJ-38488502) underwent clinical evaluation. This peptide selectively attenuated visceral pain (esophageal distension) in humans, but paradoxically decreased the threshold for skin pinch pain (Arendt-Nielsen et al. 2009), although it did not significantly alter the threshold in a human model of colonic distension (Floyd et al. 2009).

CR665 is not orally bioavailable, prompting additional modifications in an attempt to obtain orally active peptides. A series of analogs involving substitution of D-Arg^4 by modified D-Lys and D-Arg derivatives were synthesized, and several of the modified analogs exhibited antinociception when screened in the acetic acid writhing assay following oral administration; further characterization of one analog verified that it retained selectivity for peripheral KOR (Hughes et al. 2013).

The lack of oral activity and disappointing clinical results for CR665 led Cara Therapeutics to develop a third generation tetrapeptide, CR845 (difelikefalin, Fig. 6). Like CR665, the hydrophilic, small peptidic structure of CR845 limits CNS entry, facilitating peripherally selective activity. CR845 was reported to exhibit high selectivity for KOR over MOR and DOR and to produce antinociception in a number of rodent pain models, including after oral administration to rats, as well as in a mouse model of pruritus (see Albert-Vartanian et al. (2016), Lipman and Yosipovitch (2021) for summaries reviewing studies of this peptide).

Based on the promising preclinical data, difelikefalin has advanced into a number of clinical trials for pruritus and also as an analgesic (see Albert-Vartanian et al. (2016), Lipman and Yosipovitch (2021) for summaries of the clinical studies). Recently detailed results were reported for phase 2 (ClinicalTrials.gov, NCT02858726) and phase 3 (NCT03422653) clinical trials of i.v. difelikefalin showing efficacy for the treatment of pruritus in hemodialysis patients (Fishbane et al. 2020a, b). In a more recent double-blind, placebo-controlled phase 1 clinical trial, difelikefalin administered to 15 human subjects at i.v. doses 2–10 times the therapeutically effective dose (0.5 μg/kg) for treatment of pruritus did not produce respiratory depression, as measured by end-trial carbon dioxide, saturation of peripheral oxygen or respiration rate (Viscusi et al. 2021). There were no abnormalities in vital signs or electrocardiographic measures, but 12 of 15 subjects

reported dose-dependent, treatment-emergent adverse effects including paresthesia, hypoesthesia and somnolence, although all effects were mild, transient, and resolved without intervention (Viscusi et al. 2021). Based on the results of these clinical trials the Food and Drug Administration granted Priority Review status for the New Drug Application for difelikefalin for the treatment of moderate to severe pruritus in hemodialysis patients (https://www.empr.com/home/news/drugs-in-the-pipeline/priority-review-difelikefalin-korsuva-kappa-opioid-receptor-pruritus-hemodialysis/).

2.2.2 Endomorphin-1 Derivative

Another tetrapeptide derivative recently discovered to be a KOR-selective agonist was derived from the MOR-selective peptide endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2). Gentilucci and coworkers incorporated the ß^2-amino acid isoserine in position 2 followed by cyclization to yield a Freidinger lactam-like structure (Fig. 7) to impart metabolic stability (De Marco et al. 2018). While the *R,R* isomer was MOR selective, surprisingly the *S,S* isomer was selective for KOR and exhibited partial KOR agonism in a cAMP assay. In the mouse tail immersion assay this peptide exhibited significant antinociception (60% maximum possible effect after a 20 mg/kg intraperitoneal dose) lasting 30–45 min.

2.2.3 Macrocyclic Tetrapeptides

The natural product macrocyclic tetrapeptide (MTP) CJ-15,208 (*cyclo*[Phe-D-Pro-Phe-Trp], Fig. 8a), which does not possess either a basic amine or a phenol, was

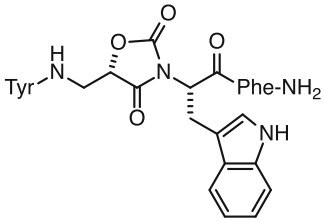


Fig. 7 KOR-selective endomorphin-1 analog

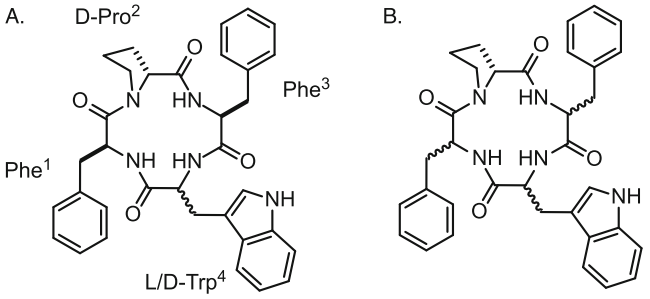


Fig. 8 (**a**) CJ-15,208 and [D-Trp]CJ-15,208 showing residue numbering used. (**b**) Stereoisomers of CJ-15,208 and [D-Trp]CJ-15,208

reported by Saito et al. to be a KOR antagonist (Saito et al. 2002). However, the stereochemistry of the Trp residue in the natural product was not determined, and therefore we and others synthesized and characterized both the L- and D-Trp isomers (Dolle et al. 2009; Ross et al. 2010). Based on optical rotation, the natural product is the L-Trp isomer (Ross et al. 2010). Both peptides bound preferentially to KOR with similar nanomolar affinities and exhibited KOR antagonist activity in the [^{35}S]-GTPγS assay (Dolle et al. 2009; Ross et al. 2010, 2012). However, the diastereomers exhibited markedly different activity profiles in vivo (Ross et al. 2012). In the mouse 55 °C warm water tail withdrawal (WWTW) antinociceptive assay the natural product unexpectedly produced robust antinociception mediated by both KOR and MOR; selective KOR antagonist activity lasting 24–48 h was demonstrated after dissipation of the antinociception. In contrast, the D-Trp isomer exhibited selective KOR antagonism with minimal antinociception.

Macrocyclic peptides are promising lead peptides compounds for potential development because of their stability to proteolytic degradation and their ability to cross biological barriers, which is facilitated by intramolecular hydrogen bonding (Rezai et al. 2006). Given the oral activity of the macrocyclic peptide cyclosporin, we examined these lead MTPs for oral activity and found that both retained opioid activity after oral (per os, p.o.) administration (Aldrich et al. 2013; Eans et al. 2013). After oral administration both peptides also antagonized a centrally administered KOR agonist, demonstrating that they could penetrate the blood-brain barrier. This is critical for the potential application of KOR ligands in the treatment of drug addiction and mood disorders which requires modulation of KOR in the CNS. Notably, both CJ-15,208 and [D-Trp]CJ-15,208 prevented stress-induced reinstatement of extinguished cocaine-seeking behavior following oral administration in a conditioned place preference (CPP) assay (Aldrich et al. 2013; Eans et al. 2013), consistent with results for other KOR antagonists. KOR agonists can prevent cocaine-primed reinstatement of extinguished cocaine-seeking behavior, and CJ-15,208 also prevented the cocaine-induced reinstatement at a time point (30 min) consistent with its agonist activity (Aldrich et al. 2013).

Exploration of the structure-activity relationships of these MTPs has revealed unexpected differences between opioid receptor interactions in vitro and the activity observed in vivo. In alanine scans, substitution of Phe^3 (see Fig. 8 for residue numbering), and especially Trp or D-Trp, by alanine in both CJ-15,208 (Aldrich et al. 2011) and its D-Trp isomer (Aldrich et al. 2014; Dolle et al. 2009) resulted in large decreases in KOR affinity. However, the alanine analogs of [D-Trp]CJ-15,208 as well as of CJ-15,208 all exhibited opioid receptor-mediated antinociception in the mouse 55 °C WWTW assay following intracerebroventricular (i.c.v.) administration. Interestingly, the Ala^3 analog of CJ-15,208 and the D-Ala^4 analog of [D-Trp]-CJ-15,208 produced the most potent antinociception of the alanine analogs in their respective series (Aldrich et al. 2011, 2014). In contrast, removal of the phenyl group from the Phe^1 side chain of either CJ-15,208 or [D-Trp]CJ-15,208 increased both KOR and MOR affinity in vitro (Aldrich et al. 2011, 2014; Dolle et al. 2009); the resulting analogs produced antinociception with similar potency to CJ-15,208 (Aldrich et al. 2011, 2014). Similar to CJ-15,208 (Aldrich et al. 2013), [Ala^1,

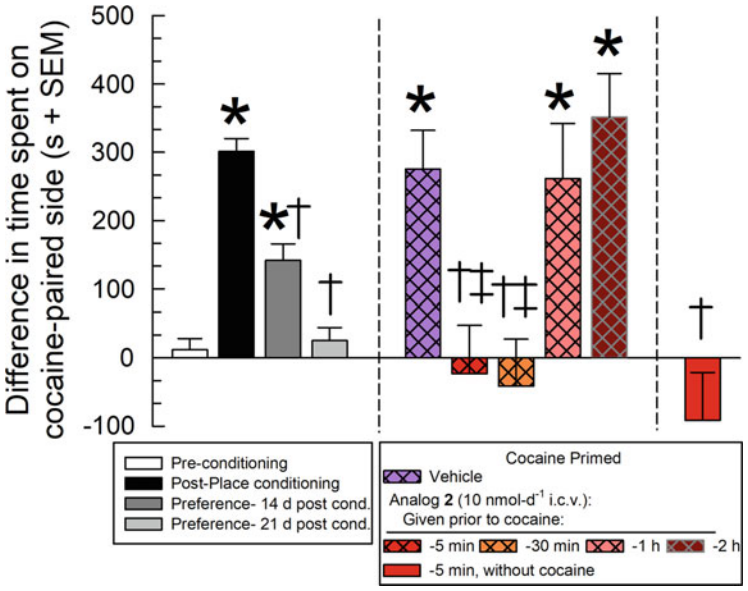


Fig. 9 Time-dependent prevention of reinstatement of extinguished cocaine-CPP following pretreatment with [Ala[1],D-Trp[4]]CJ-15,208 (**2**). The peptide prevented cocaine-primed reinstatement of cocaine-CPP when administered 5 or 30 min, but not 1 or 2 h, prior to cocaine. Pretreatment with the peptide without cocaine (rightmost bar) did not induce reinstatement by itself. Data shown are mean ± SEM ($n = 148$ mice total (left panel), distributed into groups of 16–24 mice/bar (middle and right panels)) difference in time spent on the drug-paired side. *$p < 0.05$, significantly different from preconditioning place preference response (leftmost bar); †$p < 0.05$, significantly different from post-CPP response (leftmost solid black bar); ‡$p < 0.05$, significantly different from vehicle-treated, stress-induced or cocaine-primed reinstatement of place preference response; ANOVA followed by Tukey's post hoc test. Reprinted with permission from J.V. Aldrich et al. (2014), Br J Pharmacol 171:3212–3222. Copyright (2014) John Wiley and Sons

D-Trp[4]]CJ-15,208 (analog **2**) prevented both stress- and cocaine-induced reinstatement of extinguished cocaine-seeking behavior in a time-dependent manner (Fig. 9) (Aldrich et al. 2014). All of the analogs except [D-NMe-Ala[2],D-Trp[4]]CJ-15,208 also exhibited KOR antagonist activity, albeit with varying potencies (Aldrich et al. 2011, 2014).

Gentilucci and coworkers synthesized analogs containing L- or D-Trp(1-Me) and found that both analogs lacked opioid receptor affinity, indicating the importance of the indole NH in lead MTPs to opioid receptor interaction (De Marco et al. 2016). Gentilucci and coworkers also explored analogs of CJ-15,208 in which the ring size was expanded by replacing the Phe[1] residue with either ß-alanine or γ-aminoisobutyric acid (GABA) (De Marco et al. 2016). Interestingly, while both completely lost KOR affinity, the ß-alanine[1] analog was a potent selective MOR agonist in vitro while the GABA[1] analog was a DOR antagonist with weak MOR agonist activity.

Given the differences in the opioid activity profile of CJ-15,208 and its D-Trp isomer in vivo we have also prepared stereoisomers of these MTPs (Fig. 8b)

Table 1 Summary of opioid antinociceptive activity of the stereoisomers[a]

	ED_{50} (& 95% confidence interval) values		
Stereoisomer	i.c.v. (nmol)	p.o. (mg/kg)	Receptors involved
CJ-15,208[b]	1.74 (0.62–4.82)	3.49 (1.98–5.73)	KOR, MOR
D-Phe[1]	0.75 (0.36–1.44)	7.62 (5.12–12.2)	KOR, MOR, DOR
D-Phe[3]	20.4 (10–58.7)	–	KOR, MOR
D-Phe[1,3]	1.00 (0.64–1.60)	4.12 (3.30–5.31)	KOR, DOR
D-Phe[1],D-Trp[4]	2.39 (1.40–4.56)	–	DOR, KOR
D-Phe[3],D-Trp[4]	0.56 (0.38–0.91)	4.72 (3.70–6.39)	KOR, MOR, DOR

[a]Data from Brice-Tutt et al. (2020a)
[b]From Aldrich et al. (2013); Ross et al. (2012). – Maximum antinociception was not achieved, precluding calculation of an ED_{50} value

(Brice-Tutt et al. 2020a). Similar to some of the alanine analogs of CJ-15,208 and its D-Trp isomer, these stereoisomers all exhibited substantial decreases in KOR (13- to >450-fold) and MOR affinity (8.7- to >3-fold) in vitro compared to the lead MTPs. However, these five stereoisomers with varying stereochemistry of the aromatic amino acid residues all exhibited antinociception in the mouse 55 °C WWTW assay following either i.c.v. or oral administration that was opioid receptor mediated (Table 1). While the opioid receptor contribution varied, KOR activity contributed to the antinociception for all of the isomers. However, only one of the isomers, [D-Phe[1]]CJ-15,208, retained the KOR antagonist activity exhibited by CJ-15,208 and [D-Trp]CJ-15,208; interestingly, [D-Phe[3],D-Trp[4]]CJ-15,208 exhibited weak DOR antagonist activity. The three stereoisomers of CJ-15,208 did not exhibit significant effects on respiration, while the two stereoisomers of [D-Trp]CJ-15,208 did not exhibit significant antinociceptive tolerance. The stereoisomers [D-Phe[1,3]]- and [D-Phe[3],D-Trp[4]]CJ-15,208 were examined in CPP assays following oral administration, and neither peptide produced either preference (in contrast to the MOR agonist morphine) or aversion (in contrast to the KOR agonist U50,488; Fig. 10). Thus, these stereoisomers are new lead MTPs to be explored for the potential development of safer analgesics.

We rationally designed a new MTP, *cyclo*[Pro-Sar-Phe-D-Phe], (where Sar = sarcosine, Fig. 11a), based on the reported conformations of other MTPs (Ferracane et al. 2020). This peptide adopts multiple conformations in polar solvents, as shown by NMR, but a single conformation in a nonpolar solvent (chloroform) that is stabilized by intramolecular hydrogen bonds (Fig. 11b), consistent with the proposal that such conformational flexibility is important for membrane permeability (Rezai et al. 2006). Interestingly, this peptide exhibits minimal inhibition of radioligand binding at opioid receptors in vitro and did not stimulate [^{35}S]GTPγS binding to either KOR or MOR, nor did it shift the dose-response curves for stimulation of binding by agonists at either KOR or MOR. In spite of these results, following i.c.v., intraperitoneal (i.p.) (Brice-Tutt et al. 2020b), or oral administration (Ferracane et al. 2020) the peptide produces antinociception which is mediated by KOR and MOR, and selective KOR antagonism observed after dissipation of the antinociception. It exhibits exceptionally potent KOR antagonism (at a 1 *pmol* dose)

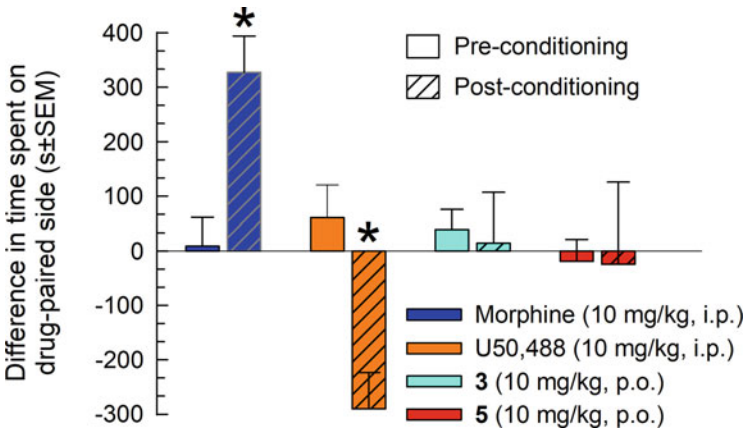


Fig. 10 Evaluation of potential rewarding or aversive properties of isomers [D-Phe^{1,3}]- (**3**) and [D-Phe^{3},D-Trp^{4}]CJ-15,208 (**5**) in the conditioned place preference assay. After determination of initial preconditioning preferences, C57BL/6J mice were place conditioned daily for 2 days with morphine (10 mg/kg, i.p.), U50,488 (10 mg/kg, i.p.), or stereoisomer (10 mg/kg, p.o.) using a counterbalanced design. Data is presented as mean difference in time spent on the drug-paired side ± SEM, with positive and negative values indicating a preference for and avoidance of the drug-paired chamber, respectively. *significantly different from matching preconditioning preference ($p < 0.05$), two-way ANOVA. $n = 14$–28 mice/compound. Reprinted from Brice-Tutt et al. (2020a)

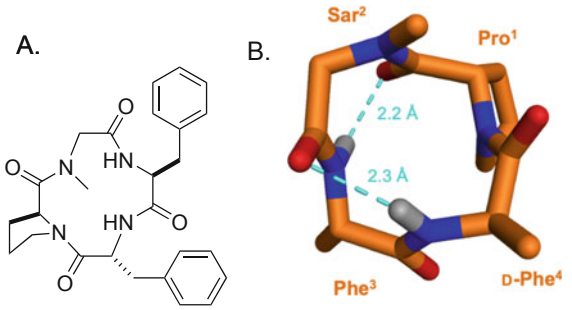


Fig. 11 (**a**) Structure of *cyclo*[Pro-Sar-Phe-D-Phe], and (**b**) its backbone conformation in nonpolar solvents. Intramolecular hydrogen bonds are shown as dotted blue lines. Reprinted with permission from M.J. Ferracane et al. (2020), ACS Chem Neurosci 11:1324–1,336. Copyright (2020) American Chemical Society

after i.c.v. administration (Brice-Tutt et al. 2020b) and can antagonize a centrally administered KOR agonist after oral administration, indicating that it can cross the blood-brain barrier (Ferracane et al. 2020). The peptide did not cause respiratory depression in wild-type mice, but KOR knock-out mice exhibited significant respiratory depression (Fig. 12), demonstrating the importance of KOR activity to the improved safety profile of this peptide (Brice-Tutt et al. 2020b). It also produced neither preference nor aversion in CPP assays following i.c.v. administration

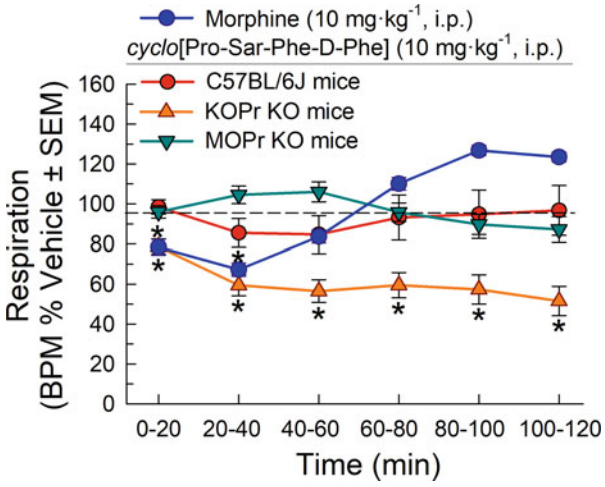
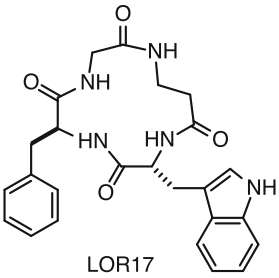


Fig. 12 Effects of *cyclo*[Pro-Sar-Phe-D-Phe] on respiration in C57BL/6J wild-type, MOR knock out (KO), or KOR KO mice tested in the CLAMS/Oxymax system. Respiration was monitored after i.p. administration of *cyclo*[Pro-Sar-Phe-D-Phe] (10 mg·kg^{-1}) or morphine (10 mg·kg^{-1}). Data presented as % vehicle response ± SEM (n = 23–27 mice/treatment group), breaths per minute, BPM. *significantly different from vehicle control response ($p < 0.05$); two-way RM ANOVA with Tukey's multiple comparison post hoc test. Reprinted with permission from A. C. Brice-Tutt et al. (2020), Br J Pharmacol 177:4209–4,222. Copyright (2020) John Wiley and Sons

Fig. 13 Structure of LOR17



(Brice-Tutt et al. 2020b). Like CJ-15,208 (Aldrich et al. 2013) *cyclo*[Pro-Sar-Phe-D-Phe] can prevent both stress- and cocaine-induced reinstatement of extinguished cocaine-seeking behavior in CPP assays in a time-dependent manner (Ferracane et al. 2020); prevention of cocaine-induced reinstatement was lost in KOR knock-out mice, verifying mediation through KOR (Ferracane et al. 2020). This peptide also prevented both stress- and drug-induced reinstatement of extinguished morphine seeking behavior in a time-dependent manner following both i.c.v. and peripheral (oral) administration (Brice-Tutt et al. 2020b; Ferracane et al. 2020), demonstrating the potential of this peptide in the treatment of opioid abuse disorder. Thus, *cyclo*-[Pro-Sar-Phe-D-Phe] showed potent and robust in vivo KOR and MOR activity, despite minimal activity detected at these receptors when tested with in vitro assays.

Bedini et al. recently reported on another macrocyclic tetrapeptide, LOR17 (Fig. 13), that is a hybrid of CJ-15,208 and the cyclic pentapeptide Cyclo EM-1 (*cyclo*[Tyr-D-Pro-D-Trp-Phe-Gly]) (Bedini et al. 2020). This peptide is a KOR-selective ligand with nanomolar KOR affinity (K_i = 1.2 nM) and potently inhibits cAMP production (IC_{50} = 3 nM), but has minimal effects on recruitment of ß-arrestin-2; it also activates early-phase ERK1/2 phosphorylation, but does not induce p38 MAPK phosphorylation, and thus is a G-protein biased agonist. In mice LOR17 displayed anti-allodynic activity in a neuropathic pain model (oxaliplatin-induced thermal hypersensitivity) after subcutaneous administration in addition to antinociceptive activity in acute pain models; it did not affect coordinated locomotion or depressant activity, in contrast to the prototypical KOR agonist U50,488.

3 Conclusions

Peptide ligands with kappa opioid receptor agonist or antagonist activity have advantages over earlier small molecules and offer significant opportunities for therapeutic development. The novel all-D-amino acid tetrapeptides and MTPs are promising peptidic ligands for KOR because of their inherent metabolic stability due to unnatural amino acids and cyclization, respectively, and the influence of their structural features on barrier (intestinal and blood-brain barrier) permeability. The oral bioavailability of peptides in both series is a major advantage over most peptides, making them promising scaffolds for development, as evident by the advancement of CR-845/difelikefalin into advanced clinical trials. The dichotomy in blood-brain barrier permeability between the two series permits different clinical issues to potentially be controlled, i.e., minimizing centrally mediated adverse effects of KOR agonists by the peripherally restricted all-D-amino acid tetrapeptides, and the potential for treating mood and substance abuse disorders requiring access to central KOR in the case of the MTPs.

For some of the MTPs there appear to be additional, and potentially new, mechanisms for their opioid activity observed in vivo. This is evident for *cyclo*-[Pro-Sar-Phe-D-Phe] (Ferracane et al. 2020) and MTP stereoisomers of CJ-15,208 and [D-Trp]CJ-15,208 (Brice-Tutt et al. 2020a) that show negligible or low (micromolar) affinity for opioid receptors while exhibiting robust opioid activity in vivo. These findings are not unique to these peptides; for example, two compounds identified from a combinatorial library exhibit potent antinociception in the mouse 55 °C WWTW assay mediated by KOR ± MOR, but only bind to opioid receptors with micromolar affinity (Reilley et al. 2010). Recent evidence is expanding the traditional definition of opioid receptor signaling, suggesting that MTPs could engage additional mechanism(s) that contribute to their activity. There is precedence for the contribution of new or multiple mechanisms to the activity of promising novel compounds. For instance, the potent MOR peptide Dmt-DALDA (Dmt-D-Arg-Phe-Lys-NH_2, Dmt = 2',6'-dimethyltyrosine) exhibits 3,000-fold higher antinociceptive potency than morphine following intrathecal administration in vivo

even though its MOR affinity is only seven-fold higher than morphine. Dmt-DALDA also inhibits norepinephrine uptake, which appears to contribute to its exceptional antinociceptive potency (Shimoyama et al. 2001). Given the complexity of the nervous system and multiple targets modulating behaviors such as pain and substance abuse, optimal combinations of different pharmacological activities could produce synergies, as demonstrated by the MTP scaffold, and provide potential advantages in future drug development.

While the potential for new mechanism(s) imparting opioid activity is exciting, it also involves challenges, particularly in efficiently screening and identifying promising new compounds for advanced studies. In vivo screening can facilitate the identification of promising new compounds with compatible pharmacokinetic properties. In fact, the use of such phenotypic screening has been a major driver in the discovery of first-in-class drugs, with a number of drugs discovered by phenotypic screening approved by the FDA without initially knowing their mechanism of action (Swinney and Anthony 2011). With their favorable in vivo activity and pharmacokinetic properties, further mechanistic insights into the KOR activity of the MTPs could expand the opioid pharmacopeia while also providing potentially valuable therapeutics with fewer liabilities of use.

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Part IV

Preclinical Studies: In Vivo Pharmacology

Dynorphin/Kappa-Opioid Receptor System Modulation of Cortical Circuitry

Hugo A. Tejeda, Huikun Wang, Rodolfo J. Flores, and Hector E. Yarur

Contents

Abstract

Cortical circuits control a plethora of behaviors, from sensation to cognition. The cortex is enriched with neuropeptides and receptors that play a role in information processing, including opioid peptides and their cognate receptors. The dynorphin (DYN)/kappa-opioid receptor (KOR) system has been implicated in the processing of sensory and motivationally-charged emotional information and is

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highly expressed in cortical circuits. This is important as dysregulation of DYN/KOR signaling in limbic and cortical circuits has been implicated in promoting negative affect and cognitive deficits in various neuropsychiatric disorders. However, research investigating the role of this system in controlling cortical circuits and computations therein is limited. Here, we review the (1) basic anatomy of cortical circuits, (2) anatomical architecture of the cortical DYN/KOR system, (3) functional regulation of cortical synaptic transmission and microcircuit function by the DYN/KOR system, (4) regulation of behavior by the cortical DYN/KOR system, (5) implications for the DYN/KOR system for human health and disease, and (6) future directions and unanswered questions for the field. Further work elucidating the role of the DYN/KOR system in controlling cortical information processing and associated behaviors will be of importance to increasing our understanding of principles underlying neuropeptide modulation of cortical circuits, mechanisms underlying sensation and perception, motivated and emotional behavior, and cognition. Increased emphasis in this area of study will also aid in the identification of novel ways to target the DYN/KOR system to treat neuropsychiatric disorders.

Keywords

Aversion · Circuits · Cognition · Dynorphin · Kappa-opioid receptor · Learning and memory · Prefrontal cortex · Reward

Abbreviations

DYN	Dynorphin
GPCR	G protein-coupled receptor
KOR	Kappa-opioid receptor
MOR	Mu-opioid receptor
PDyn	Prodynorphin
PFC	Prefrontal cortex

1 Introduction

Dynorphins (DYNs) are neuropeptides with widespread distribution and diverse functions in the central nervous system. The actions of DYNs in the central nervous system are primarily via signaling through the kappa-opioid receptor (KOR). DYN/KOR signaling is implicated in the pathophysiology of various psychiatric disorders, such as mood, substance abuse, and thought disorders. In this chapter, we provide an overview of the DYN/KOR system in regulating cortical circuitry and its implications in mediating behaviors. Briefly, this review describes (1) a brief description of the anatomical basis and cell types that constitute cortical circuits, (2) anatomical architecture of the cortical DYN/KOR system, (3) functional regulation of cortical synaptic transmission and microcircuit function by the DYN/KOR system, (4) regulation of behaviors by the cortical DYN/KOR system,

(5) implications for the DYN/KOR system for human health and diseases, and (6) future directions and unanswered questions for the field. Together, this chapter adds to an understudied area of research of the DYN/KOR system, highlighting an exciting avenue for future research that enhances our understanding of the complex function of the DYN/KOR system in cortical circuitry. Understanding the DYN/KOR system and its specific role in affective and motivational states, in addition to sensation and perception, will provide novel insights into neuropeptide systems in cortical circuits to aid the development of novel drugs for the treatment of neuropsychiatric disorders.

1.1 Cortical Circuits

Cortical circuits are specialized in processing primary sensory information and integrating such information in associative cortices to perform higher-order behavioral functions, such as motor planning, executive function, emotional control, and learning and memory. A nuanced overview of the cortical architecture and circuit function is beyond the scope of the present review. We invite the reader to the following review articles for more detailed descriptions of cortical circuitry and its function (Haider and McCormick 2009; Harris and Shepherd 2015; Briscoe and Ragsdale 2018; Adesnik and Naka 2018; Halassa and Sherman 2019). Here, we will focus on covering aspects of cortical circuits directly relevant to understanding the role of the DYN/KOR system in cortical circuits.

Cortical circuits do not act in isolation to perform their functions. They play diverse roles in behavior by integrating subcortical and intracortical (between cortical sub-regions) connections that are then processed using local circuit motifs embedded in microcircuits in a highly organized manner (Harris and Shepherd 2015). The cortex is a stratified structure consisting of up to six layers, with differences in the presence and size of individual layers among cortical sub-regions. The cortex primarily consists of glutamatergic excitatory projection neurons and several classes of GABAergic inhibitory neurons. Though not presently understood, local cortical circuit motifs are anatomically founded by extensive connections between neurons resided within the same and/or different layers wherein neurons of one layer can excite or inhibit neurons in the same and/or distinct layers (Adesnik and Naka 2018). Local circuit connectivity is cell type specific, and though common principles may hold, differences between cortical sub-regions exist. Interactions between layers are of consequence since excitatory outputs of cortical circuits tend to have layer specificity (Harris and Shepherd 2015; Adesnik and Naka 2018; Halassa and Sherman 2019). For example, corticothalamic neurons are most heavily concentrated within layer VI of the cortex, while striatal projection neurons are dispersed more superficially. Moreover, afferent inputs to the cortex also display layer specificity and, at times, show cell type-specific innervation. Cortical circuit motifs could serve several functions, including, but not limited to, signal amplification via recurrent excitatory connections, lateral inhibition via di-synaptic inhibition, generation and/or maintenance of local field potential oscillations, and mechanisms for signal convergence and divergence (Adesnik and Naka 2018). Notwithstanding,

these processes are not well understood. Thus, a deeper understanding of the organization of the cortex is critical for gaining a deep appreciation of how the architecture of the cortical DYN/KOR system shapes circuit function and behavior.

GABAergic interneurons are diverse with >28 clusters of GABAergic interneurons identified, though effects on circuit function may be limited to fewer motifs (Petilla Interneuron Nomenclature et al. 2008; Isaacson and Scanziani 2011; Kepecs and Fishell 2014; Roux and Buzsaki 2015). It is now appreciated that not all forms of inhibitory interneurons and the inhibition they exert on cortical circuits are equal. Some interneurons, such as parvalbumin (PV)- and cholecystokinin (CCK)-expressing cells, innervate the soma of pyramidal neurons and regulate the timing of pyramidal neuron outputs. Whereas other interneurons, such as somatostatin (SST)-expressing interneurons, innervate dendrites of pyramidal neurons and are hypothesized to inhibit dendritic integration of excitatory inputs (Lovett-Barron et al. 2012). Further, another motif that is fulfilled by interneurons is disinhibition, wherein interneurons inhibit other interneurons (Lee et al. 2013), thus facilitating recruitment of cortical pyramidal neurons. Like excitatory projection neurons, GABAergic interneurons play a role in information processing within cortical microcircuits by inhibiting neurons and/or dendrites within or between layers. For example, SST-expressing Martinotti cells in layer V provide extensive dendritic inhibition within superficial layers. Thus, interneurons contribute to cortical information processing via complex interactions with excitatory projection neurons and other inhibitory interneurons.

Recently, large-scale sequencing of cortical neurons demonstrated that virtually all neurons in the cortex, excitatory or inhibitory, express neuropeptides or a GPCR that binds neuropeptides, including prodynorphin (PDyn) and KOR (Fig. 1a, b) (Tasic et al. 2018; Smith et al. 2019). This highlights the importance of neuromodulators, such as the DYN/KOR system, in regulating cortical circuits. However, compared to regions such as the hypothalamus and striatum, much less is known about the role of neuromodulators within cortical circuits in regulating information processing and cortically driven behaviors. Below, we review the architecture and function of the DYN/KOR system in regulating cortical circuitry and its implications in mediating behavior.

1.2 Anatomy of the Cortical DYN/KOR System

The DYN/KOR system has been implicated in modulating cortical-dependent behaviors and is expressed in cortical regions of all mammalian species, including rodents and both human and non-human primates (Khachaturian et al. 1985; Quirion et al. 1987; Meng et al. 1993; Peckys and Hurd 2001; Martinez et al. 2019). DYNs and other neuropeptides are hypothesized to convey their signals via volume transmission where peptides diffuse far from release sites before binding to their cognate receptors (Castillo et al. 1996; Chavkin 2000). This property of DYN and neuropeptide transmission gives the impression that neuropeptide transmission is not specific. However, the specificity of signaling is conferred by the localization of receptors that bind DYNs and the enzymes that degrade DYNs to limit their

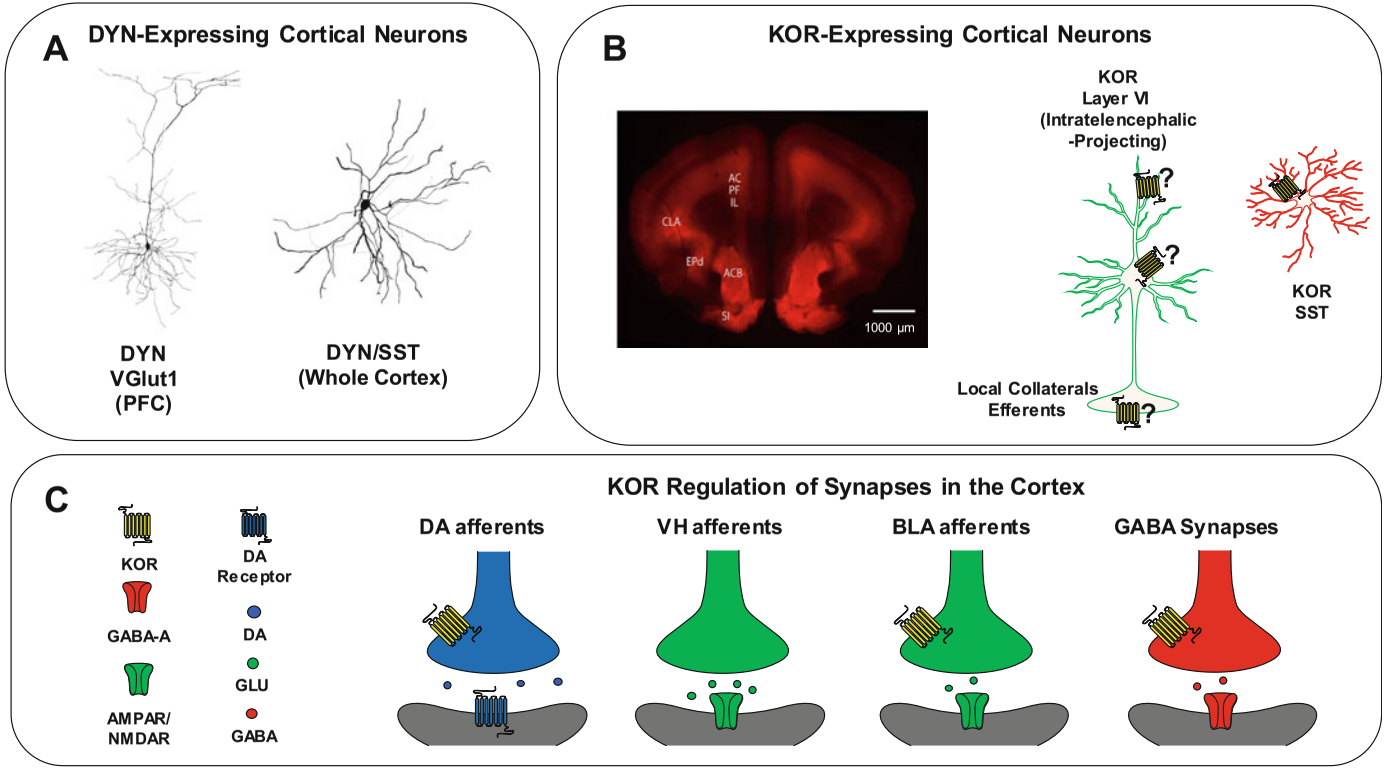

Fig. 1 Published DYN/KOR system architecture and function in cortical circuits. (**a**) DYN is expressed in SST-containing neurons throughout the cortex. DYN is also expressed in excitatory neurons in the PFC. Reconstruction of electrophysiologically-recorded, biocytin-filled excitatory and inhibitory mPFC neurons (Wang et al. unpublished observations). (**b**) KOR-TdTomato expression in a knock-in mouse line expressing KOR-TdTomato fusion protein (modified from Chen et al. 2020). KOR mRNA is present in layer VI intratelencephalic (IT) projection neurons and in SST-containing neurons and may regulate local circuit and/or long-range connections. (**c**) KOR inhibit inputs to the PFC from VTA DA neurons, the BLA, but not the ventral hippocampus (VH). KORs are also present in presynaptic GABAergic terminals and inhibit GABA release from synaptosomes

diffusion. PDyn mRNA and DYN peptide immunoreactivity are present throughout cortical regions (Khachaturian et al. 1985; Peckys and Hurd 2001; Yakovleva et al. 2006; Sohn et al. 2014). Moreover, it is likely that cortical sources of DYNs may act locally as KOR mRNA expression and significant KOR binding sites are present in cortical regions in rodents and primates (Quirion et al. 1987; Meng et al. 1993; Svingos and Colago 2002; Bazov et al. 2018). The gross organization of PDyn and KOR mRNA expression appears to have some degree of layer specificity and as such may differ between different cortical regions with differential layering (Fig. 1a, b) (DePaoli et al. 1994; Peckys and Hurd 2001; Sohn et al. 2014; Smith et al. 2019). For instance, in humans, PDyn mRNA is differentially expressed in the cingulate and dorsal lateral prefrontal cortex, while KOR mRNA expression is more similar in both regions. Additionally, KOR is differentially expressed in afferent inputs to the cortex (see Fig. 1c and Sect. 1.4), with KOR mRNA expression more concentrated in limbic regions having preferential innervation of associative prefrontal cortical regions relative to primary sensory cortices. Thus, though DYN and KOR may be present throughout cortical regions, differences in the general architecture of cortical sub-regions may translate differences in the architecture of the DYN/KOR system between cortical sub-regions. However, very little work has been aimed at elucidating the cell types within the cortex that express DYNs and KOR (Fig. 1).

In primary sensory cortices, the majority of PDyn mRNA-containing cells are GABAergic interneurons (Sohn et al. 2014; Loh et al. 2017; Smith et al. 2019). More specifically, PDyn mRNA is primarily expressed in SST-containing, but not PV- or vasoactive intestinal peptide (VIP)-expressing neurons (Fig. 1a) (Sohn et al. 2014). Interestingly, selective expression of PDyn in SST interneurons is not applicable in the prefrontal cortex (PFC), where a substantial number of PDyn-expressing neurons are glutamatergic (Fig. 1a) (Sohn et al. 2014; unpublished observations). A recent study demonstrated robust labeling of cells in superficial layers in the insular cortex of PDyn-iCre mice crossed with TdTomato reporter mice (Pina et al. 2020), suggesting that PDyn containing neurons might be restricted to specific layers in the insular cortex. We have observed substantial ectopic expression of TdTomato in PDyn mRNA-lacking neurons in the mPFC of PDyn-Cre mice crossed with tdTomato reporter mice (unpublished observations), suggesting that PDyn promoter activity may be turned on in other cell types during development that is not maintained during adulthood in naïve animals. This suggests that DYN may play a role in shaping developmental trajectories of cortical circuits. Indeed, changes in DYN/KOR signaling in the mPFC of developing rats have been reported (Sirohi and Walker 2015). Consistent with the notion that PDyn gene expression in interneurons may not be fixed but rather dynamic, a recent study demonstrated that aversive conditioning increases the number of DYN-containing SST interneurons, without changing the number of SST-positive cells (Loh et al. 2017). These results suggest that DYN expression may represent a functional state of SST interneurons rather than a hard-wired sub-type. However, further work is needed to resolve this issue. Taken together, DYN-containing cells are present in SST-positive interneurons throughout the cortex but may also be present in excitatory projection neurons within PFC circuits (Fig. 1a).

KOR-expressing cells are present in cortical circuits, suggesting that DYNs released from cortical sources may act on KOR-containing cells within cortical microcircuits (Fig. 1b). KOR mRNA expression has also been identified in bulk cortical neurons (Bazov et al. 2018). Single-cell RNA sequencing demonstrated KOR mRNA is enriched in a cluster of layer VI pyramidal neurons that project within the telencephalon (Fig. 1b) (Tasic et al. 2018; Smith et al. 2019). Consistent with the expression of KOR in cortical cells, there is a substantial amount of KOR immunoreactivity in dendrites and a marginal amount in putative glia (Svingos and Colago 2002). However, ultrastructural data suggest that the majority of KOR immunoreactivity in the mPFC is in presynaptic elements such as axonal varicosities and presynaptic terminals (Fig. 1c). Some of these synapses may be from afferent inputs expressing KOR, such as the amygdala and midbrain (see below). However, KOR immunoreactivity in presynaptic terminals could also arise from collaterals originating from KOR-expressing cortical neurons. Recent work suggests that KOR may regulate subsets of GABAergic neurons. Single-cell RNA sequencing suggests that KOR mRNA is expressed in subsets of SST interneurons (Fig. 1b, c) (Smith et al. 2019; Krienen et al. 2019, http://interneuron.mccarrolllab.org). Consistent with this notion, KOR activation decreases the excitability of GABAergic neurons in the insular cortex (IC) (Pina et al. 2020). Thus, KOR may regulate cortical circuits by directly inhibiting excitatory and inhibitory neurons and presynaptic terminals (Fig. 1b, c). What is currently not clear is how the different sources of DYN in the cortex, extrinsic or intrinsic, activate different pools of KORs.

As described above, PDyn is metabolized to various opioid-active and inactive DYN peptides and metabolites by a plethora of processing enzymes. Metabolites of DYN peptides include peptides that lack significant affinity for opioid receptors, but have been reported to act on non-opioid targets. An example of non-opioid effects of DYN metabolites are tyrosine-lacking DYNs (i.e., DYN A 2–17) that have been shown to directly modulate NMDA receptor activity (Chen et al. 1995a, b; Caudle and Dubner 1998). Differential expression of peptidases that catalyze the metabolism of PDyn and its metabolites may exist between brain regions, which is predicted to influence the profile of extracellular DYNs and their metabolites. PDyn metabolism occurs inside dense-core vesicles that contain readily releasable DYN peptides as peptidases that metabolize PDyn are expressed in dense-core vesicles (Day et al. 1998). PDyn is the most abundant product in dense-core vesicles (Yakovleva et al. 2006), suggesting a means for signals to be amplified if multiple DYN species are generated and released from a single PDyn molecule. Adding to the complexity, exogenously applied DYN B is preferentially converted to DYN B (1–7) and Dyn B (2–13) in primary somatosensory cortex and striatum, respectively (Bivehed et al. 2017). This is consistent with the hypothesis that once DYN is released, the time course of opioid-active DYN and/or engagement of non-opioid actions of DYN metabolites may be different in cortical and subcortical structures. Moreover, proprotein convertases and metalloproteinases, enzymes that process PDyn-derived peptides within cells and after release, respectively, are differentially expressed in cortical layers (Allen Brain Atlas, Lein et al. 2007). Thus, differences in the breakdown of DYN peptides may even exist within different cortical layers. Taken

together, PDyn metabolism may contribute to differential time courses and signaling by DYNs and their metabolites in cortical vs. subcortical structures.

1.3 Function of the DYN/KOR System in the Cortex

Similar to what has been described in the dorsal and ventral striatum, KORs in the PFC negatively regulates DA dynamics (Fig. 1c). Systemic injection of a KOR agonist inhibits extracellular DA levels in the mPFC (Tejeda et al. 2013). Previous research has shown that KOR within the VTA directly inhibits the excitability of DA neurons, including those that project to the PFC (Margolis et al. 2003, 2006; Ford et al. 2006; Baimel et al. 2017). However, KOR regulation of DA terminals in the PFC has been less explored. KOR activation using salvinorin A or U50,488 inhibits evoked release of [^{3}H] DA from PFC synaptosomes, and this is blocked by the KOR antagonist norbinaltorphimine (norBNI) (Heijna et al. 1990; Grilli et al. 2009). These results suggest that KORs may directly inhibits DA terminals within the mPFC (Fig. 1c). Consistent with this notion, mPFC extracellular DA levels are reduced and enhanced by local administration of KOR agonist and antagonist, respectively, indicating that KOR tonically inhibits DA levels in the mPFC (Fig. 1c). Furthermore, mPFC extracellular DA levels are not inhibited by local administration of a KOR agonist in mice lacking KOR in VTA DA neurons, unequivocally demonstrating that KOR directly inhibits DA terminals in the mPFC (Tejeda et al. 2013). Interestingly, repeated systemic administration of a KOR agonist increases evoked mPFC extracellular DA levels (Fuentealba et al. 2010), suggesting that repeated activation of this system disinhibits mPFC DA dynamics. Collectively, these results demonstrate that KOR negatively regulate DA levels in the mPFC by directly inhibiting DA neuron activity at the cell bodies and release at DA terminals within the PFC (Fig. 1c).

KOR in the PFC also inhibits excitatory transmission via a presynaptic site of action within the cortex to regulate information processing (Fig. 1c). An early study showed DYN perfusion causes inconsistent modulation of electrically evoked excitatory postsynaptic potentials (EPSPs) in frontal cortical slices (Sutor and Zieglgansberger 1984), but has no effect on exogenously applied glutamate responses. KOR activation inhibits K^+-stimulated glutamate release from frontoparietal cortex synaptosomes (Sbrenna et al. 1999). Consistent with a presynaptic site of action, the majority of mPFC KOR immunoreactive sites are localized to axon terminals of excitatory synapses (Svingos and Colago 2002). KOR activation decreases mEPSP frequency, but not amplitude, in PFC neurons in line with a presynaptic site of action (Tejeda et al. 2013). Additionally, activation of mPFC KOR inhibits local extracellular glutamate levels in freely moving animals (Tejeda et al. 2013). Presynaptic inhibition of excitatory synapses by KOR confers pathway-specific regulation, as systemic administration of a KOR agonist inhibits basolateral amygdala-evoked excitatory synaptic responses in the mPFC without altering hippocampus-evoked responses in vivo (Fig. 1c). Furthermore, local administration of a KOR agonist into the mPFC inhibits electrical and optogenetic BLA-evoked

excitatory synaptic responses in the mPFC, an effect blocked by a KOR antagonist (Tejeda et al. 2015). Taken together, KOR inhibits excitatory synapses in the mPFC via a presynaptic site of action and confers pathway-specific modulation (Fig. 1c).

Very little work has focused on the role of KOR in cortical inhibitory synapses. In the mPFC, KOR immunoreactivity in inhibitory cells has a more diverse localization, as compared to the excitatory cells (Svingos and Colago 2002). KOR immunoreactivity is present in presynaptic terminals of symmetric synapses, a feature of inhibitory synapses. This suggests that KOR may inhibit the release of GABA (Fig. 1c). In frontoparietal cortex synaptosomes, KOR activation inhibits K^+-stimulated GABA release from synaptosomes (Sbrenna et al. 1999), consistent with presynaptic KOR inhibition of GABA transmission. In the insular cortex, DYN A decreases the excitability of GABAergic interneurons, an effect blocked by KOR antagonism without directly inhibiting GABA release (Pina et al. 2020). KOR activation also inhibits glutamate-driven increases of GABA release (Tejeda et al. 2013), suggesting that KOR may also influence feedforward inhibition in cortical circuits. Taken together, evidence suggests that the DYN/KOR system may regulate inhibition within cortical circuits (Fig. 1c). However, inhibitory cortical microcircuits are complex and formed by diverse cell populations, and insight into cell type-specific regulation is currently lacking.

1.4 Behavioral Effects of the DYN/KOR System in the Cortex

As previously mentioned, multiple lines of evidence suggest that the DYN/KOR system regulates mood, motivation, stress-related behavior, as well as learning and memory. Much work has focused on the role of this system in subcortical limbic circuits. However, the specific role of the cortical DYN/KOR system in these aspects is less explored.

The DYN/KOR system mediates negative emotional states. Activation of this system leads to aversive and dysphoria-like behavior in both animal models (Mucha and Herz 1985) and humans (Pfeiffer et al. 1986). Genetic deletion of PDyn or systemic blockade of KOR in mice prevents stress-induced dysphoria (McLaughlin et al. 2003, 2006; Land et al. 2008). Notably, direct infusion of KOR agonist or a metabolically stable analog of DYN into the mPFC produces conditioned place aversion (CPA) (Bals-Kubik et al. 1993). Furthermore, the CPA induced by systemic KOR agonist is blocked by intra-mPFC microinjection of the KOR antagonist norBNI (Tejeda et al. 2013), indicating mPFC KOR signaling is necessary for KOR-mediated aversion.

Mice lacking PDyn or treated systemically with KOR antagonists display decreased anxiety-like behavior (Knoll et al. 2007; Wittmann et al. 2009; Carr and Lucki 2010; Kastenberger et al. 2012). Limited evidence from the mPFC has revealed inconsistent roles of the DYN/KOR system in anxiety-like behavior. Intra-infralimbic cortex infusion of a KOR agonist produces an anxiolytic behavioral profile on the elevated plus maze, whereas a KOR antagonist produces an anxiogenic effect in mice (Wall and Messier 2000a, b). However, another study showed that

intra-mPFC injection of a KOR antagonist had an anxiolytic effect in an open field (Tejeda et al. 2015). The discrepancy between these studies may be due to differential targeting of mPFC sub-regions, differences in mouse genetic backgrounds, and/or type of task used to assess anxiety-like behavior. Future work should be aimed at further dissecting the role of cortical KOR in regulating anxiety-like behavior.

In the forced swim test (FST) and learned helplessness test, Wistar Kyoto (WKY) rats, which have increased KOR expression in the brain, showed exaggerated depression-like behavior relative to Sprague–Dawley (SD) rats (Pare 1994; Lopez-Rubalcava and Lucki 2000; Carr et al. 2010). Systemic KOR antagonists selectively produce antidepressant-like effects in WKY rats, but not in SD rats, as assessed by reduced immobility in the FST (Carr et al. 2010). This effect may be attributed to higher levels of KOR mRNA and DYN A peptide expression in the piriform cortex and nucleus accumbens (NAcc), respectively (Carr et al. 2010). KOR antagonist treatment significantly enhances FST-induced increase of c-fos-positive neurons in the piriform cortex and NAcc shell of WKY rats. Furthermore, infusion of a KOR antagonist directly into the piriform cortex decreases immobility of WKY rats in FST, implicating the KOR system in the piriform cortex in generating depressive-like behavior in response to stress (Carr et al. 2010).

The DYN/KOR system is also implicated in mediating aversive learning and memory (Knoll et al. 2011; Cole et al. 2011, 2013; Rogala et al. 2012; Bilkei-Gorzo et al. 2012; Szklarczyk et al. 2015). Genetic deletion of PDyn or systemic antagonism of KOR in mice promotes cue-dependent fear conditioning and delays extinction of contextual fear memories (Bilkei-Gorzo et al. 2012). During the fear extinction phase, enhanced c-fos expression of infralimbic neurons observed in wild type mice is abolished in mice lacking PDyn (Bilkei-Gorzo et al. 2012). Human subjects bearing the TT allele of *PDYN* at rs1997794, which is associated with lower PDyn expression, show stronger fear reactions after conditioning, impaired extinction of the conditioned fear response, and altered PFC activity and amygdala-PFC coupling during extinction (Bilkei-Gorzo et al. 2012). Although mPFC plays a critical role in the acquisition and extinction of fear memory, direct evidence linking the mPFC DYN/KOR system to fear learning and memory is still lacking. In the primary somatosensory cortex (S1), blocking KOR activation delays the acquisition of the whisker trace eyeblink conditioning learning wherein whisker stimulation is paired with eyeball shocks (Loh and Galvez 2015). Interestingly, associative learning transiently increases PDyn expression in S1 GABAergic SST neurons without changing SST neuronal density (Loh et al. 2017). These results suggest that expression changes of PDyn in SST interneuron may represent a functional state of these neurons. Thus, DYN/KOR signaling in cortical circuits shapes aversive learning; however, it is currently unclear whether DYN/KOR signaling would produce differential effects in various cortical regions.

Physiological and psychosocial stress trigger a cascade of autonomic responses throughout the body. The mPFC is positioned to modulate the autonomic nervous system by sending direct and indirect innervations to the autonomic regions of the brain stem (Terreberry and Neafsey 1987; McKlveen et al. 2015; Kataoka et al.

2020). Emerging evidence indicates that the DYN/KOR system in the mPFC regulates stress-induced autonomic responses. Blocking KOR in either prelimbic or infralimbic cortex reduces the increase in mean arterial pressure and heart rate, but not the fall in tail temperature and increase in body temperature, induced by restraint stress (Fassini et al. 2014, 2015). Since autonomic responses mediate changes in adaptive and maladaptive responses to threats, gaining a deeper understanding of the effects of DYN/KOR signaling on autonomic and behavioral responses is warranted.

1.5 Implication for Human Health and Diseases

Post-mortem analysis suggests that cortical DYN and KOR mRNA expression are altered in patients with psychiatric disorders. Lifetime marijuana and psychostimulant use are associated with increased PDyn mRNA expression in the cortex (Peckys and Hurd 2001). In these same samples, a consistent decrease in expression level is observed across patients with depression, bipolar disorder, and schizophrenia relative to controls, though omnibus statistics fail to reach significance. Moreover, decreased PDyn mRNA expression is observed in the dorsolateral PFC of alcoholics, while Dyn B levels are unaffected (Bazov et al. 2018). Collectively, post-mortem studies suggest that life experiences and mental health states are associated with alterations in the cortical DYN/KOR system. However, assessment of DYN/KOR function in vivo is needed to advance our understanding of the role of this system in neuropsychiatric disorders. Recently, a positron-emission tomography (PET) study utilizing a radiolabeled KOR antagonist demonstrated a positive relationship between binding of a radiolabeled KOR tracer in various prefrontal and primary sensory cortical structures and cocaine choices in a self-administration paradigm following cold pressor stress (Martinez et al. 2019). Moreover, KOR availability decreased after cocaine binge in mPFC and occipital cortex, with significant levels in the temporal and parietal cortices. This effect is interpreted as increased DYN release-induced displacement of tracer. These results are consistent with post-mortem studies suggesting that drugs of abuse modify the DYN/KOR system in cortical circuits. This also highlights the utility and strength of utilizing PET imaging of radiolabeled KOR ligands to interrogate this system in vivo and its potential use to assess endogenous DYN release indirectly.

Given that the DYN/KOR system gets recruited by aversive, stressful experiences and KOR antagonists decrease stress-induced mal-adaptive behaviors in preclinical animal models of neuropsychiatric disorders, substantial efforts are aimed at the development of KOR antagonists for the treatment of negative affect and stress-induced drug-seeking behavior (Chavkin and Koob 2016; Carlezon and Krystal 2016). Unfortunately, to date, there are no FDA-approved selective KOR antagonists. However, buprenorphine, an FDA-approved partial MOR agonist and KOR antagonist, is widely utilized in clinical settings. Buprenorphine is efficacious at treating negative symptoms in patients with treatment-resistant depression (Karp et al. 2014; Yovell et al. 2016) and decreases suicidal thoughts (Yovell et al. 2016).

Recently, a combination of buprenorphine and samidorphan, a MOR antagonist, was evaluated in patients with major depression. The rationale was to isolate KOR antagonism by buprenorphine via samidorphan antagonism of partial MOR agonism by buprenorphine. These studies demonstrated that for depressive symptoms, the samidorphan and buprenorphine combination lacked clinically relevant responses to standard antidepressants (Ehrich et al. 2015; Fava et al. 2016). Ketamine has been widely utilized as an anesthetic and recently gained FDA approval for the treatment of depression and suicide ideation (Kraus et al. 2019). The antidepressant effects of ketamine were blocked by naltrexone, a non-selective opioid receptor antagonist (Williams et al. 2018), suggesting that endogenous opioid systems are critical for antidepressant effects of ketamine. However, a smaller pilot study failed to replicate these findings (Yoon et al. 2019). Many differences may explain discrepancies between the former and latter studies, but a critical distinction is that Yoon et al. (2019) administered naltrexone two-six days prior to ketamine, while Williams et al. (2018) administered naltrexone 45 min prior to ketamine. One possibility is that ketamine may be exerting therapeutic effects via actions at the KOR as ketamine is a partial agonist at the KOR (Nemeth et al. 2010), however, this appears to be unlikely mechanism because of the low affinity to KOR. Importantly, various clinical trials examining the efficacy of selective KOR antagonists for the treatment of depression and/or addiction are currently in progress or have been recently completed. Contrary to promising results outlined above, a recent pilot study failed to find effects of CERC-501 (formerly LY-2456302, JNJ-67953964, now aticaprant), a KOR antagonist, on depressive symptoms or cocaine dependence (Reed et al. 2018). However, a multi-site transdiagnostic study in humans demonstrated that daily dosing with the KOR antagonist JNJ-67953964 improved anhedonia as measured by the SHAPS inventory, but not overall depressive and anxiety symptoms, and this was associated with increased activation of the ventral striatum (Krystal et al. 2020). This finding is critical as it helps to pinpoint loci in the central nervous system wherein KOR antagonism may be conferring therapeutic efficacy. This warrants future work aimed at delineating whether cortical and other subcortical limbic regions are engaged by KOR antagonism to improve outcomes in patients. The results of these studies will be invaluable for guiding future development of KOR ligands for psychiatric disorders. Collectively, these studies demonstrate that activation of KOR in humans induces negative affective states and psychotomimetic effects reminiscent of psychiatric disorders and that KOR antagonism may be a viable treatment for negative affective states in depression and potentially other psychiatric disorders.

1.6 Unknowns and Future Directions for the Field

1.6.1 DYN/KOR Regulation of Dendritic Integration

At the cellular level, KOR may play a role beyond simply inhibiting the activity of cortical neurons. By regulating various cellular elements, such as the presynaptic terminal (i.e., via KOR-expressing inputs or local neurons, Fig. 1c) and ionic

conductance in the postsynaptic cell (i.e. via G protein-coupled inwardly-rectifying potassium channels (GIRKs) in KOR-expressing neurons, Fig. 1b), the DYN/KOR system may ultimately shape integration within cortical circuits. This brings up four ways in which DYN/KOR systems may regulate individual neurons. There are (1) cortical neurons that are solely modulated by presynaptic KOR on afferent inputs, (2) cortical neurons that are only regulated post-synaptically via direct inhibition of intrinsic excitability or metabotropic activity, (3) neurons that are regulated by KOR via pre- and post-synaptic sites of action, (4) and/or neurons that have absolutely no level of DYN/KOR regulation. One of the first issues that must be resolved is to dissect whether DYNs and KOR is regulating synaptic transmission and exerting postsynaptic responses onto the same or separate neurons and the specific afferents that are KOR-regulated.

One of the functions that the DYN/KOR system may serve in the cortex is gating of synaptic integration to modify input/output transformations in cortical circuits. As outlined above, KOR in the cortex plays a modulatory role in regulating excitatory limbic and dopaminergic inputs to the PFC via a presynaptic site of action (Fig. 1c). DA transmission is hypothesized to provide rapid changes in synaptic weights and intrinsic excitability (Yagishita et al. 2014; Iino et al. 2020; Lahiri and Bevan 2020). By directly inhibiting DA release in the PFC, KOR may inhibit many of the functions subserved by DA receptor signaling in the PFC (reviewed in Seamans and Yang 2004; Tritsch and Sabatini 2012; Arnsten 2015). Lastly, KOR inhibition of DA release is predicted to produce diverse changes within cortical ensembles as heterogeneity in DA receptor expression is present within PFC circuits, and DA inputs target deeper layers (Vander Weele et al. 2018). Additionally, since DA innervation of cortical circuits is preferentially localized to frontal cortices, DYN/KOR signaling may differentially regulate synaptic integration in PFC circuits vs. more caudal primary sensory and associative cortices.

Synaptic integration and plasticity are also gated by N-methyl-D-aspartate (NMDA) receptor activity (Major et al. 2013; Palmer 2014; Augusto and Gambino 2019). By inhibiting glutamate release, KOR may decrease the probability of NMDA receptor activation. $GABA_B$ receptors, $G_{i/o}$-coupled GPCRs similar to KOR, inhibit NMDA receptor Ca^{2+} signaling (Chalifoux and Carter 2010), which would be predicted to inhibit Ca^{2+}-regulated processes in somatodendritic compartments essential for shaping input–output transformations. Occasional co-localization of KOR and NMDA receptors in dendrites (Svingos and Colago 2002) provides an anatomical basis for interaction between KOR and NMDA receptors similar to the one with $GABA_B$ receptors. Moreover, DYNs can positively or negatively allosterically modulate NMDA receptor activity (Chen et al. 1995a, b; Caudle and Dubner 1998), an effect that may influence excitatory transmission and/or synaptic integration. Further, using a phosphoproteomic approach, KOR activation was shown to influence the mTOR pathway, which in cortical neurons modifies neurite outgrowth, spinogenesis, and synaptic plasticity (Liu et al. 2019). KOR regulation of mTOR would likewise also alter the way dendrites integrate synaptic inputs. Taken together, the diverse actions of DYN/KOR signaling may converge to gate synaptic integration of cortical neurons.

KOR inhibition of cortical DA and excitatory transmission may regulate signal-to-noise ratio in cortical circuits by ensuring that only relevant inputs capable of overcoming inhibition of synaptic integration are selected to support adaptive behavior (Fig. 1c). Studies aimed at dissecting the contributions of DYN and KOR modulation of specific circuit elements (i.e. inhibition of excitation vs. DA) will be crucial for parsing out the contribution of each component to translationally relevant behavior. Another possibility is that the concerted actions of the DYN/KOR system on cortical circuits are all simultaneously necessary to have significant changes in behavior.

As changes in dendritic morphology in prefrontal cortical circuits is critical for mediating stress-induced mal-adaptive behavior and reversing these morphological changes is necessary for antidepressant effects (Duman and Duman 2015; McEwen et al. 2016; Moda-Sava et al. 2019), KOR action on dendritic integration may be essential for the role of this system in promoting depressive-like behaviors and the locus by which KOR antagonists may exert their therapeutic effects. Taken together, our limited understanding of the cortical DYN/KOR system suggests that by impacting multiple aspects of cellular physiology (i.e., presynaptic and postsynaptic effects), cortical DYN/KOR signaling may contribute to adaptive and mal-adaptive behavior.

1.6.2 Conservation Across Species

Differences in the DYN/KOR system across species are essential to consider, especially when drawing implications for human health and diseases from preclinical results. Others have extensively reviewed the conservation of the DYN/KOR system across species, and we invite our readers to consider such work (Abbadie and Pasternak 2002; Schwarzer 2009; Tejeda et al. 2012; Tejeda and Bonci 2019). The PDYN gene encodes the PDyn peptide and is known to have four exons in the human, mouse, and rat (Horikawa et al. 1983; Douglass et al. 1989; Sharifi et al. 1999). PDyn transcription start sites appear to be similar in both human and mouse genes in exons one and four and introns 1 and 2. However, studies suggest that human-specific alternative promoter usage is in exon four and intron 2 (Nikoshkov et al. 2005; Kimura et al. 2006). Also, at least two of seven PDyn mRNA splice variants, FL1 & FL2, identified in the human brain, are conserved in the mouse and rat genomes (Horikawa et al. 1983; Telkov et al. 1998; Nikoshkov et al. 2005; Schwarzer 2009). Importantly, there appears to be conservation of the functions encoded by PDyn across species. For example, mice carrying the mutation R212W in the PDyn gene display behaviors that resemble the symptoms and cerebellar pathology of patients with spinocerebellar ataxia (Smeets et al. 2015).

The OPRK1 gene encodes the KOR and has four exons in human, mouse, and rat (Telkov et al. 1998; Yuferov et al. 2004; Liu et al. 2009). Its transcription utilizes two promoters separated by a non-coding exon whose transcription start site appears to be similar in humans and mouse. However, studies suggest that the human OPRK1 gene transcription start sites are in exon two and intron 2, whereas those of the mouse OPRK1 are in exon one and intron 1 (Liu et al. 1995; Kimura et al. 2006; Sandelin

et al. 2007). Importantly, further work characterizing the transcriptional control of PDYN and OPRK1 across species is needed.

Early investigations into the regional distribution of KOR relied on binding studies that utilized antagonists to block other receptors except for the KOR, which was then bound and labeled by radioactive ligands (Goodman and Snyder 1982a, b; Slater and Patel 1983; Mansour et al. 1987). Radioactively labeled selective KOR agonists, such as [^{3}H]U69,539 and [^{3}H]CI977, were later used. On the other hand, the characterization of the distribution of PDyn mRNA in humans and rodents is largely based on mRNA expression studies (Morris and Herz 1987; Hurd and Herkenham 1993; Hurd 1996; Merchenthaler et al. 1997; Nikoshkov et al. 2005; Lin et al. 2006). Today, it is widely accepted that DYN and KOR expression is in brain regions implicated in psychiatric disorders, such as the cortex, basal ganglia, hippocampus, amygdala, thalamus, as well as monoaminergic midbrain and brainstem structures involved in motivation, pain and pruritic processing. Importantly, studies acknowledge there are differences in the DYN/KOR system across species. For example, the density of KOR expressed throughout the brain is lower in rodents as compared to guinea pigs and humans. Regarding regional differences, humans and guinea pigs appear to display higher KOR density in the cerebellum as compared to rats. In contrast, humans and guinea pigs appear to show less KOR density in the hippocampus and substantia nigra, as compared to rodents (Maurer 1982; Robson et al. 1985; Simonin et al. 1995). These results highlight the existence of potential differences in the physiology and function of the KOR between humans and other mammals, warranting the need for studying the DYN/KOR system in humans. Nonetheless, the use of mice will still be useful for dissecting the function of this system in cortical circuits and elucidating principles in DYN/KOR transmission. Recently developed radiolabeled KOR ligands have tremendous potential to enhance our understanding of KOR availability by monitoring its responses to manipulations in a clinically relevant manner in awake individuals. Importantly, radiolabeled KOR tracers also provide experimental endpoints that are more directly comparable between animal models and patients in clinical and laboratory settings. In non-human primates and humans, radiolabeled KOR ligands have shown KOR binding sites in cortical regions and subcortical limbic regions that innervate the cortex (Tomasi et al. 2013; Vijay and Ravichandran 2016; Matuskey et al. 2019; Martinez et al. 2019), consistent with ex vivo observations. Results deriving from such work could then be directly compared with findings from studies employing novel fluorescent biosensors to monitor KOR activation dynamics in behaving animals.

Some PFC functions, such as working memory and attention selection, require DA and activation of dopamine D1 receptors (D1R) (Sawaguchi and Goldman-Rakic 1991; Granon et al. 2000; Arnsten 2015). Normal PFC performance is optimal within a range of DAergic activity. Suboptimal or excessive DA or D1R activation is detrimental for working memory. The control by DA over working memory has been found to follow an inverted-U-shaped curve with regard to working memory, but may differ for cognitive flexibility (Goldman-Rakic et al. 2000; Vijayraghavan et al. 2007; Floresco 2013). DA differentially modulates the signal-to-noise ratio in

pyramidal neurons (Vander Weele et al. 2018). Moderate activation of the D1R facilitates postsynaptic NMDA currents, which might facilitate sustained excitation of PFC circuitry during working memory (Zheng et al. 1999; Chen et al. 2004). High concentrations of DA reduce NMDA-mediated currents and postsynaptic potentials in pyramidal neurons (Castro et al. 1999; Zheng et al. 1999; Burke et al. 2018). Thus, the optimum range of DA levels must be heavily regulated in PFC. PFC DA release is inhibited by the KOR system (Tejeda et al. 2013) and mPFC KOR activation modulates working memory in the PFC (Wall and Messier 2000b, 2002). Since the KOR system modulates DA, it is possible that the KOR system limits DA levels below an optimal range to impair performance of PFC function. Conversely, in hyper-DAergic states associated with mPFC circuit dysfunction and working memory deficits, KOR system activation may buffer against deficits. Studies have also shown that aversive stimulation increases DA release in PFC of rodents, non-human primates, and humans (Jackson and Moghaddam 2004; Lammel et al. 2011; Lataster et al. 2011; Kodama et al. 2014; Vander Weele et al. 2018), but it is currently not clear how DYN/KOR might buffer these increases to prevent or exacerbate stress-induced working memory deficits. In aversive and/or stressful behaviors, the KOR system could be dysfunctional to allow an enhancement on DA release in PFC, shifting the performance of PFC to a dysregulated state. Thus, a better understanding of the mechanisms behind reduced DA release may be useful for the development of treatments for cognitive deficits.

Basolateral amygdala (BLA) hyperactivity is observed in most forms of anxiety disorders in humans, and in anxiety-like behaviors in rodents (Rauch et al. 2003; Roozendaal et al. 2009; Rosenkranz et al. 2010; Johnson et al. 2018). Optogenetic activation of BLA-PFC projection increases anxiety-like behavior (Felix-Ortiz et al. 2016). In addition, the deletion of KOR from BLA neurons increases anxiety-related behaviors (Crowley et al. 2016). This indicates that KOR dysregulation may play a role in BLA hyperactivity in the development of anxiety-related behaviors and mood disorders. Activity in the BLA-PFC pathway regulates anxiety-like behavior and conditioned fear (Senn et al. 2014; Felix-Ortiz et al. 2016; Klavir et al. 2017). Control of these behaviors might be driven by different BLA ensembles with opposing functions (Senn et al. 2014). Given that KOR stimulation inhibits BLA-evoked synaptic responses in the PFC (Fig. 1c) (Tejeda et al. 2015), it is possible that KOR may produce differential effects on anxiety-like behavior and fear depending on the functional state of the amygdala or the ensembles of cells that are regulated by presynaptic KOR. Indeed, intra-PFC blockade of KOR decreases or increases anxiety-related behaviors (Wall and Messier 2002; Tejeda et al. 2015), though examination of these effects within sub-regions of the mPFC may help to resolve this issue. Taken together, the cortical DYN/KOR system regulates anxiety-related behaviors, and it is predicted that depending on the functional state of the amygdala, KOR inhibition of the BLA to PFC pathway or desensitization of this modulation may normalize behavior or impair it, respectively. However, further work is necessary to test this hypothesis.

1.6.3 Site of Psychotomimetic Effects

The psychotomimetic and dysphoric effects of KOR agonists limit their therapeutic potentials as analgesics, antipruritics, and anti-addiction medications. Studies of selective KOR agonists in healthy volunteers have documented psychotomimetic symptoms as disturbances in the perception of space and time, visual and auditory hallucinations, racing thoughts, feelings of body distortion, and discomfort (Pfeiffer et al. 1986; Reece et al. 1994; Walsh et al. 2001; Wadenberg 2003; Addy 2012; MacLean et al. 2013; Maqueda et al. 2016). Studies on schizophrenia and other psychotic and thought disorders indicate that the perceptual disturbances are associated with hyperactivity in the associative sensory cortex and increased dopamine transmission in associative striatum (Horga and Abi-Dargham 2019). Psychoactive substances, such as ethanol, morphine, Δ9-tetrahydrocannabinol, and ketamine also have psychomimetic effects. Functional connectivity studies in humans show that subjective effects of perception by these drugs correlate significantly with the connectivity of the posterior cingulate cortex and precentral gyrus within the sensorimotor network, whereas no significant correlations were found for relaxation or dysphoria (Kleinloog et al. 2015). Interestingly, nalfurafine (TRK-820), a KOR agonist that has been used safely in Japan for treatment of uremic pruritus, shows a low rate of adverse drug reaction of hallucinations in human (Kumagai et al. 2010, 2012; Kozono et al. 2018) and is not aversive in mice (Mori et al. 2002; Liu et al. 2019). A large-scale mass spectrometry-based phosphoproteomics study indicates that compared to nalfurafine, the psychotomimetic KOR agonist U50,488 significantly regulates the mTOR signaling pathway in striatum and cortex and Wnt signaling pathway in hippocampus and cortex (Liu et al. 2019). Furthermore, inhibition of the mTOR pathway abolished U50,488-induced aversion, without affecting analgesic, anti-pruritic, and sedative effects in mice (Liu et al. 2019). These studies suggest that disturbed cortical circuitry is at least partially responsible for the psychotomimetic effects of DYN/KOR system activation. To further understand the mechanisms of KOR agonist-induced psychosis, several questions need to be addressed: (1) Are the psychotomimetic effects of KOR activation caused by direct or indirect disturbance of cortical circuitry function? (2) How is the cortex modulated in a region- and cell type-specific manner upon KOR activation? (3) Are alterations in cortical DA transmission, among other monoamines, also involved in psychotomimetic effects? (4) How does the alteration of connectivity between cortical and subcortical areas contribute to the psychosis in neuropsychiatric disorders?

In addition to activating KOR, endogenous DYNs can directly modulate the activation of glutamate NMDA receptors. DYNs (1–13, 1–17, and 1–32) shorten the mean open time and decrease the likelihood of opening of NMDA receptors in a non-KOR-dependent manner (Chen et al. 1995a, b), via des-Tyr DYN peptides. In consideration of the psychotomimetic potential of NMDA receptor antagonists, the fact that DYNs may reduce glutamate release via KOR and have KOR-independent NMDA antagonist-like properties adds to the mechanisms by which DYNs may participate in psychotic disorders associated with neuropsychiatric conditions, such as postictal seizures (Bortolato and Solbrig 2007). Although several studies show

that the mechanism of KOR-mediated hallucinations may differ from ketamine-mediated hallucinations (Butelman et al. 2004, 2010; Maksimow et al. 2006; Willmore-Fordham et al. 2007; Baker et al. 2009; Killinger et al. 2010; Ranganathan et al. 2012), further investigations are needed to evaluate the contribution of KOR-dependent and NMDA-dependent psychotomimetic effects of DYNs in psychological conditions.

1.6.4 Interactions with Other Stress-Related Peptides/Systems

Another major family of neuropeptides involved in stress responses are corticotropin-releasing factor (CRF) peptides (Henckens et al. 2016). Evidence suggests that CRF may exert some of its effects on behavior indirectly via the KOR system (Land et al. 2008; Bruchas and Chavkin 2010; Braida et al. 2011; Van't Veer et al. 2012; Beard et al. 2015; Donahue et al. 2016). Conversely, evidence suggests that the KOR system could be a downstream signal for stress and CRF action on behavioral modulation. Microinjection of CRF in the central nucleus of the amygdala (CeA) or systemic administration of a high dose of ethanol increased extracellular DYN levels, and this effect was blocked by a CRF-2 receptor antagonist (Lam and Gianoulakis 2011). CRF and the KOR system oppositely modulate GABAergic neurotransmission in the CeA (Nie et al. 2004; Kang-Park et al. 2013). KOR antagonist or KOR genetic deletion enhanced inhibitory responses in the CeA, and this was blocked by a CRF-1 receptor antagonist (Kang-Park et al. 2015). KOR or CRF-2 receptor antagonists block CRF-induced aversive behavior, but CRF-2 receptor antagonist does not prevent a KOR agonist-induced aversive behavior (Land et al. 2008). KOR antagonist in BLA prevents CRF-1 receptor-mediated anxiety-like behaviors (Bruchas et al. 2009). Taken together, CRF-1 receptors may recruit downstream DYN/KOR signaling to facilitate anxiety-like behaviors and CRF-2 receptors interact with KOR to mediate aversion.

Little is known about CRF and KOR system interaction in the cortex, but both systems are expressed in the cortex (Swanson et al. 1983; Svingos and Colago 2002; Yakovleva et al. 2006; Sohn et al. 2014; Deussing and Chen 2018). Evidence suggests a possible interaction of CRF and KOR systems in PFC as these two systems modulate similar inputs to the mPFC. Mice with genetic disruption of CRF-1 receptor in dopaminergic neurons release less DA in the PFC after footshock stress (Refojo et al. 2011), similar to the KOR system modulation of dopamine release in PFC (Margolis et al. 2006; Tejeda et al. 2013). Recent evidence shows that CRF-2 receptor has an inhibitory control over glutamatergic transmission between BLA and PFC (Yarur et al. 2020), similar to KOR inhibition of the glutamatergic BLA input to PFC (Tejeda et al. 2015). DYN and CRF neurons are in deeper and superficial layers of the PFC, respectively (Swanson et al. 1983; Peckys and Hurd 2001; Sohn et al. 2014), raising the possibility that CRF neurons and DYN neurons are engaged by stressors to regulate PFC circuits in a layer-specific manner. Further investigating potential interactions of CRF and KOR systems in cortical circuits could aid in the development of therapeutic targets for stress-related disorders.

1.6.5 Novel Approaches to Study the DYN/KOR System

The development of fluorescence-based GPCR sensors has expanded our ability to determine neuromodulator dynamics with sub-second temporal resolution, ex vivo and in vivo. A KOR-based fluorescence sensor was previously published (Patriarchi et al. 2018). This sensor is an engineered KOR with a modified third intracellular loop containing a circularly permuted green fluorescent protein (cpGFP) that inherently lacks $G_{i/o}$ G proteins- and β-arrestin-dependent signaling. Upon ligand binding to the receptor, the GPCR undergoes a conformational change that subsequently causes a conformational change in the cpGFP to induce fluorescence.

Intracerebral microdialysis is a technique that is well established for directly assessing extracellular levels of small molecules, such as neurotransmitters. However, the detection of extracellular peptides has been stymied by the lack of techniques with adequate sensitivity of peptide detection. Though few studies have utilized radioimmunoassay to detect extracellular DYNs, these approaches lack sensitivity to detect DYNs in regions that may be poor in DYN expression, require a decrease in temporal resolution to obtain larger samples, and are limited to detection of specific epitopes recognized by particular DYN antibodies (You et al. 1994; Marinelli et al. 2006). Recent breakthroughs utilizing mass spectrometry will allow for evaluation of the different DYN species present in the extracellular space and reveal biotransformation of DYNs in vivo (Al-Hasani et al. 2018). These cutting-edge approaches will be invaluable for studying the identities of DYN peptides released, the kinetics and mechanisms of peptide release in cortical circuits, the source of endogenous ligands for KOR within cortical microcircuits and to monitor endogenous peptide release time-locked to behaviorally relevant events.

Other approaches to detect DYN/KOR signaling are to utilize biological readouts of KOR activation. For instance, KOR undergo phosphorylation upon agonist binding. Immunoreactivity against this phosphorylated KOR has been successfully utilized to examine activation of KOR (Appleyard et al. 1997; Land et al. 2008; Chiu et al. 2017; Liu et al. 2019). With the continued development of tools to monitor intracellular signaling cascades downstream of G-protein-mediated KOR signaling, it is possible to manipulate either KOR function/expression or DYN expression and determine how this impacts signaling. One such candidate is protein kinase A, a known downstream target of GPCRs that can be assayed using fluorescence imaging approaches (Chen et al. 2014). The use of KOR-specific nanobodies will also allow for determining functional states of KOR and provide sub-cellular resolution of KOR signaling (Che et al. 2018, 2020). Similarly, KOR activation produces well-established physiological effects, such as the inhibition of neurotransmitter release or activation/inhibition of ion channels in postsynaptic compartments. This provides an additional endpoint that can be used to monitor peptide release and receptor activation using electrophysiological approaches. Furthermore, the use of single-cell recordings coupled with transgenic and viral approaches to express optogenetic actuators in specific elements of cortical circuitry will provide the means to dissect how DYN/KOR signaling is embedded within microcircuits.

Lastly, incorporation of established genetic approaches such as transgenic animals, CRISPR-based gene editing, RNA-based genetic ablation will be crucial

to casually link observed changes in the DYN/KOR system to changes in circuit function and behavior. Transgenic approaches include the use of animals expressing recombinases under the control of either the DYN or KOR promoter to gain genetic access to molecularly defined neurons within discrete circuits. With advances in intersectional approaches capitalizing on the expression of different recombinases to selectively express transgenes or ablate expression of endogenous opioid peptides/receptors (Fenno et al. 2014), further refinement of cell populations based on molecular identity and/or connectivity will be possible. Generation of mice expressing KOR fused to fluorescent proteins as has been published for other opioid receptors (Chen et al. 2020; Scherrer et al. 2006) will reveal how KOR are distributed in networks and are trafficked ex vivo and in vivo in response to behavioral experiences. Development of knock-in or CRISPR-edited mice endogenously expressing mutated KOR that lack specific components of intracellular signaling will allow the role of downstream consequences of KOR signaling on circuit dynamics and behavior. In conjunction with behavioral and in vivo and ex vivo physiological approaches, as well as those outlined above, advances in techniques will provide a means to causally link the role of the DYN/KOR system in shaping cortical dynamics.

Thus, as a field, we are finally positioned to start to utilize well-established techniques in conjunction with novel, cutting-edge techniques to dissect how DYN/KOR signaling modifies circuit function to influence behavior.

2 Conclusions

In conclusion, here we provide an overview of the literature on the cortical DYN/KOR system (Fig. 1). Less is known about this system in the cortex relative to its subcortical counterparts. Furthering our understanding of this system will be invaluable for identifying how neuropeptide systems regulate information processing in cortical circuits and associated behaviors. Further, capitalizing on novel approaches and translational work will help elucidate novel treatments for neuropsychiatric disorders.

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Molecular Genetics of Kappa Opioids in Pain and Itch Sensations

Pang-Yen Tseng and Mark A. Hoon

Contents

Abstract

The opioid peptides and their receptors have been linked to multiple key biological processes in the nervous system. Here we review the functions of the kappa opioid receptor (KOR) and its endogenous agonists dynorphins (Goldstein A, Tachibana S, Lowney LI, Hunkapiller M, Hood L, Proc Natl Acad Sci U S A 76:6666–6670, 1979) in modulating itch and pain (nociception). Specifically, we discuss their roles relative to recent findings that tell us more

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about the cells and circuits which are impacted by this opioid and its receptor and present reanalysis of single-cell sequencing data showing the expression profiles of these molecules. Since the KOR is relatively specifically activated by peptides derived from the prodynorphin gene and other opioid peptides that show lower affinities, this will be the only interactions we consider (Chavkin C, Goldstein A, Nature 291:591–593, 1981; Chavkin C, James IF, Goldstein A, Science 215:413–415, 1982), although it was noted that at higher doses peptides other than dynorphins might stimulate KOR (Lai J, Luo MC, Chen Q, Ma S, Gardell LR, Ossipov MH, Porreca F, Nat Neurosci 9:1534–1540, 2006). This review has been organized based on anatomy with each section describing the effect of the kappa opioid system in a specific location but let us not forget that most of these circuits are interconnected and are therefore interdependent.

Keywords

Dynorphin · Itch · Kappa opioid receptor · Nociception · Pain · Pruriception · somatosensory

Abbreviations

BLA	Basolateral amygdala
BNST	Bed nucleus of the stria terminalis
CeA	Central nucleus of the amygdala
CGRP	Calcitonin gene related peptide
CNS	Central nervous system
CPA	Conditioned place aversion
DRG	Dorsal root ganglion
DRN	Dorsal raphe nucleus
GRP	Gastrin-releasing peptide
Grpr	Gastrin-releasing peptide receptor
KOR	Kappa opioid receptor
LTMR	Low threshold mechanoreceptor
Mrgpra3	Mas-related G-protein coupled receptor A3
Nppb	Natriuretic polypeptide B
PAG	Periaqueductal gray
PbN	Parabrachial nucleus
RVM	Rostral medial medulla
TG	Trigeminal ganglion
VMH	Ventral medial hypothalamus

1 The Kappa Opioid System in Pain

The opioids enkephalin and endorphin are the superstars of pain treatment, possibly because they were identified many years before dynorphins (Kieffer 1999), and mu receptor agonists are the most clinically used opioids. Nevertheless, it is now recognized that dynorphins have many effects on how nociceptive signals are

modulated and some of these effects are as dramatic as those produced by enkephalin and endorphin. This has led to the re-evaluation of KOR agonists as potential therapeutics. Similar to the other opioid peptides, dynorphins and their receptors are expressed in neurons along the pain neural-axis and this is reflected in the multiple different effects of kappa opioids (Corder et al. 2018). However, the expression patterns of the different opioids and their receptors are distinct from each other suggesting that they elicit separate influences on pain sensory perception (Mansour et al. 1994; Neal et al. 1999).

1.1 Peripheral Effects of Dynorphins (DRG and TG Neurons)

Peripherally selective KOR agonists can inhibit nociception showing that they attenuate behavioral responses at the level of sensory neurons (Kivell and Prisinzano 2010; Haley et al. 1990; Vanderah 2010), although it has been suggested that agonist decreases pain/itch-stimulated behaviors through non-selective decrease in motivated behavior (Lazenka et al. 2018). Given this fact, it was suggested that KOR is expressed in nociceptors (Ji et al. 1995) and recently this was directly shown using transgenic mice engineered to drive the expression of genetic reporters under the control of the KOR gene (Snyder et al. 2018). These studies established that KOR is expressed in an array of dorsal root ganglion (DRG) and trigeminal ganglion (TG) cell-types. There is prominent, about 2/3 of positive neurons, co-expression of KOR with CGRP and other vasodilatory neuropeptides (Fig. 1a, d). The remaining KOR expressing neurons are mostly low threshold mechanoreceptors (LTMR) (Fig. 1b, e). Also, interestingly it was shown that a substantial proportion of KOR expressing neurons innervate viscera, including innervating the colon and bladder (Fig. 1c, f). This result agrees with the increased visceral chemical nociceptive sensitivity phenotype exhibited by KOR null mice (Simonin et al. 1998) and the known efficacy of kappa agonists to attenuate visceral pain (Junien and Riviere 1995). Analysis of results from single-cell sequencing (Zeisel et al. 2018) supports this distribution of KOR expression in sensory neurons (Fig. 1d–f). The KOR is a $G_{\alpha i/o}$-linked G-protein coupled receptor, which upon activation leads to inhibition of neural responses. This inhibition was reported to be presynaptic on central projections of primary afferents in the spinal cord and probably occurs through inhibition of voltage-gated calcium currents (Bean 1989; Snyder et al. 2018) (Figs. 1c and 3a). Importantly, when the KOR is genetically eliminated, or when KOR agonists are administered, basal nociceptive responses, to evoked noxious thermal and mechanical stimuli, are normal. By contrast, after injury KOR null mice display hypersensitivity and kappa-agonist produces analgesia (Vanderah 2010; Cowan et al. 2015; Simonin et al. 1998), suggesting that the effects of KOR are context dependent. In addition to acting on presynaptic transmission, dynorphins can attenuate neurogenic inflammation (Fig. 1a). This inflammatory response is a hallmark of release, from peripheral nerve endings of nociceptors at target organs, of various peptides including CGRP and substance P (in the skin and various visceral organs) which cause vasodilation and a resultant extravasation reaction (Fig. 1a, d). The KOR is predominantly expressed by peptidergic neurons and consequently

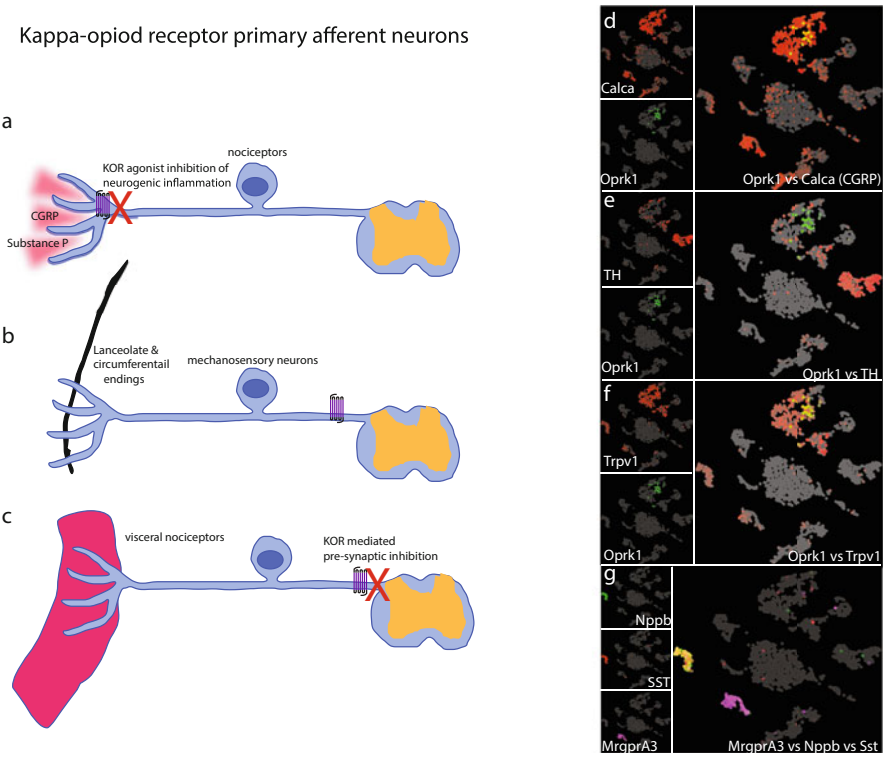

Fig. 1 KOR is expressed in multiple classes of DRG neurons. (**a–c**) Schematics of the potential mechanisms by which KOR modifies nociception in primary sensory afferents. (**a**) The majority of KOR expressing neurons co-expresses the neuropeptide CGRP and substance P. Upon activation these neurons can release these peptides from their nerve terminals. KOR agonists can attenuate this release and thereby suppress neurogenic inflammation. (**b**) Mechanosensory neurons also express KOR. These neurons have terminal specializations that surround hair follicles including lanceolate and circumferential endings. (**c**) A large number of KOR positive afferents project to visceral targets and kappa agonists reduce nociception from these fibers via presynaptic inhibition. (**d–g**) Single-cell RNA-sequencing is a powerful method to comprehensively identify and classify neuronal subtypes. We re-analyzed publically available single-cell RNA-sequencing data from dorsal root ganglia (Zeisel et al. 2018). To visualize these high-dimensional datasets, we used UMAP plots which cluster cells with the most similar gene expression with each other and separate them from other clusters. Therefore, in this representation DRG neurons segregate into clusters where neurons with common transcriptomes are closest. Each cell in this analysis is represented as a dot (gray) with colored (red or green or magenta) gene expression superimposed. The individual gene name is displayed in the left-hand of each panel and co-expression is shown in the merged right panels. (**d**) KOR (OprK1) and CGRP (Calca), (**e**) KOR and tyrosine kinase (TH), a marker of a subset of mechanosensory neurons, (**f**) KOR and Trpv1, a marker of nociceptors, are co-expressed. (**g**) Nppb and somatostatin (SST) are co-expressed, but are distinct from Mrgpra3-expressing neurons and neither of these two classes of primary afferent express KOR

attenuating KOR activity in sensory neurons has a major influence on extravasation by reducing the release of vasodilatory peptides (Snyder et al. 2018). Together these findings highlight that KOR can participate in the modulation of nociception; however, under physiological conditions it is still unclear under what circumstances dynorphins are released and might influence neurogenic inflammation and modulate presynaptic signaling. It is also still not known the sources of dynorphins (there are no major exogenous sources outside the CNS, although some glia and immune cells may release dynorphins (Pannell et al. 2016; Wahlert et al. 2013) which could control the activity of peripheral nociceptors, but like for enkephalins and endorphins, dynorphins are expressed in a subset of spinal cord interneurons and this may be the source of released peptide (see next section).

1.2 Dynorphin Neurons in the Spinal Cord

Unlike the peripheral nervous system, the spinal cord has both neurons which express KOR and dynorphins (Sardella et al. 2011). The suggested actions of dynorphins and KOR in the spinal cord are predicated by the fact that intrathecal administration of dynorphin produces both mechanical and thermal allodynia (Laughlin et al. 1997; Vanderah et al. 1996). In addition, during persistent injury, levels of dynorphins increase dramatically in the ipsilateral spinal cord (Iadarola et al. 1988) and the increased dynorphins encompass spinal cord territories outside those innervated by injury (Malan et al. 2000). This upregulation of dynorphins appears to be mainly in the spinal cord (Parra et al. 2002), although there are other supraspinal regions with heightened expression (see later sections). The genetic elimination of dynorphins results in a mild increase in sensitivity to noxious stimuli in naïve mice. After injury, for a short period, normal increased sensitization was observed in KOR null mice (Wang et al. 2001). However, while in wild-type mice sensitization remains after nerve injury, in animals lacking KOR, at latter times, sensitization does not continue but instead nociceptive responses return to baseline. Altogether, these results have been interpreted to suggest that during injury, in the spinal cord, dynorphins appear to act to increase nociceptive sensitivity while paradoxically in naïve conditions, they act tonically to suppress nociceptive signals. These opposing actions of dynorphins, as inhibitors in naïve conditions and as facilitators of nociception in persistent pain, are likely due to the known plastic circuit changes in the spinal cord brought about during central sensitization which alter the spinal circuitry and thereby alter the action of dynorphins (Melzack and Wall 1965; Woolf and Chong 1993), as well as the increased expression of dynorphins (see summary comments).

To examine more about the role of dynorphins in the spinal cord, the cells which express dynorphins and their synaptic connections have been investigated (Duan et al. 2014). Using molecular genetics, mice were engineered where the intersection of the recombinases for Cre and Flpo driven by prodynorphin and Lbx1-promoters, respectively, was used to ablate neurons and mark subsets of interneurons that represent a neuronal lineage in the spinal cord of dynorphin expression during

development. This means that not only cells which express dynorphin in the adult spinal cord but also those which express dynorphin transiently during development were examined in these studies. The phenotypes of the resulting dynorphin-cell-lineage ablated animals were remarkable (Duan et al. 2014). These mutant mice exhibited profoundly heightened sensitivity to mechanical stimulation (but not to thermal stimuli), similar to that found during spared nerve injury (a model of neuropathic pain). Together with extensive characterization of the electrophysiological properties of the inputs and outputs to these neurons, these results suggest that the dynorphin/Lbx1 lineage of neurons is required to gate mechanical pain (Melzack and Wall 1965) and do this through inhibition of excitatory somatostatin-expressing interneurons. In other studies, the role of neurons expressing dynorphins rather than neurons transiently expressing *dynorphin* was investigated (Huang et al. 2018). The behaviors displayed by mice in which dynorphin neurons were chemogenetically activated revealed that these cells can sensitize mice to mechanical, but not thermal, stimuli and showed that these neurons are important in controlling itch (see later section). The contradictory findings of these two studies (Huang et al. 2018; Duan et al. 2014) are likely because of differences in the neurons which were manipulated in the two reports. A further complication is that the neurons which express dynorphin in adult spinal cord are also heterogeneous. Recently, neurons in the spinal cord were divided into different classes based on various genes they express. The sequencing of single spinal cord cells has led to their classification defined by their molecular characteristics. Analysis of this data revealed 15 classes of inhibitory and 15 types of excitatory neurons (Haring et al. 2018). This report suggests that dynorphin is expressed in 3 classes of inhibitory neurons, implying that the neurons expressing this neuropeptide are heterogeneous. However, this sequencing study did not reveal the whole story as it was reported that there is a class of excitatory dynorphin neurons (Duan et al. 2014; Huang et al. 2018; Gutierrez-Mecinas et al. 2019; Boyle et al. 2017) which are clearly absent from this sequencing study. Therefore, in future it will be important to determine which developmental class (es) of dynorphin-lineage neurons are required for gating mechanical pain and which neurons control signals for other sensory modalities including itch.

1.3 Functions of the Dynorphin/KOR System in the Parabrachial Nucleus

The parabrachial nucleus (PbN) is a brainstem nucleus which receives direct inputs from spinal cord projection neurons (Chiang et al. 2020; Barik et al. 2018) and other brain regions (Kim et al. 2020; Alhadeff et al. 2018) (Fig. 2). In addition to serving as a center where many different sensory inputs are received, the PbN is known to modulate pain signals and produce tiered responses to noxious stimuli by directing nociceptive signals to other brain regions (Campos et al. 2018; Han et al. 2015; Barik et al. 2018; Huang et al. 2019). These connections include those to the amygdala

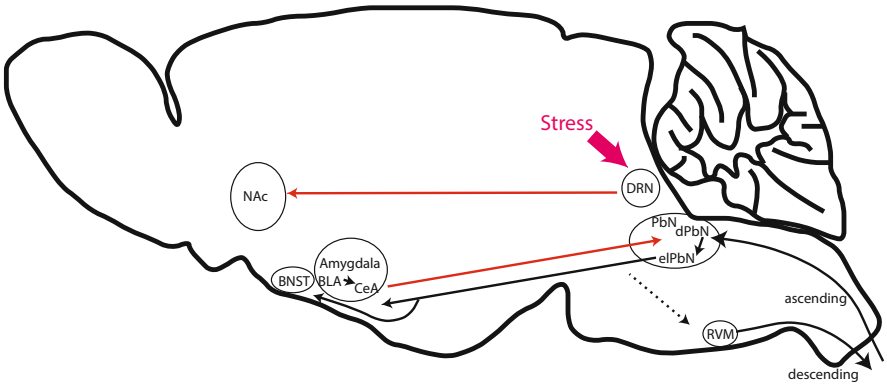

Fig. 2 Anatomy of the kappa opioid system controlling pain perception. Schematic diagram of the pain pathways which are modulated by kappa opioids. Red arrows indicate connections known to use dynorphin and those in black are links that are known to originate from either cells expressing KOR or dynorphins. The main inputs to these pathways are from spinal cord projection neurons or stress responses from the mid- and forebrain. Abbreviations, nucleus accumbens (NAc), bed nucleus of the stria terminalis (BNST), basolateral amygdala (BLA), central nucleus of the amygdala (CeA), dorsal raphe nucleus (DRN), parabrachial nucleus (PbN), dorsal PbN (dPbN), external lateral PbN (elPbN), and rostral ventral medulla (RVM)

(reciprocal), the rostral medial medulla (RVM), the periaqueductal gray (PAG), the ventral medial hypothalamus (VMH), and the bed nucleus of the stria terminalis (BNST). The PbN can be divided into several distinct sub-nuclei and a dynorphin ensemble of neurons is concentrated in the dorsal lateral region (dPbN) (Fig. 2). This ensemble has received attention in terms of their projections and their contribution to pain escape behavior and memory of pain stimuli (Chiang et al. 2020). Dynorphin neurons send projections to the external lateral PbN (elPbN), although they also send them to the PAG, PVT, and various nuclei in the hypothalamus (Chiang et al. 2020; Kim et al. 2020). It was shown that dynorphin neurons trigger aversive memory, but do not affect escape behaviors or analgesic responses to nociceptive stimulation, suggesting their major projections that control responses to nociceptive signals are directed to the CeA and BNST (Chiang et al. 2020). The dynorphin population of neurons may not specifically tuned for noxious stimuli as they have also been shown to serve functions in temperature homeostasis (Geerling et al. 2016) and control ingestion responses to mechanical stimuli (Kim et al. 2020). This suggests the dPbN dynorphin neurons are part of a network that can generate a variety of appropriate behavior based on coordination with other brain nuclei, some of which may be in the parabrachial nucleus. Alternatively, the dynorphin neurons might be heterogeneous with different sub-populations serving distinct functions.

1.4 Role of Kappa Opioids in Pain-Induced Stress Responses in the Dorsal Raphe Nucleus, Amygdala, Rostral Ventral Medulla, and Nucleus Accumbens

Stress responses are strongly associated with pain and dynorphin has been shown to mediate critical elements of these reactions (Bruchas et al. 2010; Wittmann et al. 2009). For instance, stress induced by forced swim results in attenuated tail withdrawal to heat that is dependent on kappa signaling (McLaughlin et al. 2003). Using an elegant in vivo assay, brain regions, where KOR is activated by stress, were uncovered (Land et al. 2008; Bruchas et al. 2007, 2008). This approach showed, in response to stress, there is activation of KOR in the dorsal raphe nucleus (DRN), the basal lateral amygdala, the hippocampus, the ventral palladium, the ventral tegmental area, nucleus accumbens (NAc), and BNST. These results were the basis for a series of investigation aimed at understanding the contribution of the kappa opioid system in pain related stress responses and other effects of KOR.

The limbic centers in the brain are concerned with motivation, learning, and emotion, driving such behaviors as anxiety, depression, and fear. Since the limbic system receives and processes stress signals, it is one of key centers concerned with controlling negative affect associated with pain. The amygdala is at the center of the limbic brain and is divided into several nuclei which are believed to serve distinct functions. The central nuclei of the amygdala (CeA) have a large number of dynorphin-expressing inhibitory neurons. In the CeA, specific classes of neurons are inhibited by nerve injury or are silenced by general anesthetics (as well as other stimuli) (Wilson et al. 2019; Hua et al. 2020). Activation of one of these classes of inhibitory neurons attenuates injury-induced pain related behaviors. The attenuation of nociceptive behaviors by these neurons is thought to occur through a disinhibitory CeA–PbN circuit which under normal conditions suppresses nociception (Wilson et al. 2019). And at least in part, the CeA dynorphin neurons are likely a component of this CeA–PbN circuit (Kissiwaa et al. 2020; Raver et al. 2020). This is also in line with the high expression of the KOR by PbN-neurons (Meng et al. 1993) (Fig. 2). The CeA is also known to influence descending modulation of diffuse noxious inhibitory control through a KOR dependent process (Navratilova et al. 2019; Phelps et al. 2019). The exact pathways producing this effect is unknown, however, it has been suggested this inhibition may occur via a PbN to the rostral ventral medulla (RVM) circuit (Navratilova et al. 2019; Phelps et al. 2019) (Fig. 2).

Aversive responses are evoked by exposure to pain and can generate conditioned place aversion (CPA). Several brain nuclei are known to be required for CPA, one of which is the dorsal raphe nuclei (DRN) and mice exposed to stress show dynorphin release and increased KOR activation in this major serotonergic nucleus (Land et al. 2008). It was demonstrated that CPA responses can be induced by injection of KOR agonists in the DRN (Land et al. 2009). In addition, genetic elimination of KOR and blockade of KOR in the DRN prevent the development of these aversive responses (Land et al. 2009). The DRN sends projections broadly throughout the brain and is involved in modulating many different processes. The specific projections of DRN neurons to the NAc are required to induce CPA (Fig. 2). In addition, supporting this

circuit in modulating responses to painful stimuli, mice lacking central serotonergic neurons lack KOR agonists-induced analgesic responses (Zhao et al. 2007).

KOR agonist has long been recognized to produce dysphoria (Land et al. 2008). Some of the depressive effects of dynorphins, including those associated with pain, have been linked with changes in regulation of dynorphin expression in the NAc (Land et al. 2009). The NAc is responsible for integrating both positive and negative valence associated with stimuli (Al-Hasani et al. 2015). Chronic pain states are well known to be comorbid with many types of negative affect. Linking these phenomena, it was shown that KORs in a subregion of the NAc, the ventromedial shell, are plastically regulated by pain states (Massaly et al. 2019; Liu et al. 2019). In normal conditions there is a relatively low kappa tone in the NAc and, through presynaptic inhibition, KOR minimally lowers the release of dopamine, serotonin, and glutamate reward signals in this nucleus. By contrast, during persistent pain, when dynorphin expression increases (and perhaps in other centers in the hypothalamic and in the ventral palladium), elevated dynorphins increase KOR activity and there is a consequent reduction in the release of reward transmitters by the NAc (Massaly et al. 2019; Liu et al. 2019).

1.5 Summary and Future Direction of Kappa Signaling in Control of Pain

In summary, the kappa opioid system is found in many different areas of the brain which are important for pain perception. However, the known location where kappa signaling has effects is likely not the only places where this system operates and there are several other brain regions implicated in pain processing which express dynorphins/KOR that have not yet been investigated. Among these are the nucleus of the solitary tract (NTS), the locus coeruleus, the medial thalamic nuclei (parafiscular nucleus), the agranular insular cortex, and several hypothalamic nuclei. In the future, exploration of these centers and their targets should be fertile ground for the discovery of novel aspects of kappa opioid influence on pain signaling.

2 Kappa Opioids in Itch

Even though historically more has been studied about the interaction of the kappa opioid system with the pain system, recently there were many insightful advances which highlight a distinct role for dynorphins in pruriception (itch). Interestingly, in contrast to morphine which stimulates itch (Ballantyne et al. 1988), KOR agonists suppress itch. In fact, these divergent outcomes, on itch responses, illuminate one of the many places where these two different opioids have different effects in the nervous system (Sakakihara et al. 2016).

2.1 Dynorphin Spinal Cord Interneurons Inhibit Itch

The importance of spinal cord inhibitory neurons in itch was first prominently revealed by the spontaneous itch phenotype exhibited by Bhlhb5 (Bhlhe22) knockout mice (Ross et al. 2010). Bhlhb5 is a transcriptional factor that mediates neuronal cell fate determination and loss of Bhlhb5 leads to programmed cell death of many of the neurons in which it is expressed. Specifically, Bhlhb5 null mice developed self-inflicted skin lesions and display augmented itch responses upon pruritogen challenge. Selective knockout of Bhlhb5 in the forebrain (dorsal telencephalon), DRG, or spinal cord, revealed that the phenotype is due to the loss of interneurons in dorsal horn of the spinal cord, particularly neurons in lamina I and II and not caused by the loss of expression of Bhlhb5 in other brain areas. Additional studies showed that 75% of Bhlhb5 derived interneurons are inhibitory (Pax2+) while 25% are excitatory neurons. Further, the selective ablation of inhibitory, but not excitatory, Bhlhb5 interneurons was sufficient to recapitulate the spontaneous itch phenotype exhibited by global Bhlhb5 knockout animals (Ross et al. 2010). These results strongly suggest that a subpopulation of inhibitory interneurons in the spinal cord dorsal horn acts to gate chemical itch.

Since Bhlhb5 is transiently expressed during early development and its expression level in adult interneurons is low, molecular markers of these neurons were unclear. It is known that about 50% of inhibitory neurons in spinal cord dorsal horn selectively express somatostatin receptor type 2 (Sstr2) and these interneurons can be subclassified based on the expression of the neuropeptides dynorphin and galanin, and based on the presence of neuronal nitric oxide synthase (Nos1) (Boyle et al. 2017; Polgar et al. 2013a, b) (Fig. 3a, b). Immunohistochemistry uncovered that inhibitory Bhlhb5 neurons are a subpopulation of Sstr2 inhibitory neurons that co-express galanin and dynorphin (Kardon et al. 2014) (Fig. 3c). The fact that Bhlhb5 neurons co-express dynorphin was intriguing because it had been previously shown that kappa opioid agonists can inhibit itch evoked by various pruritogens including histamine, chloroquine, bombesin, and compound 48/80 (Cowan et al. 2015; Inan and Cowan 2004; Kumagai et al. 2010; Togashi et al. 2002). Therefore, it was hypothesized that the spontaneous itch phenotype displayed by Bhlhb5 knockout mice might be due to the loss of dynorphin inhibition in a spinal cord itch pathway. This predicted that the disruption of endogenous dynorphin-kappa opioid receptor signaling should increase itch sensitivity. As proposed, the pharmacological blockage of kappa opioid receptor by intrathecal injection of antagonists norbinaltorphimine (norBNI) or 5́-guanidinonaltrindole (5́-GNTI) resulted in increased itch responses to chloroquine (Kardon et al. 2014). However, the elimination of the dynorphin gene (Pdyn) did not produce a spontaneous itch phenotype and did not trigger exaggerated itch responses to pruritogens, suggesting that dynorphin neurons predominately communicate with downstream excitatory neurons with other neurotransmitters such as GABA or glycine (Cowan et al. 2015; Kardon et al. 2014), at least in the experimental conditions used.

Although dynorphins seem dispensable, multiple lines of evidence support the concept that dynorphin interneurons dampen itch sensation. As previously

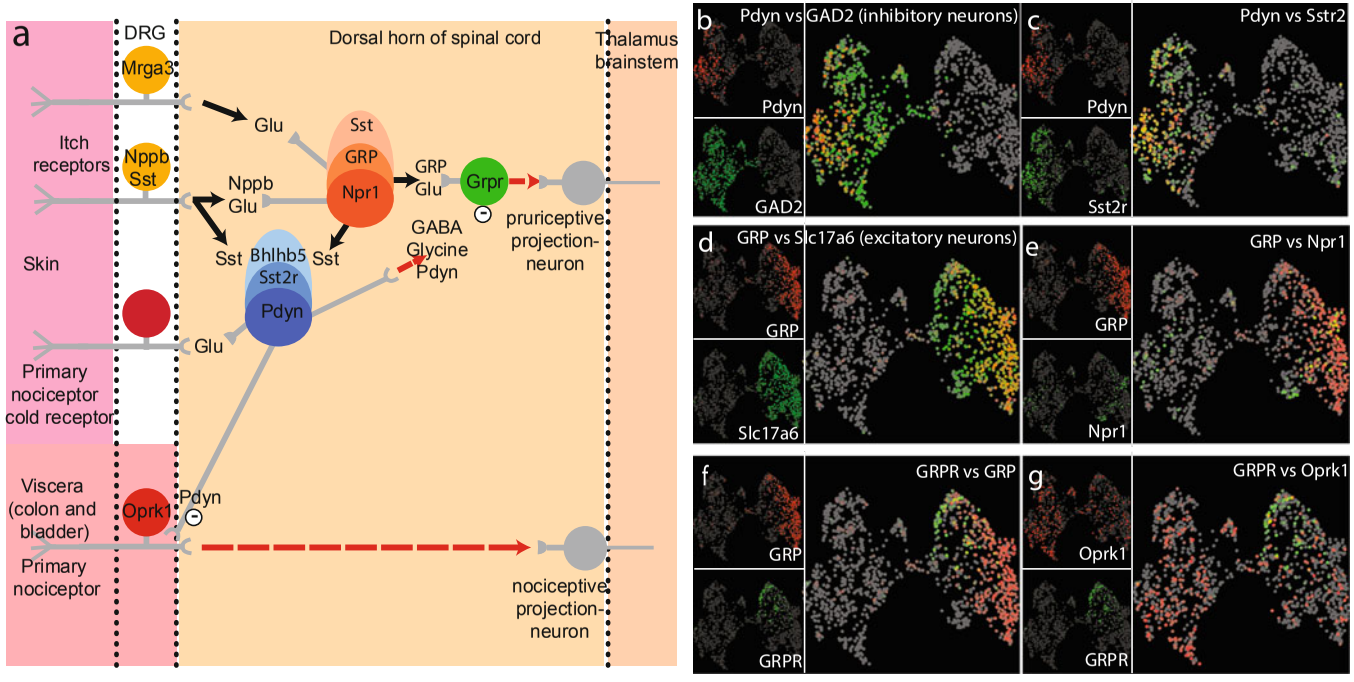

Fig. 3 Spinal cord itch circuit and KOR signaling. (**a**) Schematic representation of the neuronal circuit underlying itch. Input from DRG neurons (left-hand side) is transmitted to spinal cord interneurons (center), and then onto the brain (right-hand side). Key transmitters and cell-types are indicated, dotted arrowed lines are speculative connections. (**b**–**g**) Single-cell RNA-sequencing (GSE103840, Haring et al. 2018) was re-analyzed and UMAP plots (see Fig. 1 for details) of spinal cord neurons reveal that cells can be broadly classified into two groups, GABAergic inhibitory neurons (**b**) and excitatory glutamatergic neurons (**d**), based on their expression of *glutamate decarboxylases 2* (*GAD2*) and vesicular glutamate transporter 2 (Slc17a6), respectively. (**b**, **c**) Many inhibitory neurons express dynorphin (Pdyn) and somatostatin receptor 2 (Sstr2), respectively. (**d**) By contrast excitatory neurons express GRP. (**e**) In turn a subset of GRP-neurons co-expresses Npr1. (**f**) GRP and GRPR are expressed by separate populations of interneurons. (**g**) KOR (Oprk1) is expressed at low levels in both excitatory and inhibitory neurons and is expressed by GRPR-neurons

mentioned, dynorphin neurons co-express somatostatin receptor. This receptor is coupled to inhibitory G-protein alpha subunit (G_i) to inhibit adenylyl cyclase and calcium influx. Indeed, applying somatostatin on Bhlhb5 neurons caused strong hyperpolarization and mice receiving intrathecal injection of somatostatin analog octreotide trigger vigorous itch behaviors (Kardon et al. 2014). Furthermore, the chemogenetic activation (DREADD hM3Dq) of dynorphin-expressing neurons (Pdyn-cre::AAV-flex-DREADDq) in lumbar spinal cord dorsal horn effectively reduced scratching responses evoked by pruritogens including histamine, chloroquine, and octreotide (Huang et al. 2018). These results showed that inhibiting the dynorphin interneurons causes disinhibition of itch, while activating these neurons causes attenuation of itch (Fig. 3a). The ablation of dynorphin-lineage neurons (see Section: dynorphin neurons in the spinal cord) did not eliminate itch responses (Duan et al. 2014). However, not all dynorphin neurons were eliminated in the mice used in this study and many other types of inhibitory neurons were also ablated in these complex transgenic animals used in this study making it hard to interpret the results from this report (Duan et al. 2014; Huang et al. 2018).

2.2 Neuronal Circuits for Itch

Itch stimuli are detected by peripheral DRG and TG sensory neurons that innervate skin (Bautista et al. 2014; Hoon 2015; Mishra and Hoon 2015). Recent studies revealed two distinct sub-populations of DRG neurons that preferentially convey itch signals to the spinal cord dorsal horn. One class of cells co-express Mas-related G-protein coupled receptors A3 (Mrgpra3) and C11 (Mrgprc11) which can be activated by chloroquine (Han et al. 2013; Liu et al. 2009) and pruritic peptides BAM8–22, SLIGRL, respectively (Liu et al. 2009, 2011). The other subpopulation is a class of cells which express the neuropeptide, natriuretic polypeptide B (Nppb) and express multiple itch-receptors (Solinski et al. 2019b) (Figs. 1g and 3a). This raises the unexpected finding that KOR expression is not found in these neurons (Snyder et al. 2018; Zeisel et al. 2018) (Fig. 1g) despite the fact that peripherally restricted KOR agonist can suppress itch (Inan and Cowan 2004; Cowan et al. 2015). Perhaps there is modest CNS penetration of these KOR agonists (see next section).

When Nppb is released from itch neurons, it activates spinal cord interneurons expressing its receptor, natriuretic peptide receptor 1 (Npr1) (Mishra and Hoon 2013; Solinski et al. 2019a). Interestingly, Nppb-neurons also co-express somatostatin (~99% overlap) (Huang et al. 2018) (Fig. 1g). Given that dynorphin interneurons express somatostatin receptor and can be inhibited by somatostatin or its analog (Huang et al. 2018; Kardon et al. 2014), it is likely that somatostatin DRG neurons, and perhaps somatostatin-expressing interneurons, are the upstream afferents for dynorphin interneurons. Indeed, optogenetic activation of somatostatin-neurons (Sst-cre::ChR2) in the TG preferentially evoked robust itch, but not pain behaviors (Huang et al. 2018). However, somatostatin is expressed in both peripheral sensory neurons and spinal cord excitatory interneurons (Fig. 3a). To pin down the source of somatostatin, mice with conditional knockout of somatostatin

in either peripheral afferent neurons (Trpv1-cre::$Sst^{fl/fl}$), spinal cord dorsal horn (Lbx1-cre::$Sst^{fl/fl}$), or both (Wnt-cre::$Sst^{fl/fl}$) were tested. Only the mice lacking somatostatin in both peripheral and spinal cord neurons exhibited itch deficits to pruritogens including histamine, chloroquine, compound 48/80, serotonin, and endothelin (Huang et al. 2018). These results suggested that dynorphin/Sstr2 interneurons receive presynaptic inputs from both peripheral afferents and dorsal horn interneurons.

In the dorsal horn of the spinal cord, a subpopulation of excitatory neurons expressing gastrin-releasing peptide receptor (Grpr) is critical for itch sensation (Fig. 3a, d). Mice lacking Grpr or Grpr-expressing neurons displayed profound deficits in itch responses to various pruritogens (Sun and Chen 2007; Sun et al. 2009). In addition, activation of Grpr-neurons requires release of Gastrin-releasing peptide (Grp) from Grp-expressing interneurons (Pagani et al. 2019) (Fig. 3f) and some Grp interneurons co-express Nppb receptor, Npr1 (Fig. 3a, e). Targeted-toxin ablation of these neurons (by administration of Nppb-saporin conjugate) greatly reduced itch responses evoked by histamine or by intrathecal administration of Nppb, suggesting that these neurons transmit itch signals from Nppb primary afferents (Mishra and Hoon 2013). Interestingly, Grp interneurons also form monosynaptic connections with Mrgpra3 primary afferents (Albisetti et al. 2019; Sun et al. 2017). These studies suggest that Grp-expressing neurons receive inputs from both Mrgpra3 and Nppb primary afferents and this Grp–Grpr axis in spinal cord could be the major pathway for itch signal transmission. Itch evoked by intrathecal injection of Grp can be greatly attenuated by kappa opioids (nalfurafine), suggesting that kappa opioids and perhaps endogenous dynorphins act on or downstream of Grpr-expressing neurons (Kardon et al. 2014). Indeed, about 50% of Grpr-expressing interneurons co-express kappa opioid receptor (KOR) and kappa opioids inhibited calcium influx induced by applying Grp on spinal cord neurons (Munanairi et al. 2018) (Fig. 3g). These results indicate that Grpr-neurons receive excitatory inputs from Grp-expressing neurons but receive inhibitory inputs from dynorphin-expressing neurons. Most Grp-expressing neurons co-express somatostatin (Gutierrez-Mecinas et al. 2014), and some of them co-express Npr1 (Mishra and Hoon 2013). While dynorphin-expressing neurons co-express somatostatin receptor (Sstr2) (Kardon et al. 2014), suggesting a possible communication between Grp-neurons and dynorphin neurons via somatostatin. Indeed, Sstr2 agonist, octreotide, potentiated itch responses evoked by intrathecal administration of Nppb or Grp, while CYN154806, a somatostatin receptor antagonist, attenuated these itch responses. Furthermore, KOR agonist, ICI199441, attenuated Nppb, octreotide, and histamine-induced itch; however, CYN154806 could not attenuate KOR-antagonist (norbinaltorphimine) evoked itch (Huang et al. 2018). These results indicate that somatostatin can potentiate Nppb and Grp-evoked itch responses by disinhibition of dynorphin neurons. To determine the cellular cascade for itch signal transmission, conjugated toxins that selectively ablate Npr1- or Grpr-neurons were used. Ablation would only impact signals from upstream but not downstream components in this pathway. Ablation of Grpr-neurons with Grp-saporin as well as Grpr antagonist treatment profoundly reduced itch evoked by octreotide, KOR-antagonist, and

histamine. By contrast, ablation of Npr1-neurons by Nppb-saporin attenuated histamine-induced itch, but not octreotide or KOR-antagonist evoked itch. Together these results suggest that Npr1-neurons are upstream of somatostatin and dynorphin neurons, while Grpr-neurons are the downstream (Fig. 3a).

2.3 Summary and Future Direction for Itch Research

The role of neuropeptides in the control of itch signaling is remarkably complex involving multiple transmitters (Fig. 3a). In the spinal cord, dynorphin is at the core of a disinhibition pathway. Disruption of disinhibition with kappa receptor agonists is now being used as a therapeutic basis for treatment of some types of chronic itch (Pereira and Stander 2018). The physiological role of this circuit is still not understood although it has been suggested to be a mechanism by which counter stimuli may attenuate itch (Kardon et al. 2014). In the future it will be interesting to explore the exact inputs to this pathway to better understand how counter-stimulus mechanistically attenuates itch sensory signaling.

3 Common Mechanisms of Action of the Kappa Opioid System

Kappa opioid-mediated signaling, for both itch and pain, is multifaceted with manifold ways in which this GPCR transduction cascade produces different effects in different circuits. Another aspect of the kappa opioid system is the context-dependent complexity of it use. Despite these complications, there appears to be three themes which overall explain the mechanisms by which the kappa opioid system works, see points below.

- KOR engagement can lead to reduction in neuronal activity through inhibition of voltage-gated calcium channels and other pathways; for instance, by attenuating the activity of peripheral nociceptors (Snyder et al. 2018).
- KOR activates an inhibitory signaling cascade causing hyperpolarization and within disinhibitory circuits this generates a net activation, for example, in response to stress a disinhibitory circuit is engaged in the NAc (Massaly et al. 2019).
- In different states, many neural systems show plastic expression of dynorphins and/or KOR and undergo changes in circuitry. In turn, these changes can alter the valence of circuits, for example, in the spinal cord during chronic pain dynorphin levels increase dramatically and there are changes in the circuitry (Vanderah et al. 2001) which alters the effects of dynorphin from inhibitory to excitatory.

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Antipruritic Effects of Kappa Opioid Receptor Agonists: Evidence from Rodents to Humans

Saadet Inan and Alan Cowan

Contents

Abstract

Centrally administered bombesin induces scratching and grooming in rats. These behaviors were blocked by early benzomorphan kappa opioid receptor (KOR) agonists as reported by Gmerek and Cowan in 1984. This was the first evidence that KORs may be involved in the sensation of itch-like behaviors. Subsequent development of additional animal models for acute and chronic itch has led to important discoveries since then. For example, it was found that (a) gastrin-releasing peptide (GRP), natriuretic polypeptide b and their cognate receptors are keys for the transmission of itch sensation at the spinal cord level, (b) dynorphins (Dyns), the endogenous KOR agonists, work as inhibitory neuromodulators of itch at the spinal cord level, (c) in a mouse model for acute itch, certain KOR antagonists elicit scratching, (d) in mouse models of acute or

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chronic itch, KOR agonists (e.g., U50,488, nalfurafine, CR 845, nalbuphine) suppress scratching induced by different pruritogens, and (e) nalfurafine, CR 845, and nalbuphine are in the clinic or in clinical trials for pruritus associated with chronic kidney disease and chronic liver disease, as well as pruritus in chronic skin diseases.

Keywords

5′-GNTI · Animal models of itch · Difelikefalin · Itch · Kappa opioid receptor agonist · Kappa opioid receptor antagonist · Nalbuphine · Nalfurafine · norBNI

1 Introduction

There have been great advances in the field of itch research since its first-time description by the German physician, Samuel Hafenreffer (1587–1660), in a 1660 dermatology textbook as "an unpleasant sensation that provokes the desire to scratch." Historically, written medical mention of itch was first recorded in an Egyptian papyrus (Mueller et al. 2020). When the word "itch" was searched in PubMed, 26,136 studies were listed (until June 2020). Figure 1 summarizes the number of publications, both clinical and preclinical, reported in 10-year periods, beginning from 1940. The figure clearly shows that by the beginning of the1990s interest in itch research intensified. This might have been due to (a) a mouse model for itch developed by Kuraishi et al. (1995), (b) the founding of the International Forum for the Study of Itch (IFSI) in 2005, a scientific organization that brings together clinical and preclinical researchers studying itch, (c) advances in genetic

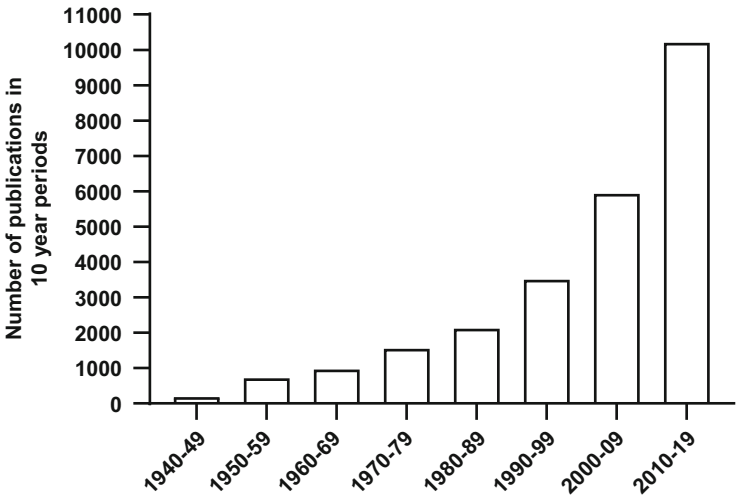

Fig. 1 Number of publications for itch found in PubMed for every 10-year period beginning from 1940. Significant increases have been observed since the beginning of the 1990s

and molecular techniques, and (d) appreciation of how chronic itch affects nearly 15% of the population (Matterne et al. 2011) and lowers the quality of life.

Advances in the last two decades can be outlined as:

1. Transmission of itch sensation: (a) Discovery of pruriceptors (nociceptors for itch) and transmission of itch sensation by myelinated A-delta and unmyelinated C-fibers, (b) demonstration of two pathways, one with mechano-insensitive C fibers for histamine-dependent transmission and a second with mechano-sensitive C and A-delta fibers for non-histamine-dependent transmission, (c) Discovery of different pruritogens and their receptors, (d) Validation of GRP and its cognate receptor GRPR as parts of the itch circuitry in the spinal cord as well as the role of the neurotransmitter natriuretic polypeptide b in releasing GRP in the spinal cord, (e) Discovery of inhibitory B5-I interneurons in the spinal cord, as well as the roles of glycine, gamma-amino butyric acid (GABA) and Dyn in inhibition of itch sensation in the spinal cord, and (f) Detection of a spinothalamic pathway and brain regions that are involved in the transmission and inhibition of itch. We encourage readers to peruse a review on physiology and pathophysiology of itch by Cevikbas and Lerner (2020).
2. Pathophysiology of itch: (a) Understanding of the role of the immune system in the pathogenesis of itch, at least in part, (b) Acceptance of itch of unknown origin, and its addition to classifications (Weisshaar et al. 2019), and (c) Acceptance of chronic itch as a syndrome or disease and recognition as a disease like chronic pain (Ständer 2019).
3. Treatment of itch: Important results with KOR agonists have been achieved. Nalfurafine (previously known as TRK 820, trade name Remitch) is approved in Japan for the treatment of chronic itch in kidney dialysis patients and chronic liver disease patients. Phase 3 clinical trials for the peripherally restricted KOR agonist, difelikefalin (previously known as CR 845, trade name Korsuva), against chronic itch in hemodialysis patients have yielded promising results (Fishbane et al. 2020). Additionally, Korsuva is in Phase 2 clinical trials for atopic dermatitis and itch in chronic liver disease. (https://www.caratherapeutics.com/pipeline-technology/our-pipeline/). Nalbuphine (trade name Nubain), a KOR agonist and mu opioid receptor antagonist, is in Phase 2b/3 clinical trials for chronic itch in prurigo nodularis and chronic itch associated with chronic liver disease as well as Phase 2 clinical trial for chronic itch in patients with chronic kidney disease (https://www.trevitherapeutics.com/pipeline/).

   Substance P/neurokinin 1 receptor antagonist, Serlopitant, shows promising results for pruritus in epidermolysis bullosa (Chiou et al. 2020), psoriasis (Pariser et al. 2020), and chronic pruritus of unknown origin in Phase 2 clinical studies.

   (http://www.menlotherapeutics.com/newsroom/menlo-therapeutics-announces-results-from-phase-2-trial-of-serlopitant-in-patients-with-chronic-pruritus-of-unknown-origin/). Targeting the immune system is also giving promising results for pruritus in atopic dermatitis (AD). Results of a Phase 2b study of nemolizumab, a humanized monoclonal antibody that targets alpha subunit of IL-31 receptors, showed that nemolizumab significantly improved skin lesions

and pruritus in AD patients (Silverberg et al. 2020). Another Phase 2b study in AD patients with lebrikizumab, a monoclonal antibody against IL 13, reported significant improvement in skin lesions and pruritus scores, compared to placebo (Guttman-Yassky et al. 2020).

This chapter provides a brief introduction to the field of itch and reviews evidence from animal studies from our and other laboratories for the involvement of KORs in inhibiting the sensation of itch as well as developments of KOR agonists for the treatment of itch clinically.

2 A Brief Introduction to the Field of Itch

2.1 Terminology and Classification of Itch

A guideline written by IFSI described itch as "a sensation that provokes the desire of scratch" and accepted that pruritus and itch are synonyms (Weisshaar et al. 2019). Itch lasting less than 6 weeks is described as acute and itch lasting 6 weeks or more is described as chronic. When itch is chronic, it can be very annoying and is associated with negative effects on the quality of life for patients, as mentioned below:

> I have an area on my neck that itches really badly several times a year. It is worse at night and this time I have had to use Benadryl topical cream eight times a day and take the pills also twice a day. It is only in one area. It has been ongoing for the last 5 years. It is getting worse and lasting longer with each episode. (https://comments.medicinenet.com/itch/patient-comments-907.htm)

Two types of itch have been classified: clinical- and etiological-based. According to the clinical-based classification, itch is grouped as follows:

- Group I. Pruritus in primarily diseased, inflamed skin
- Group II. Pruritus in primarily normal, non-inflamed skin and
- Group III. Pruritus with chronic secondary scratch lesions (Ständer et al. 2007; Weisshaar et al. 2019).

According to the etiological-based classification, itch is grouped as follows (Ständer et al. 2007; Hashimoto and Yosipovitch 2019; Rinaldi 2019):

- Pruritoceptive itch is due to dermatological diseases of the skin. Itch is induced by pruritogen activation of sensory fibers. Dermatological diseases like atopic dermatitis, contact dermatitis, scabies, prurigo nodularis, infections are causative examples of pruritoceptive itch.
- Neurogenic itch originates from organs other than skin, without nerve damage or a psychiatric disorder. Systemic diseases like chronic kidney failure, liver

diseases, hyperthyroidism, polycythemia vera, Hodgkin's and non-Hodgkin's lymphoma, and pruritus gravidarum are examples of neurogenic itch.
- Neuropathic itch is associated with nerve damage in peripheral or central nervous systems. Multiple sclerosis, post-herpetic neuralgia, and neoplasms are examples that may cause neuropathic itch.
- Psychogenic itch originates from psychiatric and psychosomatic disorders. Examples are itch associated with depression, obsessive compulsive disorder, and schizophrenia.

2.2 Animal Models of Itch

Several animal models for both acute and chronic itch have been established to study mechanisms and evaluate compounds for treating itch. Attempts to develop an experimental animal model began following a report that screened pruritogenic agents in humans by Arthur and Shelley in 1955. They discovered that *Mucuna pruriens,* known as cowhage, induces non-histamine dependent scratching. Joglekar et al. (1963) applied 5% cowhage ointment topically to dogs to provoke scratching.

Gmerek and Cowan (1983) introduced a rat model that allowed quantitative measurements of scratching behavior by intracerebroventricular administration of bombesin, a tetradecapeptide originally isolated from frog skin. Bombesin elicited dose-dependent excessive grooming and scratching of the face, head, and neck with the hind paws. It was known that bombesin is a homolog of mammalian GRP, subsequently identified as a mediator for itch (Sun and Chen 2007). Ko and Naughton (2000) developed a scratching model in monkeys by administering morphine intrathecally (i.t.), as is the case in humans (Baraka et al. 1981; Chaney 1995).

Kuraishi and his colleagues described an easily applicable experimental model in mice in 1995. They injected either a pruritogen or algesic subcutaneously (s.c.) behind the neck of mice and observed the animals for scratching bouts directed to the neck with hind legs. While pruritogens elicited scratching behavior, algesics did not. Figure 2 shows a mouse scratching after a pruritogen injection in the nape of the neck. This model has allowed the identification of neurotransmitters, neuromodulators, and receptors involved in the sensation of itch as well as possible therapeutic agents. Several substances were shown to induce scratching: serotonin (Yamaguchi et al. 1999; Nojima and Cartens 2003; Tian et al. 2016; Ostadhadi et al. 2017); chloroquine, an antimalarial drug that induces itch in certain black Africans (Inan and Cowan 2004); SLGRL, a proteinase-activated receptor-2 agonist (Shimada et al. 2006; Liu et al. 2011); Interleukin-31 (IL-31), a TH2 cell-derived cytokine (Cevikbas et al. 2014); and phoenixin, a brain-gut-skin peptide (Cowan et al. 2015). Some of their receptors were subsequently identified. For example, later studies with chloroquine implicated Mas-related G-protein coupled receptors (Mrgprs) in non-histaminergic itch (Liu et al. 2009). Recent studies indicate that Mrgprs are involved in the mechanism of cholestatic pruritus (Meixiong et al. 2009a, b).

Fig. 2 A scratching mouse following injection of a pruritogen in the nape of the neck. Mice are acclimated into observation cages with bedding for an hour. Following an injection of pruritogen agent, after briefly licking hind paw, the mouse scratches behind the neck area (Courtesy of Saadet Inan)

Shimada and LaMotte (2008) also described an experimental mouse model that enables differentiation of pain-induced behavior from itch-induced behavior by injecting agent intradermally (i.d.) to the cheek of the rodents. While an algogen induced wiping of cheek with forelimbs, an itch inducer agent caused scratching of cheek with hind limbs.

Animal models for diseases were also developed. Bile duct ligation or resection (Bergasa et al. 1992; Inan and Cowan 2005) and chronic administration of ethinylestradiol (Trauner et al. 2005; Inan and Cowan 2006) have been used to study cholestatic pruritus in rodents. NC/Nga mice, which have increased IgE levels and TH-2 cell-derived cytokines and have AD-like skin lesions, were utilized to study atopic dermatitis (Suto et al. 1999; Vestergaard et al. 1999; Takaoka et al. 2006; Grimstad et al. 2009; Arai et al. 2013). Also, topical application of certain compounds was used to induce AD-like pathogenesis, for example, oxazolone (Man et al. 2008), vitamin D3 (Li et al. 2006), ovalbumin (Yatsuzuka et al. 2007), and 2,4-dinitrofluorobenzene (DNFB) (Terakawa et al. 2008). Chronic topical application of DNFB or squaric acid dibutylester to the skin of a mouse produces an allergic reaction, which has been used as a model for allergic dermatitis (Fua et al. 2014; Zhang et al. 2015). An animal model of psoriasis was developed by application of imiquimod, a toll-like receptor 7 agonist, to the back of mice for 7 days (Sakai et al. 2016). A model for dry skin was established by 5-day application of acetone/ether (1:1 mixture) to the nape of the neck of mice (Miyamoto et al. 2002).

3 KORs Are Involved in the Sensation of Itch

Evidence for the involvement of KORs in the sensation of itch goes back to the 1980s. For example, Gmerek and Cowan (1984) reported that systemic administration of early benzomorphan KOR agonists (e.g., bremazocine, cyclazocine,

ketocyclazocine, and pentazocine) significantly reduced bombesin-induced grooming and scratching in rats in a dose-dependent manner. In another study, during withdrawal from chronic administration of the KOR agonist U50,488, excessive scratching was observed in monkeys (Gmerek et al. 1987). It had been postulated that an imbalance in the expression of mu receptors and KORs as well as their endogenous agonists might play an important role in itch. Mu opioid receptor antagonists suppress experimentally induced itch in primates and patients. Ko and Naughton (2000) reported that the MOR antagonist nalmefene abolished scratching induced by i.t. morphine in monkeys. When naloxone is administered s.c. to cholestatic patients, there is a reduction of itching as well as a precipitation of morphine withdrawal-like reactions, suggesting that upregulation of endogenous mu opioid function occurs during itch (Jones et al. 2002). In a rat model of cholestasis, decreased Dyn A-promoted [^{35}S]GTPγS binding in the hypothalamus and decreased serum Dyn A levels were described (Inan and Cowan 2005). One of the most exciting discoveries was that Dyn A acts as a neuromodulator for inhibiting itch in the dorsal horn of the spinal cord (Kardon et al. 2014). The next two sections summarize evidence for the role of KOR and its ligands in itch.

3.1 Kappa Opioid Receptor Antagonists Evoke Scratching in Mice

Kamei and Nagase (2001) reported for the first time that norbinaltorphimine (norBNI), the KOR antagonist, elicited scratching in a dose-dependent manner when given i.d. behind the neck of mice. We selected 5′-guanidinonaltrindole (5′-GNTI), as a more selective KOR antagonist, to further study KOR antagonist-induced scratching in mice. 5′-GNTI (0.03–3 mg/kg, s.c.) induced vigorous scratching in a dose-dependent manner and was more potent than norBNI, (A_{50} values of 0.16 mg/kg vs 7.0 mg/kg) (Cowan et al. 2002). Following injection of a submaximal dose (0.3 mg/kg) of 5′-GNTI, the maximal scratching occurred at +10–30 min and then faded gradually at +30–80 min. Tolerance did not develop to the scratch-inducing effect of 5′-GNTI when it was given once a day for 8 consecutive days (Cowan and Inan 2009). Further studies with 5′-GNTI showed that (a) administration of this agent to the right cheek of mice induced only scratching and grooming, (b) pretreatment with lidocaine (i.d.) inhibited 5′-GNTI-induced scratching and c-fos expression in the superficial layer of the dorsal horn of the spinal cord, (c) nalfurafine, a KOR agonist, suppressed 5′-GNTI-elicited scratching as well as c-fos expression in the superficial layer of the dorsal horn of the spinal cord, (d) pretreatment with GRP receptor antagonists, either RC-3095 or [DPhe^6]bombesin(6–13) methyl ester, did not suppress 5′-GNTI-induced scratching, (e) pretreating mice (i.t.) with McN-A-343, a muscarinic M1 receptor agonist, reduced 5′-GNTI-induced scratching (Inan et al. 2009a, b, 2011), and (f) surprisingly, pretreatment with norBNI (3 mg/kg, s.c. at −20 h) did not have any effect on 5′-GNTI-elicited scratching. Importantly, 5′-GNTI-induced scratching in KOR knock out (KO) mice was of a similar extent as that seen in wild type litter mates (Inan 2010). Thus, both pharmacological and genetic approaches to turn off

KORs did not suppress 5′-GNTI-induced scratching in our experiments. Morgenweck et al. (2015) reported that scratching induced by norBNI and 5′-GNTI was decreased by about 50–60% in KOR KO mice compared to wild type littermates, suggesting that KOR plays a role, but is not the only component, contributing to scratch-inducing effects of these compounds. The reasons for the discrepancy between our study and that of Morgenweck et al. (2015) are not clear.

Similar results were obtained with zyklophin, a chemically different KOR antagonist. Zyklophin (0.1–1 mg/kg, s.c.), a short-acting cyclic peptide KOR antagonist, induced dose-related scratching in mice (DiMattio et al. 2014). The maximum response was observed at +15 min and the scratching was over by +30 min. Pretreating mice with norBNI (3 mg/kg, at −20 h) had no antagonizing effect on zyklophin-induced scratching. KOR KO mice given zyklophin displayed a similar number of scratching bouts to the wild type littermates. Also, it should be noted that scratching starts a few min after the injection of these three chemically divergent KOR antagonists. However, KOR antagonism effects of norBNI and 5′-GNTI start at the earliest 2 h and 30 min following injection of the compounds (Endoh et al. 1992; Inan et al. 2009c). Concordant results with these three chemically different KOR antagonists, norBNI, 5′-GNTI and zyklophin, suggest that scratching behavior induced by KOR antagonists may not be through the KOR.

Notably, the itch-inducing effect of KOR antagonists is not only limited to mice. Modest pruritus was reported as a side effect of repeated administration of the novel short-acting KOR antagonist Opra kappa (also known as: LY2456302, CERC-501, JNJ-67953964), in ex-cocaine volunteers (Reed et al. 2018). Opra kappa is the first KOR antagonist given in humans for possible treatment of cocaine abuse. Further studies are required to understand the mechanisms behind how selective KOR antagonists induce scratching in mice and in humans.

3.2 Antipruritic Effects of Kappa Opioid Receptor Agonists Against Different Pruritogens

Cowan and Gmerek (1988) showed that the KOR agonists tifluadom and U50,488 were effective against bombesin-induced scratching and grooming in rats. Both enadoline and ICI 204,448 (a peripherally restricted KOR agonist) effectively inhibited compound 48/80-induced scratching in mice (Cowan and Kehner 1997). ICI 204,448 suppressed the scratching over 2 h and the effect was blocked by pretreatment with norBNI. ICI 204,448 also inhibited chloroquine-elicited scratching in mice (Inan and Cowan 2004). When ICI 204,448 was given once daily for 5 consecutive days, tolerance developed against the anti-scratch activity of ICI 204,448; however, tolerance was not observed when it was given every other day for 5 days.

Nalfurafine, CR 845, and nalbuphine are summarized separately below since they are in clinical trials and in the clinic.

Nalfurafine (Formerly TRK820, Remitch® Capsules)

Nalfurafine was introduced as an antinociceptive, structurally different, moderately selective, and potent KOR agonist by Nagase et al. in 1998. The first anti-scratch activity of nalfurafine was reported by Togashi et al. (2002) against histamine- and substance P-elicited scratching in mice. The anti-scratch effect was reversed by norBNI. In our studies, we found that nalfurafine was an effective anti-scratch agent against scratching induced by chloroquine (Inan and Cowan 2004), compound 48/80 (Wang et al. 2005; Liu et al. 2019), and 5′-GNTI (Inan et al. 2009b). In these studies we have shown that (a) its effects are dose-dependent (b) tolerance does not develop against the antipruritic effect (following once a day injection for 10 days), (c) nalfurafine is effective not only as pretreatment but also as post treatment, (d) nalfurafine did not affect locomotion at the doses producing the anti-scratch effect, and (e) nalfurafine inhibits c-fos expression induced by compound 48/80 and 5′-GNTI in the dorsal horn of mouse spinal cord. In addition, nalfurafine at the doses producing effective anti-scratch effect did not cause conditioned place aversion and induce only a low degree of motor incoordination, unlike typical KOR agonists such as U50,488 (Liu et al. 2019).

Phoenixins (PNX-14 and PNX-20), the recently discovered brain-gut-skin peptides (Cowan et al. 2015), were shown to have physiological roles in the reproductive system (Yosten et al. 2013). Cowan et al. (2015) showed PNX immunoreactivity in the dorsal root ganglia and superficial layers of the dorsal horn of the spinal cord as well as in skin. In addition, PNX-14 and PNX-20 induce scratching in mice and nalfurafine inhibits scratching elicited by PNXs.

The antipruritic effect of nalfurafine was also shown in disease models of itch in rodents. Nalfurafine suppressed the whole-body scratching in a dose-dependent manner in rats with cholestasis due to chronic injections of ethinylestradiol (Inan and Cowan 2006). Orally administered nalfurafine significantly reduced spontaneous scratching in MRL/lpr mice that were suggested as a model for autoimmune disease-associated pruritus (Umeuchi et al. 2005). Orally administered (Nakao et al. 2008) and topically applied (Elliott et al. 2016) nalfurafine was effective in pruritus of AD in mice. Nalfurafine inhibited dry skin-induced spontaneous scratching in mice (Akiyama et al. 2015). Intrathecally administered nalfurafine dose-dependently reduced i.t. morphine-induced scratching in mice (Sakakihara et al. 2016). Further, in primates, nalfurafine was effective against i.t. morphine-induced scratching (Wakasa et al. 2004; Ko and Husbands 2009).

The first clinical study to examine possible antipruritic effects of nalfurafine was reported in patients with pruritus associated with chronic kidney failure by Wikström et al. (2005). This study was followed by more open-labeled studies in uremic patients (Kumagai et al. 2010, 2012; Ueno et al. 2013). These reports showed that both i.v. and orally administered nalfurafine are effective antipruritics. Nalfurafine (Remitch® 2.5 and 5 μg tablets) was approved in Japan for treatment of pruritus in hemodialysis patients and in chronic liver disease patients. However, the application for use of injectable form of nalfurafine in Europe was withdrawn by Committee for Medicinal Products for Human Use of European Medicines Agency due to insignificant effectiveness compared to placebo (see the chapter "Clinical Profiles of

Table 1 Nalfurafine is an effective antipruritic against different pruritogens. The list shows studies that reported for the first time nalfurafine suppressing itch in rodents, non-human primates and humans

Pruritogen	Route (nalfurafine)	Species	Reference
Substance P, histamine	p.o.	Mouse	Togashi et al. (2002)
Chloroquine	s.c., p.o.	Mouse	Inan and Cowan (2004)
Autoimmune	s.c.	MRL/lpr mouse	Umeuchi et al. (2005)
Compound 48/80	s.c.	Mouse	Wang et al. (2005)
Ethinylestradiol -induced cholestasis	s.c.	Rat	Inan and Cowan (2006)
Atopic dermatitis	s.c.	NC/Nga mouse	Nakao et al. (2008)
5′-GNTI	s.c.	Mouse	Inan et al. (2009b)
Morphine	i.t.	Monkey	Ko and Husbands (2009)
Phoenixin	s.c.	Mouse	Cowan et al. (2015)
Dry skin	s.c.	Mouse	Akiyama et al. (2015)
Atopic dermatitis	Topical	Mouse	Elliott et al. (2016)
Morphine	i.t.	Mouse	Sakakihara et al. (2016)
Chronic renal failure	i.v.	Human	Wikström et al. (2005)

Nalfurafine Hydrochloride for the Treatments of Pruritus Patients"). More clinical studies are underway for long-term treatment of pruritus in liver diseases (Kamimura et al. 2017; Yagi et al. 2018). Antipruritic effects of nalfurafine are summarized in Table 1. A detailed review on nalfurafine in the clinic is given in the chapter "Clinical Profiles of Nalfurafine Hydrochloride for the Treatments of Pruritus Patients."

CR 845 (Difelikefalin, Korsuva™)

CR 845 (4-amino-1-(D-phenylalanyl-D-phenylalanyl-D-leucyl-D-lysyl) piperidine-4-carboxylic acid), a peripherally restricted KOR agonist, was developed by Cara Therapeutics for pain and itch (Vanderah et al. 2008). CR 845 was shown to inhibit compound 48/80- and 5′-GNTI-induced scratching in mice by Spencer et al. (2015). In this study, it was also reported that CR 845 has a long duration of action. Twelve hours following one i.v. dose administration, CR 845 still significantly decreased scratching induced by compound 48/80. Results of a Phase 3 study for pruritus in

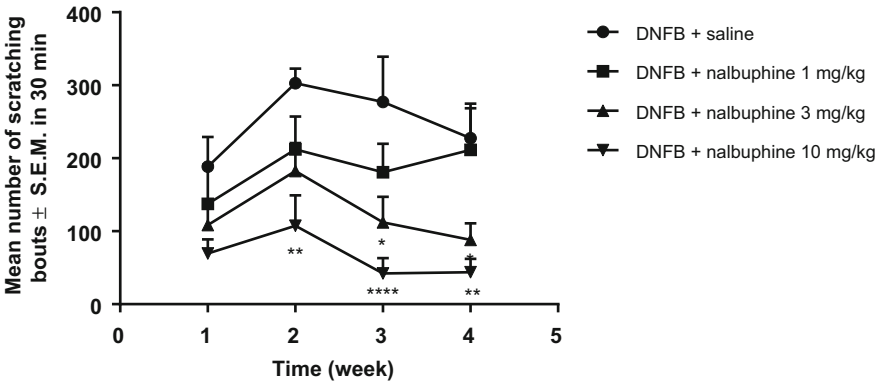

Fig. 3 Nalbuphine inhibits scratching in a time- and dose-dependent manner. Nalbuphine at 3 and 10 mg/kg significantly reduced scratching compared to saline (Two-way ANOVA (time and treatment) followed by Dunnett's multiple comparison test, $*P < 0.05$; $**P < 0.01$; $****P < 0.0001$; $n = 6–8$). Reprinted from European Journal of Pharmacology; 864/172702; Saadet Inan, Alvaro Torres-Huerta, Liselotte E Jensen, Nae J Dun, Alan Cowan; Nalbuphine, a kappa opioid receptor agonist and mu opioid receptor antagonist attenuates pruritus, decreases IL-31 and increases IL-10 in mice with contact dermatitis; 2019 with permission from Elsevier

hemodialysis patients have recently been published for i.v. Korsuva™ (Fishbane et al. 2020). Patients given treatment had a significant decrease in itch intensity and a significant increase in their quality of life, compared to patients given placebo. Phase 2 studies for oral Korsuva™ continue for pruritus in chronic kidney disease, chronic liver disease, and AD (https://www.caratherapeutics.com/pipeline-technology/our-pipeline/).

Nalbuphine (Nubain)

Nalbuphine, a KOR agonist and mu opioid receptor antagonist, has been in clinical use as an analgesic (Beaver and Feise 1978, 1981) as well as for reversal of centrally administered morphine-induced itch (without affecting analgesia) (Alhashemi et al. 1997; Tubog et al. 2019) since the beginning of the 1980s. Nalbuphine was demonstrated to be a highly effective agonist/antagonist analgesic with minimal respiratory depression or inhibition of gastrointestinal transit, minimal analgesic tolerance or physical dependence, and minimal psychotomimetic potential (dysphoria only at doses beyond the therapeutic range) (Schmidt et al. 1985). Nalbuphine has been approved for use in clinical practice for mild to moderate pain relief (https://www.accessdata.fda.gov/drugsatfda_docs/label/2016/018024s041lbl.pdf). It is the only narcotic agonist/antagonist currently unscheduled.

Hawi et al. (2013) described an anti-scratch effect of nalbuphine using a mouse model of scratching induced by substance P. We studied whether nalbuphine would be an antipruritic in mice against chronic scratching (Inan et al. 2019). For this aim, we chose chronic DNFB-induced contact allergic dermatitis. Mice were sensitized two times a week for 4 weeks and they were observed once a week following the second sensitization of the week. Nalbuphine was given 20 min (s.c.) before DNFB

Table 2 The list shows studies that report for the first time that nalbuphine suppresses itch in mice and humans

Pruritogen	Route (nalbuphine)	Species	Reference
Substance P	s.c.	Mouse	Hawi et al. (2013)
Morphine	i.v.	Human	Alhashemi et al. (1997)
Uremic pruritus	p.o.	Human	Hawi et al. (2015)
Prurigo nodularis	p.o.	Human (phase 2b/3)	TREVI therapeutics
Contact allergic dermatitis	s.c.	Mouse	Inan et al. (2019)

and the number of scratching bouts counted for 30 min following DNFB. As seen in Fig. 3, nalbuphine at 3 mg/kg and 10 mg/kg significantly decreased scratching. Nalbuphine at 10 mg/kg had no significant effect on locomotion suggesting that scratch inhibition is not through sedation. We next examined whether nalbuphine induced an anhedonic-like effect, a side effect of KOR agonists, using the sucrose splash test (Yalcin et al. 2008; Butelman et al. 2019). An anhedonic-like effect was measured using the splash test which quantitates self-grooming following 10% sucrose sprayed to flank areas of mice. During a 10-min observation, grooming was reduced by only 20% in mice injected with nalbuphine compared to saline-injected controls ($P < 0.05$). In addition, we found that nalbuphine decreased IL-31 and increased anti-inflammatory IL-10 levels in neck skin collected at the end of the fourth week. In the same tissue samples, we also measured a wide range of cytokines and chemokines and found that nalbuphine during that period increased certain chemokines and cytokines that play roles in skin healing. Inducible nitric oxide synthase, a marker for M1-type monocyte/macrophages, was also increased in nalbuphine-treated mice compared to saline-treated mice in these skin samples. Taken together, these findings suggest that nalbuphine promotes healing of inflamed skin. Collectively, in addition to suppression of scratching, this outcome may be an advantage. Table 2 lists the first reports of nalbuphine suppressing scratching in mice and humans. Promising results with nalbuphine ER tablets (Haduvio™) have been reported against pruritus of prurigo nodularis (Phase 2b/3) and uremia (Phase 2) (https://www.trevitherapeutics.com/pipeline/).

4 Conclusions

The journey that started to explore the role of KORs in itch and to find effective therapeutic agents, beginning in the 1980s, continues. The inhibitory role of Dyns on transmission of itch sensation at the spinothalamic level was discovered. It was shown that the anti-scratch activity of KOR agonists is through KORs in mice. The effectiveness of KOR agonists on acute and chronic pruritus was reported not only in rodents but also in non-human primates and humans. Studies will continue to find

new kappa opioid therapeutic agents – either peripherally selective or G-protein biased – to minimize central side effects of this class of pharmacological agent.

Other than systemic administration, there is an increased interest in using antipruritic agents topically. For example, janus kinase inhibitors and phosphodiesterase 4 inhibitors are in clinical trials for topical application in AD patients (Soeberdt et al. 2020). Topical administration of nalfurafine in a mouse model of AD was found effective for anti-scratch activity (Elliott et al. 2016). Further, W0L071-007, a KOR agonist, is in a Phase 1b clinical trial for topical application in AD (Soeberdt et al. 2020).

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Antinociceptive Effects of Kappa-Opioid Receptor Agonists

Matthew F. Lazenka

Contents

Abstract

Preclinical models that assess "pain" in rodents typically measure increases in behaviors produced by a "pain stimulus." A large literature exists showing that kappa opioid receptor (KOR) agonists can decrease these "pain-stimulated behaviors" following many different pain stimuli. Despite showing apparent antinociceptive properties in these preclinical models, KOR agonists failed as analgesics in clinical trials. Recent studies that assessed decreases in behavior due to a pain stimulus show that KOR agonists are not effective in restoring these "pain-depressed behaviors" to normal levels, which agrees with the lack of effectiveness for KOR agonists in clinical trials. One current explanation for the failure of previous KOR agonists in clinical trials is that those agonists activated beta-arrestin signaling and that KOR agonists with a greater bias for G protein signaling will be more successful. However, neither G protein-biased agonists nor beta-arrestin-biased agonists are very effective in assays of pain-depressed behavior, which suggests that novel biased agonists may still not be effective analgesics. This review provides a concise account of the effectiveness of KOR agonists in preclinical models of pain-stimulated and pain-depressed behaviors

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following the administration of different pain stimuli. Based on the previous results, it may be appropriate to include both behaviors when testing the analgesic potential of KOR agonists.

Keywords

Analgesia · Antinociception · Kappa-opioid receptor · Pain · Preclinical models

1 The Difficulty in Assessing Pain with Preclinical Models

Pain is a natural consequence of living, and about one in five Americans live with chronic pain (https://www.cdc.gov/mmwr/volumes/67/wr/mm6736a2.htm). Analgesics are often provided for the treatment of pain in the USA, which has led to the use of prescription opioids to treat pain. The widespread use of prescription opioids has contributed to an epidemic that has claimed hundreds of thousands of lives (Chang et al. 2014a; Koepke et al. 2018; Manchikanti et al. 2012). While this epidemic has encouraged researchers to develop novel analgesics, it has not encouraged researchers to question why these analgesics do not already exist. In the case of κ-opioid receptor (KOR) agonists, the issue does not involve the reproducibility of results, which has become a concern in biomedical research (Wen et al. 2018); instead, the problem may be more related to the validity of preclinical pain models and/or the interpretation of results (Gonzalez-Cano et al. 2020).

In preclinical models, subjective pain reports are not possible, and models rely on utilizing a "pain stimulus" that produces a "pain response" (Le Bars et al. 2001; Negus 2019). In this case, a noxious stimulus is utilized, which is defined as something that is "damaging or threatens damage to normal tissues" (http://www.iasp-pain.org/Education/Content.aspx?ItemNumber=1698). This noxious stimulus activates nociceptive pathways in the nervous system that may capture only one aspect of pain in humans. Drugs that modulate behaviors following this stimulus are presumed to exhibit antinociceptive properties. While there may be concerns regarding what constitutes appropriate pain stimuli, this review will focus on pain responses. The majority of preclinical models tend to measure "pain-stimulated behaviors," which represent an increase in the intensity, duration, rate, or frequency of behaviors following the presentation of a noxious stimulus (Negus et al. 2010a). The overall success of KOR agonists to treat nociception in preclinical models that utilized these pain-stimulated behaviors was followed by the failure of these agonists to prove effective in treating pain in clinical trials. By continuing to focus only on pain-stimulated behaviors, novel compounds targeting the KOR may again fail in clinical trials. The incorporation of "pain-depressed behaviors," defined as a decrease in the intensity, duration, rate, or frequency of behaviors following the presentation of a noxious stimulus, may complement typical pain-stimulated behaviors and help improve the screening of novel analgesics in preclinical models (Negus 2013; Negus et al. 2006, 2010a).

2 KORs and Antinociception in Pain-Stimulated Behaviors

Acute noxious stimuli used in preclinical models often include mechanical, thermal, or chemical manipulations that activate nociceptors and exhibit face validity when compared to clinical measures (Negus 2019). Assays of noxious mechanical stimulation typically involve the application of increasing pressure to the paw or tail until the animal withdraws or produces a vocalization (Deuis et al. 2017). For example, filaments of increasing diameter that exert a constant pressure (known as von Frey or Semmes-Weinstein filaments) are applied to the paw until the rodent withdraws the paw. The pressure at which the animal withdraws its paw is referred to as the paw-withdrawal threshold, and there are several methods used to determine these thresholds (Mills et al. 2012). Similarly, an analgesy-meter is used to apply pressure, and thresholds are determined with the Randall–Selitto method (Anseloni et al. 2003). A simpler method involves pinching the tail or paws with forceps, and this is often used to assess whether animals are fully anesthetized during surgical procedures (Tsukamoto et al. 2018). In addition to paw- or tail-withdrawal thresholds, vocalizations may also be measured. Under normal conditions (absent any inflammatory or neuropathic stimulus), KOR agonists can effectively increase paw- or tail-withdrawal thresholds and reduce vocalizations (Hayes et al. 1987; Leighton et al. 1988; Seguin et al. 1995; Tyers 1980). The effects for paw-withdrawal thresholds seem to be mediated by supraspinal KORs in rats since intrathecal (i.t.) administration does not increase paw-withdrawal thresholds, but intracerebroventricular (i.c.v.) injections do (Leighton et al. 1988). For pressure applied to the tail, the peripherally restricted KOR agonist EMD 61753 was only effective at centrally penetrating doses (Barber et al. 1994a).

Assays of acute thermal stimulation typically involve the use of a heat source (e.g., a hot plate, warm water, or a focused light beam) to elicit a paw- or tail-withdrawal response. To avoid confusion, it may be appropriate to refer to these tasks as "hot plate paw withdrawal" and "radiant-heat tail flick," which provides both the stimulus used and the behavior observed (Negus 2019). Unlike mechanical stimulation of the paw, these assays typically measure latency to withdraw the paw (often followed by licking) or the tail. Overall, KOR agonists can be effective in increasing the withdrawal latency; however, the potency of KOR agonists can vary widely across thermal tasks. Effectiveness depends on the temperature applied, the task utilized, and whether rats or mice are used (Hayes et al. 1987; Millan 1989; Piercey and Einspahr 1989; Schmauss et al. 1983; Tyers 1980; Vonvoigtlander et al. 1983). For instance, higher doses of U50,488 are needed in rats for a tail flick in response to a beam of light compared to a tail flick following tail immersion in warm water (Vonvoigtlander et al. 1983). In contrast, similar doses of U50,488 are effective in mice, and similar doses of morphine are effective in both mice and rats (Vonvoigtlander et al. 1983). Similar to mechanical stimulation of the paw, i.t. administration of U50,488 is not effective in rats when testing hot plate paw withdrawal or radiant-heat tail flick (Leighton et al. 1988; Schmauss et al. 1983). In contrast, intraspinal injection is effective in mice (Piercey and Einspahr 1989). KOR agonists are also effective when cold water is used as the noxious stimulus and tail

withdrawal as the behavior. For rats, the endogenous KOR ligand dynorphin A (1-17) is effective at increasing tail withdrawal latency following cold, but not hot, water immersion (Tiseo et al. 1988). Spiradoline (primarily through U63640) also increases tail-withdrawal latency following cold water immersion (Adams et al. 1994; Briggs et al. 1998).

Chemical noxious stimuli can take many forms and can represent a transition from acute noxious stimuli to more sustained inflammatory stimuli because they can both directly activate primary nociceptors and also mimic endogenous inflammatory mediators and/or produce local inflammation. Most studies utilize acid as an acute chemical noxious stimulus (e.g., acetic or lactic acid), which mimics tissue acidosis during inflammation and increases tissue concentration of protons that directly excite nociceptors by activating transient receptor potential channels (e.g., TRPV1) or acid-sensing ion channels (ASICs). In one family of procedures using acid as a noxious stimulus, mice or rats are given an intraperitoneal (i.p.) injection of dilute acid to cause a contraction of the abdominal muscles and extension of the hind legs. This behavior is referred to as "writhing" or "stretching" (Le Bars et al. 2001). KOR agonists are effective at reducing this behavior in a dose-dependent manner, and norbinaltorphimine (norBNI) can block the agonist-induced decrease (Broadbear et al. 1994). Administration of KOR agonists at all levels of the nervous system reduce writhing (Barber et al. 1994a; Porreca et al. 1987; Przewlocki et al. 1983; Qi et al. 1990; Rogers et al. 1992), although peripherally restricted drugs may only be effective at doses that penetrate the central nervous system. Evidence to support this is that the peripherally restricted KOR agonist GR94839 is more potent following i.c.v. injection compared to subcutaneous (s.c.) injection (Rogers et al. 1992). In contrast, the centrally penetrating drug, GR103545, is equipotent through both routes of administration.

Another commonly used acute chemical noxious stimulus is formalin administered via intraplantar (i.pl.) injection into the hindpaw. This i.pl. formalin injection causes licking, tonic flexion, and jerking of the limb associated with the injected paw, and these behaviors are typically expressed in two phases. The first phase is from approximately 0–10 min after injection and, after a brief interphase interval, the second phase from approximately 15–60 min after injection (Capone and Aloisi 2004). The first phase is thought to represent effects of direct nociceptor activation by formalin, whereas the second phase is thought to represent a process of central sensitization in the spinal dorsal horn produced by the initial nociceptor barrage, and as such, drug effects on these two phases of formalin-elicited behaviors are thought to reflect drug effects on these distinct processes. In general, KOR agonists are effective in both phases. An i.c.v. injection of the KOR agonist U50,488 can produce dose-dependent decreases in formalin-dependent behaviors at doses that do not produce locomotor depression (Calcagnetti et al. 1988). Systemic administration of KOR agonists also produces antinociception in the formalin test at doses that do not produce locomotor suppression, and this is blocked by norBNI (Nakazawa et al. 1990). An i.t. injection of U50,488 also produces antinociception in the formalin test (Pelissier et al. 1990). Inhibition of these spinal sites by norBNI also indicates an important role for a spinally mediated reduction in

formalin-induced behaviors (Nakazawa et al. 1991). Similar to writhing, peripherally restricted KOR agonists can be effective in the formalin assay, but there is some evidence that these drugs are more effective in the second phase (Kumar et al. 2005; Rogers et al. 1992; Vanderah et al. 2004). For example, GR94839 had a lower potency to reduce licking in the first phase compared to the second phase, even though the centrally penetrating GR103545 was equipotent in both phases (Rogers et al. 1992). In contrast to writhing, i.c.v. administration of a KOR agonist is far more potent than i.t. administration in the formalin assay (Murray and Cowan 1991).

Inflammatory stimuli typically include injections of molecules that are recognized by the immune system and induce an inflammatory response. Some of these agents include carrageenan, lipopolysaccharide, complete Freund's adjuvant (CFA), or pro-inflammatory cytokines (Czlonkowski et al. 1993; Negus 2019). These agents are often injected into the paw or joint. Paw incisions are also used to model postsurgical inflammatory responses. After the initial stimulus, a second stimulus is used (often mechanical or thermal) to measure allodynia (pain due to a stimulus that is not normally painful) or hyperalgesia (increased pain from a normally painful stimulus) (Negus 2019).

For example, following administration of carrageenan into the paw, U50,488 produces antinociception when injected systemically but not i.t. (Hylden et al. 1991; Idanpaan-Heikkila et al. 1994). Similarly, when carrageenan and kaolin are injected into the knee joint, U50,488 produced weak antinociceptive effects following i.t. injection but produced robust effects when injected directly into the joint. Spiradoline and PD117302 produced similar effects regardless of the route (Nagasaka et al. 1996). Subsequent studies with peripherally restricted KOR agonists indicate a strong role for peripheral KORs in mediating the antinociceptive effects of KORs in inflammatory pain models (Auh and Ro 2012; Bileviciute-Ljungar and Spetea 2001; Binder and Walker 1998; Machelska et al. 1999). While the expression of KORs on nociceptors is likely the major contributor, recent studies suggest that KORs are also expressed on leukocytes (Celik et al. 2016; Schreiter et al. 2012), and inhibition of KORs on leukocytes reduces the antinociceptive effects of NSAIDs (Silva et al. 2016). Despite the effectiveness in inflammatory models, one concern regarding KORs is that they may contribute to hyperalgesia induced by nerve growth factor (Apfel et al. 1995).

Neuropathic models also involve inflammatory cascades. These models involve selectively injuring peripheral nerves or central neurons but mostly spare surrounding tissue. Several models have been developed to study neuropathy and include models designed to reflect disease states. Nerve ligation attempts to mimic the effects of peripheral nerve injury through chronic constriction injury (Bennett and Xie 1988), partial sciatic nerve ligation (Seltzer et al. 1990), ligation of L5/L6 spinal nerves in rats (Chung et al. 2004; Kim and Chung 1992), or ligation of L4/L5 spinal nerves in mice (Ye et al. 2015). Once ligated, mechanical or thermal stimuli can be applied to the paw in the same way as inflammatory models. These manipulations also produce spontaneous pain behaviors (e.g., spontaneous foot lifting) that can be observed with effects lasting several weeks. KOR antagonists

enhance both mechanical and thermal allodynia after nerve ligation (Obara et al. 2003; Xu et al. 2004), suggesting a physiological role for the KOR system in reducing allodynia or hyperalgesia. Systemic administration of a KOR agonist produces antinociception following nerve ligation without the development of tolerance (Sounvoravong et al. 2004). Even when morphine tolerance is present, the antinociceptive effect of KOR agonists occurs regardless of whether agonists cross the blood–brain barrier or are peripherally restricted (Catheline et al. 1996; Walker et al. 1999). In a sciatic nerve ligation model in mice, i.t. but not i.c.v. administration of U50,488 produced antinociception, and tolerance did not develop with i.t. administration (Sounvoravong et al. 2004). Research suggests that KORs in brain areas, like the amygdala and nucleus accumbens, play a role in the development of chronic neuropathic pain. In the nucleus accumbens, a decrease in KOR mRNA expression is seen following spared nerve injury (Chang et al. 2014b), and, following spinal nerve ligation, microinjection of norBNI in the central amygdala blocked the aversive aspects of this stimulus (as measured by conditioned place preference), but did not block mechanical allodynia (Navratilova et al. 2019).

While neuropathic pain results from damage or injury to peripheral nerves, similar neuropathy can develop due to diabetes, arthritis, following chemotherapy, or develop spontaneously without a clear cause, such as with complex regional pain syndrome or fibromyalgia. While the etiology of pain in these models is not completely known, several models have been developed to mimic symptoms seen in patients (Negus 2019). For instance, animals treated with streptozotocin develop diabetes as well as diabetic neuropathy. In this model, mechanical or thermal allodynia/hyperalgesia is typically measured. U50,488 is not only effective in increasing withdrawal thresholds in this model, but it has also been shown to be more potent in streptozotocin-induced diabetic rats at reducing behaviors induced by some acute noxious stimuli (Kamei et al. 1992; Suzuki et al. 2001). The increased potency was seen with s.c. and i.t. injections but not with i.c.v. injections (Suzuki et al. 2001). The peripherally restricted drug asimadoline is also effective in alleviating tactile allodynia in a streptozotocin-induced diabetic model (Jolivalt et al. 2006). These effects could be blocked by i.pl. injection of norBNI, suggesting that peripheral KORs are involved. In this study, diabetic rats were also given a formalin injection into the paw, and phase 2 flinching behavior was assessed. Asimadoline was more potent in reducing flinching behavior induced by formalin than in reducing tactile allodynia in the streptozotocin-induced diabetic rats alone. Asimadoline at 1 and 5 mg/kg was effective against formalin-evoked flinching during phase 2, but 15 mg/kg was necessary to produce antinociceptive effects equivalent to gabapentin when measuring tactile allodynia. These results suggest that both central and peripheral KOR agonists are effective in this model.

Injection of formalin produces acute nociception, but it also produces persistent allodynia/hyperalgesia. Salvinorin A, a KOR agonist used recreationally as a hallucinogen, and U50,488 increase mechanical thresholds following the injection of formalin in the paw (Guida et al. 2012). In contrast, peripherally restricted KOR agonists only appear to produce acute antinociception (Machelska et al. 1999). In chronic inflammatory states, the antinociceptive effects of KOR agonists may

depend on central nervous system activity since salvinorin A reduced the frequency of firing of spinal neurons following mechanical stimulation. Both norBNI and the cannabinoid receptor type 1 antagonist AM251 can block the antinociceptive effects of a KOR agonist in this inflammatory model, and this may depend on glial activation in the spinal cord, which is reduced by repeated KOR agonist treatment (Guida et al. 2012).

Arthritic models also represent a chronic disease state. One example of this model is the injection of inflammatory agents (e.g., CFA) into the joint (Fischer et al. 2017). KOR agonists are effective in different arthritic models (Walker et al. 1995), and KOR antagonists exacerbate the hyperalgesia (Millan et al. 1987). This anti-arthritic effect is seen with peripheral administration of KOR agonists (Wilson et al. 1996) but does not seem effective with i.c.v. injection (Antic et al. 1996). The onset of treatment is also important since KOR agonist administration at later time points appears less effective (Binder and Walker 1998). While this study did not specifically focus on pain-depressed behaviors, it did show that asimadoline improved motor activity but only when given for the first 3 days.

3 KORs and Antinociception in Pain-Depressed Behaviors

The previous section provides strong evidence that KOR agonists may be ideal clinical analgesics; however, no KOR agonist is approved as a clinical analgesic due either to a failure to produce analgesia above placebo or due to adverse effects. For instance, the centrally acting KOR agonist enadoline failed to produce an analgesic effect above placebo following surgical extraction of impacted molar teeth (Pande et al. 1996a) at doses that were well tolerated. Enadoline was also tested for surgical pain. While the doses tested were effective in producing analgesia similar to morphine (although of shorter duration), these doses also produced adverse effects (Pande et al. 1996b). A comparison of enadoline with hydromorphone and butorphanol revealed that enadoline produced a constellation of adverse effects that differed from these other opioids; it produced sedation, confusion, visual distortions, and depersonalization (Walsh et al. 2001). Similarly, spiradoline was not as effective as morphine in producing analgesia and produced adverse effects such as sedation and dysphoria (Wadenberg 2003).

To avoid the adverse effects of centrally active KOR agonists, peripherally restricted KOR agonists were predicted to have the same analgesic effectiveness in humans without adverse effects (Albert-Vartanian et al. 2016). For peripherally restricted KOR agonists, clinical studies with fedotozine and asimadoline (EMD 61753) did not show much promise; asimadoline even produced hyperalgesia in postoperative pain patients (Lembo 2006; Machelska et al. 1999). While ADL 10-0101 was effective in reducing pain in patients with acute pancreatitis (Eisenach et al. 2003), clinical trials were discontinued. Asimadoline also reduced pain in irritable bowel syndrome patients; however, the primary endpoint of the number of months of adequate pain relief was not met (Mangel et al. 2008). Similarly, CR665 had some effect on increasing pain thresholds following a visceral pain stimulus

(esophageal distension) but exacerbated pain caused by skin pinching (Arendt-Nielsen et al. 2009). The most recent attempt at determining the analgesic effects of peripherally restricted KOR agonists comes from CARA Therapeutics. Its drug candidate, CR845, only showed significant analgesia for hip pain above placebo at the highest dose (5.0 mg) in a Phase 2b trial (http://ir.caratherapeutics.com/news-releases/news-release-details/cara-therapeutics-announces-top-line-results-phase-2b-trial-oral). Since phase 3 trials are ongoing, CR845 could be approved as an analgesic. The failure of KOR agonists to produce effective clinical analgesia has led to the development of newer preclinical models that assess pain-depressed behaviors instead of pain-stimulated behaviors (Gonzalez-Cano et al. 2020; Negus 2019; Negus et al. 2010a). Studies that have tested KOR agonists against depression of behavior following a noxious stimulus have failed to show consistent antinociception, and in some instances, KOR agonists will further depress behavior. Compared to preclinical studies that utilize pain-stimulated behavior, preclinical studies of pain-depressed behaviors more closely match the results of clinical studies.

Before the use of pain-depressed behaviors, the adverse effects of KOR agonists were often tested in preclinical models by using a rotarod. In many cases, depending on the noxious stimulus, these studies found that KOR agonists were more potent at producing antinociception than producing sedation. For example, Hayes et al. (1987) reported that U50,488 in mice produced an increase in tail withdrawal latency caused by a beam of light at much lower doses than needed to decrease latency on the rotarod. However, this potency difference was not as large when comparing the doses of U50,488 necessary to increase the latency to lick the paw caused by heat. When comparing the ID50s of morphine, U69,593, and enadoline in mice to reduce rotarod latency to the ID50s for antinociception produced in both phases after formalin injection, Seguin et al. (1995) found similar ratios (~8-fold) for these drugs and comparable results for other noxious stimuli. One interpretation of these results would be that KOR agonists are equally effective as MOR agonists at doses that do not produce adverse effects. However, Vanderah et al. (2004) compared the effects of U50,488 and FE200041 in both the rotarod and the acetic acid writhing test in mice. In this case, there was only a twofold shift in the potency for U50,488 to produce a decrease in rotarod performance. For the peripherally restricted agonist FE200041, there was an 84-fold shift. These results are less favorable than those previously reported for centrally active KOR agonists and indicate that a better interpretation of the rotarod test is that it can screen for whether KOR agonists cross the blood–brain barrier. Table 1 shows that despite effectiveness in pain-stimulated behaviors at doses that do not produce sedation, KOR agonists are ineffective at treating pain-depressed behaviors. Instead of testing for overall motor impairment in the absence of a pain stimulus, it may be better to test candidate analgesics in pain-depressed behaviors that could detect both drug-induced dysphoria and sedation.

Several assays of pain-depressed behavior are easy to establish in the laboratory and involve the same amount of training as typical assays measuring pain-stimulated behavior. For instance, depression of nest building occurs following surgery (Arras

Table 1 Comparison between a purported β-arrestin-signaling-biased KOR agonist (U69,593), a purported G protein-signaling-biased KOR agonist (nalfurafine), and a peripherally restricted KOR agonist (ICI204448) in an assay measuring pain-stimulated behavior, an assay measuring sedation, and an assay measuring pain-depressed behavior

Preclinical assay	Drug	Effective dose(s)	Animal model (route of administration)	Reference
Acid-induced writhing/ stretching	U69,593	ID_{50} 0.16 mg/kg	Mouse (s.c.)	(Seguin et al. 1995)
	U69,593	0.56 mg/kg	Rat (i.p.)	(Negus et al. 2010b)
	Nalfurafine	ED_{50} 0.0033 mg/kg	Mouse (s.c.)	(Endoh et al. 1999)
	Nalfurafine	0.01–0.032 mg/kg	Rat (i.p.)	(Lazenka et al. 2018)
	ICI204448	10–32 mg/kg	Rat (i.p.)	(Negus et al. 2012)
Rotarod	U69,593	ID_{50} 0.68 mg/kg	Mouse (s.c.)	(Seguin et al. 1995)
	Nalfurafine	ED_{50} 0.027 mg/kg	Mouse (s.c.)	(Endoh et al. 1999)
	ICI204448	>100 mg/kg	Rat (s.c.)	(Barber et al. 1994b)
Acid-induced depression of intracranial self-stimulation	U69,593	Not effective	Rat (i.p.)	(Negus et al. 2010b)
	Nalfurafine	Not effective	Rat (i.p.)	(Lazenka et al. 2018)
	ICI204448	Not effective	Rat (i.p.)	(Negus et al. 2012)

s.c. subcutaneous, *i.p.* intraperitoneal

et al. 2007; Gaskill et al. 2013; Jirkof et al. 2013; Oliver et al. 2018), lipopolysaccharide administration (Aubert et al. 1997), CFA injection (Negus et al. 2015), lactic acid injection (Lewter et al. 2017; Negus et al. 2015), acetic acid injection (Lewter et al. 2017), and formalin injection (Ulker et al. 2020). Following treatment with a noxious stimulus, mice will exhibit a reduction in nest-building behavior and certain endpoints (e.g., the complexity of the nest, time to build the nest, or removal of nesting material from specific zones) can be measured. Drugs that

are effective in preclinical pain-stimulated behaviors and clinically, such as non-steroidal anti-inflammatory drugs (NSAIDs) and morphine, are effective in either completely or partially restoring nesting behavior following a noxious stimulus.

In one study, the centrally active KOR agonist U69,593 did not restore nesting behavior (defined as the number of zones cleared of nesting material) following treatment with lactic acid or CFA; instead, U69,593 depressed nesting on its own and further depressed nesting after lactic acid and CFA treatment (Negus et al. 2015). In the absence of a noxious stimulus, the KOR antagonist JDTic was able to block the depression of nesting by U69,593, and, in agreement with its lack of antinociceptive properties, JDTic did not affect the depression of nesting following the administration of either lactic acid or CFA. While it is not clear whether this depression of behavior by U69,593 is due to sedation or dysphoria, it is clear that its effects were not similar to clinically available analgesics. For example, ketoprofen was able to restore nesting behavior following treatment with lactic acid or CFA without having any effect on nesting behavior when given in the absence of a noxious stimulus. Morphine also dose-dependently restored nesting behavior at doses that did not affect nesting when given in the absence of a noxious stimulus. These results suggest that, in contrast to ketoprofen and morphine, U69,593 would not be an effective analgesic.

While more technically challenging, intracranial self-stimulation (ICSS) is a powerful tool that not only provides the ability to assess potential analgesic drugs in pain-depressed behavior but also allows for the evaluation of the abuse potential of drugs. Studies of ICSS utilize operant conditioning whereby rodents press a lever under a fixed-ratio schedule to receive electrical stimulation to the medial forebrain bundle (Negus and Miller 2014). Drugs with abuse liability (e.g., morphine) will increase responding for electrical stimulation, and drugs that produce sedation or dysphoria (e.g., KOR agonists), as well as noxious stimuli, will decrease responding for electrical stimulation. In initial studies utilizing ICSS to assess pain-induced depression of behavior, the KOR agonist U69,593, the KOR antagonist norBNI, and morphine were tested against lactic acid-induced stretching and lactic acid-induced depression of ICSS (Negus et al. 2010b). Both U69,593 and morphine produced a reduction in lactic acid-induced stretching. However, in ICSS, U69,593 further reduced responding for electrical stimulation following lactic acid treatment, whereas morphine restored responding to baseline levels. The KOR antagonist norBNI had no effect on lactic acid-induced depression of ICSS at a dose that blocked U69,593-induced reductions of lactic acid-induced stretching.

Other studies utilizing ICSS to measure pain-depressed behavior have demonstrated that KOR agonists are not effective at restoring behavior. For instance, the peripherally restricted KOR agonists d-Phe-d-Phe-d-Ile-d-Arg-NH2 (ffir) and ICI204448 were assessed in both lactic acid-induced stretching and lactic acid-induced depression of ICSS (Negus et al. 2012). Both drugs were able to reduce lactic acid-induced stretching; however, neither drug was able to block lactic acid-induced depression of ICSS. Agreeing with its lack of access to the central nervous system, ICI-204448 did not produce a significant decrease in percent baseline

stimulations in control ICSS or further reduce percent baseline stimulations following lactic acid. Although ffir decreased percent baseline stimulations in control ICSS, this was at a dose higher than was necessary to reduce acid-induced stretching. The centrally active KOR agonist, salvinorin A, produced decreases in responding for control ICSS and further decreased responding after lactic acid treatment, which agreed with the previous findings with the centrally active U69,593. This study also demonstrated that the NSAID ketoprofen was effective in decreasing acid-induced stretching as well as restoring ICSS to baseline following lactic acid treatment.

The role of the KOR system in pain-depressed ICSS has also been determined following i.p. lactic acid, i.pl. formalin, and i.pl. CFA (Leitl et al. 2014a, b). Both lactic acid and U69,593 alone decreased ICSS and dopamine levels in the nucleus accumbens, which provides a possible mechanism for why U69,593 is not effective in restoring acid-induced depression of ICSS (Leitl et al. 2014a). In contrast, neither ketoprofen nor morphine depressed nucleus accumbens dopamine when given alone, and both restored acid-induced depression of nucleus accumbens dopamine and ICSS. The restoration following acid administration was seen at doses that did not alter nucleus accumbens dopamine or ICSS when either drug was given alone. As expected, norBNI was not effective in either case. With regard to formalin and CFA, both produced mechanical hypersensitivity and increased paw width for 7 days, but only CFA caused sustained weight loss (Leitl et al. 2014b). These results differed from ICSS. With ICSS, CFA only produced an acute depression of responding for electrical stimulation, whereas formalin produced a decrease in ICSS that lasted for 14 days. While morphine was capable of restoring ICSS responding to pre-formalin levels, norBNI was not. Consolidating the results from both studies, acid, formalin, and CFA did not change KOR or prodynorphin mRNA levels in brain regions involved in the mesolimbic dopamine pathway except for a delayed increase in prodynorphin mRNA following acid (Leitl et al. 2014a).

While there are currently no highly selective KOR agonists available clinically in the USA, nalfurafine (TRK-820) is a centrally active KOR agonist available clinically in Japan to treat uremic pruritus and systemic itch associated with chronic liver diseases. One study assessed the ability of nalfurafine to alter pain-depressed ICSS following lactic acid as well as itch-depressed ICSS utilizing intradermal serotonin as a pruritic agent (Lazenka et al. 2018). In this study, nalfurafine reduced both acid-induced stretching and serotonin-induced scratching. In ICSS, nalfurafine decreased control ICSS and exacerbated both lactic acid-induced and serotonin-induced depression of ICSS. The KOR antagonist JDTic blocked the depression of ICSS by nalfurafine, but not morphine. The failure of nalfurafine to alter serotonin-induced depression of ICSS is not entirely consistent with the fact that it is clinically approved for treating pruritus; however, this could be that intradermal serotonin is not a good model of uremic pruritus. More importantly, this study showed that ketoprofen and morphine were also not effective in treating serotonin-induced depression of ICSS, which is consistent with their lack of antipruritic effects. This provides further evidence that the ability of ketoprofen and morphine to restore acid-induced depression of ICSS is likely due to antinociceptive effects.

Other models of pain-depressed behaviors suggest that KOR agonists are not effective analgesics. For example, GR89,696 was assessed in several different behaviors following the application of capsaicin to the face of rats, one of which included a reward-conflict operant paradigm (Neubert et al. 2007). For this task, rats could access a sweetened condensed milk solution, but licking would result in the delivery of a thermal stimulus. Following capsaicin treatment, intake of sweetened water decreases along with other outcome measures. Pretreatment with morphine can restore these outcome measures to baseline (Neubert et al. 2005), but GR89,696 is not effective. For the acetic acid-induced conditioned place aversion task in mice, ketoprofen and morphine were effective in preventing aversion (exploration of the environment was not decreased), but U50,488 was not (Bagdas et al. 2016). In another study, constriction of the sciatic nerve in mice decreased marble burying and digging behavior (Wilkerson et al. 2018). Again, morphine restored these behaviors to baseline, but U69,593 was not effective.

Other models of pain-depressed behavior could help determine the effectiveness of KOR agonists. Wheel running is a procedure that rodents will actively partake in even though it does not increase survival in a laboratory setting. Wheel running can be depressed following monosodium iodoacetate injected into the joint (Stevenson et al. 2011), injection of CFA in the paw (Kandasamy et al. 2016), and injection of allyl isothiocyanate onto the dura mater (Kandasamy et al. 2017). While a KOR agonist has not been tested in wheel running following a noxious stimulus, the effect of KOR agonists on wheel running has. In this study, the development of tolerance to KOR agonists for both antinociception and wheel running was tested (Suzuki et al. 2004). Both nalfurafine and U50,488 were more potent at producing antinociception (assessed with acetic acid-induced writhing) than at producing depression of wheel running. The effects on acid-induced writhing were similar for all KOR agonists tested and produced similar effects as morphine. Therefore, wheel running may be a useful "pain-depressive behavior" test for evaluation of analgesic effect.

Burrowing is another behavior that can be depressed by several pain models (Andrews et al. 2012; Chartier et al. 2020; Huang et al. 2013; Jirkof et al. 2010), and several stimuli have been tested against this behavior. This behavior is easy to measure and involves the evolutionarily conserved behavior of rodents that need to seek shelter. In the laboratory setting, this procedure involves a burrow (plastic tube) filled with objects, such as food pellets or pea shingles (Deacon 2006, 2009). While burrowing has been tested after many different treatments (e.g., prion disease and systemic inflammation), one of the first to assess nociception involved the effects of laparotomy in mice (Jirkof et al. 2010). In this case, laparotomy increased latency to burrow, and the NSAID carprofen restored burrowing latency to near control levels. Despite being a useful model, one concern with burrowing is the variability of the procedure, which may be greater than pain-stimulated behaviors (Muralidharan et al. 2016; Wodarski et al. 2016).

4 A Biased Future for KOR Agonists

Since KORs are G protein-coupled receptors (GPCRs), drugs can be designed to either activate G proteins or recruit β-arrestins (Mores et al. 2019). This has led to efforts to determine whether biased KOR agonists can produce analgesia without dysphoric effects. Currently, there are no biased KOR agonists in clinical trials, and only recently has the Food and Drug Administration approved a μ-opioid receptor (MOR) biased agonist (Markham 2020). Similar to MOR agonists, it has been proposed that the G protein signaling pathway is responsible for the antinociceptive effects of KOR agonists, and the β-arrestin pathway is responsible for sedation and dysphoria (Bohn and Aubé 2017; Bruchas et al. 2007). However, recent studies suggest that both determining the bias of a compound in vitro and predicting its effects in vivo can be complicated by several factors. These can include variability across labs, cell lines used, downstream pathways tested, pharmacokinetics, and various other technical issues (Cao et al. 2020; Dunn et al. 2019; Kaski et al. 2019; Liu et al. 2019; Mores et al. 2019; White et al. 2015).

Several of the drugs previously tested and presented here have been reported to be more β-arrestin biased or unbiased at mouse KORs: U69,593, U50,488, salvinorin A, and ICI-199,441 (DiMattio et al. 2015; White et al. 2014). Although the β-arrestin pathway appears to contribute primarily to sedation, these drugs exhibit antinociceptive activity in pain-stimulated behaviors and, depending on the assay, only produce sedation (as tested in the rotarod) at higher doses than are necessary for antinociception. The only exception is ICI-199,441 (Endoh et al. 1999), which is also purported to be G protein biased (Kaski et al. 2019). Both U69,593 and salvinorin A support the hypothesis that agonists with a bias for the β-arrestin pathway would not make good candidate analgesics since they are not effective in assays of pain-depressed behaviors. In contrast, nalfurafine does not support this hypothesis since it is reported to be G protein biased (Kaski et al. 2019; Schattauer et al. 2017). While there is some evidence to suggest that nalfurafine may be unbiased (Liu et al. 2019) or β-arrestin biased (Dunn et al. 2019), it is this inconsistency across studies that makes it difficult to determine how G protein biased a KOR agonist must be before it can be considered a good analgesic candidate.

Several compounds that may have greater G protein bias than nalfurafine have been developed, and these include RB-64 (White et al. 2015) and triazole 1.1 (Brust et al. 2016). At the doses tested for both drugs, antinociception was found using pain-stimulated behaviors, and neither drug produced major deficits in rotarod or baseline ICSS. Unlike nalfurafine, triazole 1.1 did partially restore acid-induced depression of ICSS to baseline values. RB-64 was not tested in ICSS following a noxious stimulus, but it did produce conditioned place aversion in β-arrestin knock-out mice. One concern regarding the analgesic potential of these drugs is that ketoprofen was more effective than triazole 1.1 in restoring acid-induced depression of ICSS to baseline. Most recently, the G protein biased agonist 16-Bromo SalA was shown to be effective in several pain-stimulated behaviors (Paton et al. 2020). In agreement with the reduced adverse effects of G protein biased agonists, 16-Bromo

SalA did not have anxiogenic effects (measured with marble burying and elevated zero maze), although it did reduce latency to fall off the rotarod at the highest dose tested. Since this drug was not tested in an assay of pain-depressed behavior, it is unclear whether this drug has greater analgesic potential than previously studied KOR agonists.

5 Conclusion

Based on preclinical models that measure pain-stimulated behaviors, it was predicted that KOR agonists would be effective clinical analgesics; however, this hypothesis has not been supported by clinical data. Centrally active KOR agonists fail to produce analgesia at doses that do not also produce sedation or dysphoria. In the case of peripherally restricted KOR agonists, they fail to produce sufficient analgesia. In contrast to pain-stimulated behaviors, assays utilizing pain-depressed behaviors have produced results that are more similar to clinical data. Although biased agonists appear promising, it is suggested that novel KOR agonists be tested in both assays of pain-stimulated and pain-depressed behaviors to avoid false positives. In preclinical studies, a KOR agonist with true analgesic potential should reduce pain-stimulated behaviors and completely restore pain-depressed behaviors to baseline.

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Kappa Opioid Signaling at the Crossroads of Chronic Pain and Opioid Addiction

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Contents

Abstract

Pain is complex and is a unique experience for individuals in that no two people will have exactly the same physiological and emotional response to the same noxious stimulus or injury. Pain is composed of two essential processes: a sensory component that allows for discrimination of the intensity and location of a painful stimulus and an emotional component that underlies the affective, motivational, unpleasant, and aversive response to a painful stimulus. Kappa opioid receptor (KOR) activation in the periphery and throughout the neuroaxis modulates both of these components of the pain experience. In this chapter we focus on recent findings that KORs contribute to the emotional, aversive nature of chronic pain, including how expression in the limbic circuitry contributes to anhedonic states and components of opioid misuse disorder. While the primary focus is on preclinical pain models, we also highlight clinical or human research where there is strong evidence for KOR involvement in negative affective states associated with chronic pain and opioid misuse.

Keywords

Amygdala · Analgesia · Anhedonia · Antinociception · Biased agonist · Chronic pain · Dopamine · Dynorphin · Emotional component of pain · Endogenous opioid · Inflammatory pain · Kappa opioid receptor · Mesolimbic circuitry · Negative affect · Neuropathic pain · Nociception · Nucleus accumbens · Opioid misuse · Opioid use disorder · Pain · Pain aversion · Peripherally-restricted · Ventral tegmental area

Abbreviations

ACTH	Adrenocorticotropic hormone
BLA	Basolateral amygdala
BNST	Bed nucleus of the stria terminalis
CeA	Central nucleus of the amygdala
CPA	Conditioned place aversion
CPP	Conditioned place preference
CRF	Corticotropin-releasing hormone
DOR	Delta opioid receptor
GABA	Gamma-aminobutyric acid
GPCRs	G-protein coupled receptors
ICSS	Intracranial self-stimulation
JDTic	$C_{28}H_{39}N_3O_3$ kappa opioid antagonist
KOR	Kappa opioid receptor
LiCl	Lithium chloride
MAP	Mitogen-activated protein
MK801	Dizocilpine, NMDA antagonist
MOR	Mu opioid receptor
nor-BNI	Nor-binaltorphimine, kappa opioid antagonist
NMDA	Nucleus accumbens NAc
ORL1	Opioid receptor-like/nociceptin receptor
PAG	Periaqueductal gray
VTA	Ventral tegmental area

1 Introduction

The family of opioid receptors includes four homologous 7 transmembrane spanning G protein-coupled receptors (GPCRs) denoted kappa, mu, delta, (KOR, MOR, DOR, respectively), and the opioid receptor-like/nociceptin receptor (ORL1). Opioids are also involved in a myriad of other physiological, defensive, and behavioral processes, including autonomic regulation, control of breathing, immune function, gut transit, and itch (Bodnar 2018). In the early years of opioid receptor exploration, researchers understood the addiction liability associated with MOR agonists, and hoped that the identification of compounds targeting the alternate opioid receptors, including KOR, might lead to non-addictive alternatives for pain relief. Unfortunately, while acute activation of DOR and KOR does result in analgesia in preclinical models, KOR agonists produce strongly aversive emotional states. Subsequent research has implicated both dynorphin peptides and KOR activation in the acute and chronic impact of pain, stress, and addiction, though their contribution to each of these issues is complex and likely changes over time. There is now consensus that KOR agonists can produce dysphoria, depressive-like symptoms, and psychotomimetic effects in humans (Kumor et al. 1986; Pfeiffer et al.

1986; Wadenberg 2003) and elicit place aversion and depressive-like behaviors (Shippenberg and Herz 1986; Shippenberg et al. 1993; Bruchas et al. 2010; Knoll and Carlezon 2010; Chavkin and Koob 2016) as well as stimulate drug-seeking (Valdez et al. 2007; Grella et al. 2014; Nygard et al. 2016; Lê et al. 2018) in rodents. Activation of the KOR system also elicits signs of anxiety and fear in animals and humans (Chartoff and Mavrikaki 2015; Chavkin and Koob 2016; Darcq and Kieffer 2018); however, spinal administration of KOR agonists produce antinociceptive effects in various preclinical pain models. The characterization of the crystal structures of inactive and active states of KOR has provided insights for drug–receptor interactions allowing new concepts for novel drug design (Wu et al. 2012; Che et al. 2018). This structural characterization together with identification of the signaling events that elicit antinociceptive versus dysphoric and psychotomimetic effects has provided extensive advancement in novel chemical entities that hold promise as new pain treatments with minimal aversive effects including depressive or addictive properties. In addition, peripherally restricted compounds, low efficacy ligands, and compounds with mixed mechanisms of action have shown promising results in early clinical trials. In this chapter, we will discuss (1) the involvement of KOR systems in the brain that contribute to pain and negative affective states associated with ongoing persistent pain and (2) the potential involvement of KOR systems in opioid medication misuse and opioid use disorder in the context of concomitant occurrence with chronic pain, given the recent evidence that KOR agonists can trigger drug relapse and that a systematic review confirm that chronic pain patients have high rates (13–38%) of opioid misuse (defined as opioid use contrary to the directed or prescribed pattern of use (Vowles et al. 2015).

2 Functional Upregulation of KOR Systems in Chronic Pain States

One of the first studies to report that dynorphin expression is increased in chronic pain states used a model of polyarthritis produced by intradermal tail injection of *Mycobacterium butyricum* that caused a gradual development of arthritic limbs, hypophagia (reduction of food intake), hypodipsia (reduced thirst), and reductions in thermal and mechanical sensory thresholds. This arthritic model was associated with a significant upregulation of immunoreactive dynorphin in the spinal cord that correlated with both the intensity and time course of mechanical hyperalgesia (Fig. 1) (Millan et al. 1985) and KOR antagonism potentiated this hyperalgesic response (Millan et al. 1987). KOR antagonism also enhanced pain hypersensitivities in a model of neuropathic pain (Xu et al. 2004). Together these data suggest that the dynamic endogenous tone of dynorphin at KORs is an adaptive process to suppress nociceptive transmission associated with tissue injury. Constitutive (global) KOR knockout produced enhanced pain hypersensitivity in a model of chronic inflammatory pain, but interestingly this enhancement was modality specific where mechanical, but not thermal, hypersensitivity was exaggerated in mice lacking KORs (Gavériaux-Ruff et al. 2008). Constitutive KOR knockout mice also exhibited

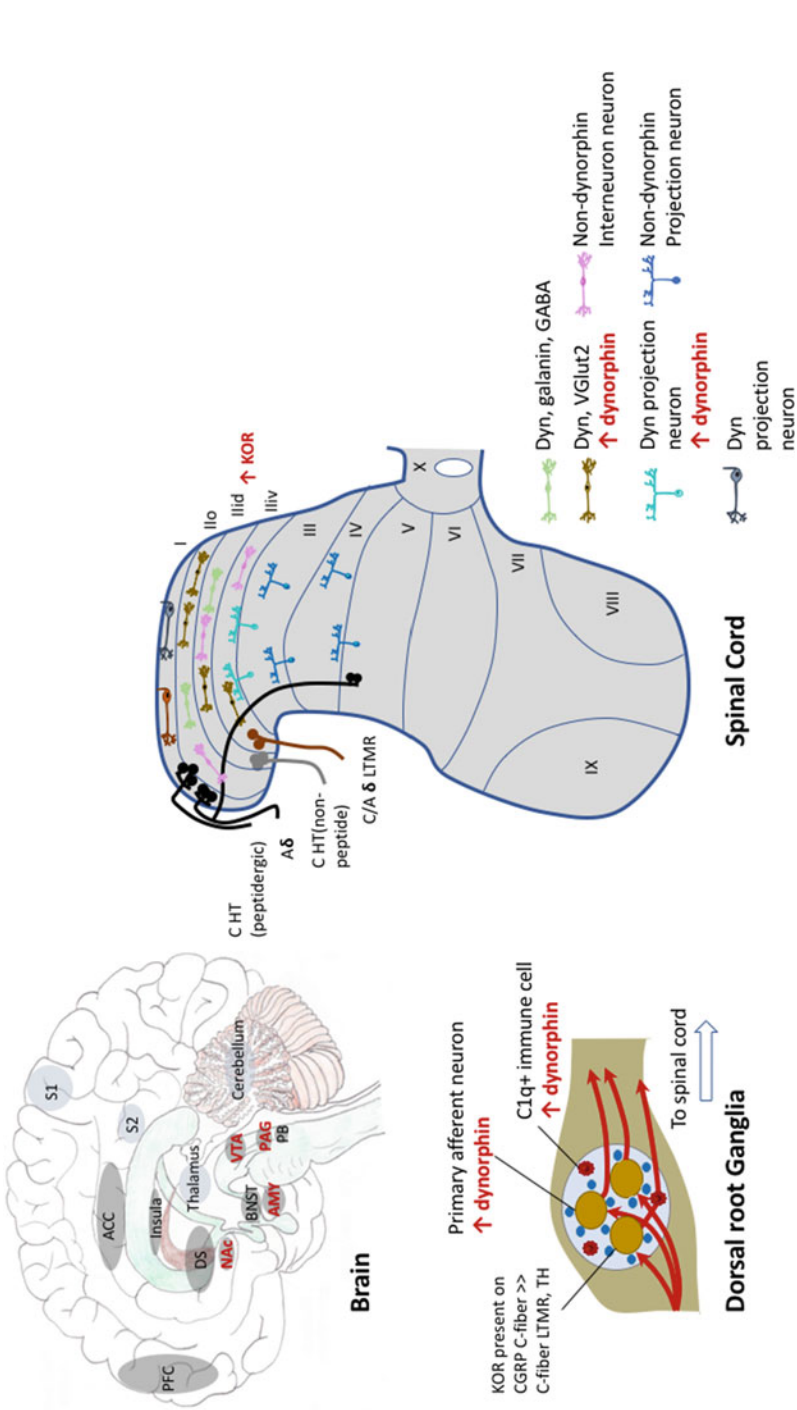

Fig. 1 Evidence for increased dynorphin or KOR function or expression in various peripheral or central nervous system brain regions in chronic pain states. *Dorsal Root Ganglia:* Following nerve injury, dynorphin is present in primary afferent neurons, where it is not expressed under pain-naïve conditions (Sapio

Fig. 1 (continued) et al. 2020), is produced by C1q+ immune cells (Mika et al. 2010). *Spinal Cord*: Neurons in the spinal cord are organized into laminae, where ascending projection neurons are enriched in lamina I and throughout lamina II-V, whereas lamina II primarily consists of interneurons, including dynorphin containing inhibitory or excitatory neurons. Projection neurons make up 5 distinct circuits that relay sensory information to the brain. The spinothalamic pathway is known for transmitting pain information to the thalamus, and subsequently to the somatosensory cortex (S1) to provide information about pain intensity and location. Nociceptive projection neurons in lamina I relay pain information to specific brainstem (e.g., lateral parabrachial nucleus) and thalamic nuclei, which further project to areas involved in the affective, emotional component of pain such as the amygdala and hypothalamus. Primary afferent nociceptors including C-fiber and A-delta HT (high threshold) project to lamina I and outer lamina II (IIo), while low-threshold mechanoreceptor (LTMR) neurons project to inner lamina II. Prodynorphin immunoreactivity is present in galanin-positive, GABAergic interneurons in lamina I/II (Sardella et al. 2011) and glutamatergic (VGlut2+) (Nahin et al. 1992) interneurons that synapse with projection neurons in lamina III, that also contain dynorphin, where a recent study showed that the dynorphin containing neurons was 88% GABA to 12% glutamatergic (Duan et al. 2014). Dynorphin is upregulated following tissue or nerve injury in excitatory dynorphin-containing interneurons and dynorphin-containing projection neurons (Iadarola et al. 1988a; Ruda et al. 1988; Sapio et al. 2020). It is unclear whether dynorphin expression within dynorphin projection neurons in lamina I is modified in chronic pain states. There is an increase in KOR expression and function within the spinal cord have also been described. *Brain*: Many brain regions show increased KOR function in chronic pain states (highlighted in red text). *ACC* Anterior cingulate cortex, *AMY* amygdala, *BNST* bed nucleus of the stria terminalis, *DS* dorsal striatum, *NAc* nucleus accumbens, *PAG* periaqueductal gray, *PB* parabrachial nucleus, *PFC* prefrontal cortex, *S1* somatosensory cortex, *S2* secondary somatosensory cortex, *VTA* ventral tegmental area. A detailed description of spinal neurons in nociceptive transmission has been described (Todd 2010; Peirs and Seal 2016)

enhanced nociceptive responses in a model of chemical visceral pain (intraperitoneal acidic acid) where abdominal constrictions were significantly greater, although there was no effect of KOR deletion on formalin-induced nocifensive (licking, flinching, guarding) behaviors (Simonin et al. 1998).

Many inflammatory agents (carrageenan, phorbol ester, yeast, and complete Freund's adjuvant) that rapidly induce edema and thermal hyperalgesia increased dynorphin mRNA within 8h and dynorphin A (1-8) peptide in the spinal cord within 48h following tissue injury (Iadarola et al. 1988a). Spinal dynorphin is upregulated in models of preclinical models of neuropathic and inflammatory chronic pain (Fig. 1) (Millan et al. 1985, 1986, 1988; Iadarola et al. 1988a, b; Kajander et al. 1990; Draisci et al. 1991; Malan et al. 2000; Rosén et al. 2000; Wang et al. 2001), as well as elevated in the cerebrospinal fluid of chronic pain patients (Vaerøy et al. 1991; Samuelsson et al. 1993). Moreover, dynorphin peptides can remain elevated for several months concurrent with persistent pain (Iadarola et al. 1988a; Kajander et al. 1990; Malan et al. 2000). Dynorphin is also present in primary afferent nociceptors and dynorphin neuropeptides were shown to be upregulated in dorsal root ganglia neurons in models of chronic pain (Fig. 1) (Calzà et al. 1998; Mika et al. 2010).

KOR agonists have been shown to produce antinociceptive effects in various visceral, inflammatory, and neuropathic pain models, although KOR agonists were found to have no effect on nociceptive responses in models of chronic musculoskeletal pain such as intramuscular acid injection (Sluka et al. 2002). Considering KOR agonists produce motor impairment and hypo-locomotion (Castellano et al. 1988; Bakshi et al. 1990), it is important to dissociate changes in reflexive nociceptive behaviors from potential confounds of motor impairment. The KOR agonist SA14867 (more than 31,000 and 2,200-fold higher affinity for KOR compared to mu and delta opioid receptors) elicited a large therapeutic window of antinociceptive effects relative to sedative/motor effects (as measured by rotarod) compared to other KOR agonists (Tsukahara-Ohsumi et al. 2011), demonstrating that indeed changes in threshold evoked sensory responses are not solely attributed to changes in motor performance.

2.1 Dynorphin Peptides Can Exacerbate Nociceptive Transmission via Non-opioid Mechanisms

Spinal dynorphin peptides have also been associated with exacerbation of pain outcomes. Spinal administration of KOR agonists produce significant changes in the excitability of some superficial nociceptive neurons, where both facilitation and inhibition of excitability and expansion of receptive fields were reported (Hylden et al. 1991). Later studies concluded that inhibitory effects of spinal dynorphins and KOR agonists on C-fiber excitability were mediated via KOR activation, whereas the facilitation induced by dynorphin peptides was not opioid receptor-mediated (Knox and Dickenson 1987). The prodynorphin precursor can be cleaved into various dynorphin peptides, where some peptides (e.g., dynorphin (1-17)) were

reported to facilitate NMDA-mediated currents via direct excitatory effects on this ionotropic receptor (Caudle and Dubner 1998). Spinal dynorphin A (2-13) peptide also interacts with bradykinin receptors to promote hyperalgesia (Lee et al. 2015), where blockade of spinal bradykinin receptors also reverses neuropathic pain, but only when there was elevated spinal dynorphin A (2-13) peptides (Lai et al. 2006). Similarly, spinal dynorphin peptides dynorphin A (1-17), dynorphin A (2-17), dynorphin A (2-13), and dynorphin (11-17) produced long-lasting allodynia (painful response to a stimulus that is not normally painful), where effects persisted up to 70 days after a single injection (Vanderah et al. 1996; Laughlin et al. 1997). In these studies naloxone did not reverse the pronociceptive effects of dynorphin peptides, but rather were blocked by NMDA receptor antagonists, and the longer duration of action after a single injection suggests that dynorphin peptides can produce the phenomenon of spinal wind-up that contributes to central sensitization. Additionally, pain hypersensitivity associated with nerve injury recovered in prodynorphin knock-out mice, consistent with the report that intrathecal dynorphin antiserum reversed neuropathic pain (Wang et al. 2001). Similarly, neutralization of dynorphin peptides with antibodies was also shown to attenuate pain hypersensitivities associated with neuropathic pain (Malan et al. 2000; Gardell et al. 2004). These data suggest that the injury-induced upregulation of dynorphin produces pronociceptive effects and is required for the maintenance of persistent neuropathic pain. Indeed, a review argued that a dynorphin targeted gene silencing strategy to block dynorphin upregulation is a rational drug approach for treatment of chronic pain (Podvin et al. 2016). However, generation of mice that specifically ablated dynorphin containing inhibitory (but not excitatory) interneurons enhanced static and dynamic mechanical sensory thresholds in the absence of injury, demonstrating that these neurons normally act to prevent low-threshold mechanical stimuli from activating pain transmission neurons (Duan et al. 2014).

There is the possibility that KORs can also facilitate nociception, as this receptor is expressed on astrocytes, whereby KOR activation in these glial cells triggers their hypertrophy (Xu et al. 2007). Hence, in a model of neuropathic pain, KOR activation on astrocytes was shown to produce proliferation of spinal cord astrocytes via activation of p38 MAP kinase (Xu et al. 2007). The activation and hypertrophy of spinal astrocytes contributes to the maintenance of persistent pain states as well as MOR analgesic tolerance (Eidson and Murphy 2019; Ji et al. 2019; Donnelly et al. 2020). Taken together, spinal dynorphin peptides can either inhibit or facilitate nociceptive transmission; however, the facilitating effects of dynorphins are not mediated by neuronal KORs. Whether they engage astrocyte KORs that contributes to enhanced nociceptor excitability remains unknown.

2.2 Chronic Pain Changes KOR Function and Expression in Supra-Spinal Sites

While early research focused on dynorphin peptides and KORs in peripheral nociceptors and the spinal cord, chronic or persistent pain also increases KOR

function in various brain structures. In an arthritic pain model, KOR binding increased in the dorsomedial and dorsolateral periaqueductal gray (PAG) (Millan et al. 1987). However, it is unknown what the functional relevance is of the chronic pain-induced KOR upregulation in the dorsal PAG. The PAG is a key structure in descending modulation (both facilitation and inhibition) of nociception, risk assessment triggering sympathetic and emotional responses as well as the learning and action of defensive and aversive behaviors (Lefler et al. 2020; Mokhtar and Singh 2020). Activation of the dorsolateral sub-region of the PAG produces emotional arousal and a stress response (increased heart rate, blood pressure, and alertness), whereas the ventrolateral sub-region is known for its involvement in modulating nociceptive transmission and opioid-mediated antinociception (Bagley and Ingram 2020). Given that KOR activation in the dorsal PAG causes anxiogenic effects and escape behavior (Maraschin et al. 2017), it would be of interest to determine the extent KOR activation in the ventrolateral region may contribute to aspects of negative affective states associated with chronic pain. Kappa ORs present in the ventrolateral PAG and are partially responsible for oxytocin-induced analgesia (Ge et al. 2002). An elegant study recently demonstrated that KOR activation in the ventrolateral PAG reduced GABAergic transmission onto PAG dopamine neurons, suggesting that KOR activation leads to disinhibition of these neurons to modulate pain transmission (Li and Kash 2019). These ventrolateral PAG dopamine neurons project to the extended amygdala (BNST and CeA), areas known to regulate stress and anxiety where they contribute to Pavlovian fear conditioning (Matthews et al. 2016) and were proposed to be responsible for aberrant fear memory formation in PTSD patients (Torrisi et al. 2019). In addition, they also contribute to wakefulness where they have reciprocal connections with the sleep–wake regulatory system (Lu et al. 2006). Hence, there is a possibility that KOR modulation of PAG neurons in both the dorsal and ventral regions may contribute to the emotional, affective dimension of the pain response. The subsequent sections in this chapter will focus on how chronic pain alters KOR expression and function in mesolimbic circuitry and the functional consequences of this enhanced KOR system.

It was identified that a group of GABAergic prodynorphin positive neurons (tyrosine hydroxylase negative) in the brain stem (present in both humans and rodents) are poised to regulate pain transmission. LJA5 (lateral pons, juxta A5) projects to the lateral and ventrolateral PAG, the lateral parabrachial nucleus and to lamina I of the spinal cord, and receives input from many stress and sensory areas including the somatosensory and insula cortices, the paraventricular nucleus of the hypothalamus, the dorsomedial nucleus and lateral hypothalamus, central nucleus of the amygdala, periaqueductal gray and lateral parabrachial nucleus (Agostinelli et al. 2021). Whether chronic pain changes the expression of prodynorphin transcript in this specific brain region has yet to be assessed, but these neurons are the only known inhibitory neurons that project directly and selectively to innervate lamina I of the spinal cord, and appose dynorphin neurons in lamina I that project to the parabrachial nucleus (Standaert et al. 1986).

An important component of the pain experience is the negative, affective, emotional component of pain. Narita and colleagues were the first to report the

occurrence of an anxiogenic phenotype in mice with chronic pain and that the time line of resolution of negative affect correlated with recovery of sensory pain hypersensitivity (Narita et al. 2006). Subsequent studies by Yalcin and colleagues elegantly reported the timeline history/development of various anxiogenic and depressive-like behaviors associated with neuropathic pain in mice (Yalcin et al. 2011). Chronic inflammatory, but not neuropathic pain, produced a significant increase in KOR agonist stimulated [^{35}S]GTPγS binding in membranes prepared from the amygdala at 4 weeks post-injury. In the amygdala, KOR is thought to contribute to anxiety-like behavior, as intracerebral amygdala injection of dynorphin A precipitated an anxiogenic-like phenotype (Narita et al. 2006). Given the overlap in circuitry is involved in pain processing, emotional learning, and fear, it is perhaps not surprising that chronic pain causes an upregulation of KOR systems in limbic brain structures. We recently demonstrated that KOR and dynorphin mRNA transcript were upregulated in the nucleus accumbens (NAc) and ventral tegmental area (VTA) of chronic pain mice, compared to sham controls (Liu et al. 2019), while others also report an upregulation of KOR mRNA in the locus coeruleus (Llorca-Torralba et al. 2020) and prefrontal cortex (Palmisano et al. 2018).

To confirm to what extent the upregulation in transcript expression within mesolimbic circuitry translates into an increased function of the receptor, we showed using *ex vivo* autoradiography that KOR agonist-induced [^{35}S]GTPγS autoradiographic binding was increased in the NAc and VTA of chronic pain mice (Liu et al. 2019). Enhanced KOR agonist stimulated [^{35}S]GTPγS was also reported in the prefrontal cortex and somatosensory cortex one month after induction of neuropathic pain (Llorca-Torralba et al. 2020). The enhanced KOR activity was also evidenced by an increase in the phosphorylated state of the KOR in the NAc in the absence of exogenous agonist administration (Liu et al. 2019). Prodynorphin mRNA was increased in the NAc, the prefrontal cortex and anterior cingulate cortex 2 weeks after nerve injury model of neuropathic pain (Palmisano et al. 2018; Liu et al. 2019). Interestingly, which peptide is cleaved from the prodynorphin peptide was suggested to be brain region specific. Hence, bioconversion of dynorphin B produced DYN B (1-7) in cortical areas, whereas it produced dynorphin B (2-13) in the striatum (Bivehed et al. 2017). These data suggest that prodynorphin cleavage to various peptides within specific brain regions can influence whether KOR or non-opioid mediated effects are produced and would presumably influence various aspects of the pain experience.

2.3 Kappa OR Agonist-Induced Place Aversion Is Enhanced in Chronic Pain States

Kappa OR-mediated dysphoric states are evaluated in rodent models using agonist-induced conditioned place aversion (CPA). CPA is evident in [pain-naïve] rodents when conditioned to KOR (unbiased) agonists, non-specific opioid antagonists such as naloxone, or lithium chloride (LiCl, which produces gastrointestinal distress, emesis, and vomiting in humans). KOR agonist-induced CPA can be elicited

following systemic administration or microinjection directly into the VTA, NAc, prefrontal cortex, and lateral hypothalamus, but not the dorsal striatum or substantia nigra (Fig. 2a) (Bals-Kubik et al. 1993; Tejeda and Bonci 2018). Microinjection of KOR antagonists into the prefrontal cortex or dorsal raphe nucleus blocked CPA produced by systemic administration of a KOR agonist, suggesting a critical role for these brain regions in the aversive state (Land et al. 2009; Tejeda et al. 2013). Using conditional knockout mice, KORs on dopamine neurons were shown to be sufficient to produce a place aversion (Chefer et al. 2013; Ehrich et al. 2015b) and re-expression of KOR in dopamine neurons using a viral approach recovered KOR-mediated place aversion suggesting that KOR on dopamine neurons is sufficient for KOR-mediated place aversion (Chefer et al. 2013). The activation of KORs on mesolimbic dopamine neurons that are responsible for KOR agonist-induced aversion was subsequently identified to require activation of p38 MAP kinase (Ehrich et al. 2015b), although there was a dissociation between aversion and changes in dopamine release within the ventral striatum.

Despite this strong evidence for KOR on dopamine neurons projecting to the NAc being responsible for KOR-mediated aversion, re-expression of KORs in the dorsal raphe using lentivirus approaches in the KOR knockout mouse also restored KOR agonist-induced aversion, whereas re-expression of a mutant receptor that failed to activate p38 MAP kinase did not restore aversion (Land et al. 2009). Indeed, subsequent studies also reported that KOR activation in serotoninergic dorsal raphe neurons is both necessary and sufficient to mediate KOR agonist aversive behavioral effects as well (Land et al. 2009; Ehrich et al. 2015b). It remains unclear whether the dopamine and serotonin systems integrate to produce the aversive state associated with KOR activation or if there are multiple brain regions that are sufficient to elicit this aversive state. Considering that KORs are expressed on terminals of dopamine neurons projecting to various targets including (but not limited to) the basolateral amygdala (BLA), NAc, and prefrontal cortex, it is not unreasonable to question whether the inhibitory effects on dopamine release to other targets may also contribute to KOR-mediated aversion, although there are conflicting reports of KOR regulation of dopamine neurons within each of these brain regions (Tejeda and Bonci 2018; Margolis and Karkhanis 2019). KOR agonists also appear to modulate other circuits (independent of monoamines such as dopamine) to produce aversion. Inhibition of glutamate and GABA synaptic transmission in the NAc shell by KOR agonists (Hjelmstad and Fields 2001) was also proposed to contribute to KOR agonist-induced aversion.

Considering chronic pain is a stressor in itself, it is unknown if the associated KOR upregulation is generalizable to all stressors. Stress induced by maternal separation and social isolation increased KOR expression in the amygdala (Nakamoto et al. 2020). However, chronic stress induced by social defeat *decreased* dynorphin transcript in the NAc (Donahue et al. 2015), although acute social defeat stress increased it in this brain region and additionally induced KOR-mediated stress-induced analgesia. Despite the lack of change in prodynorphin transcript in the chronic stress state, this latter study reported that ablating KORs from dopamine neurons using a conditional knockout approach delayed the development of chronic

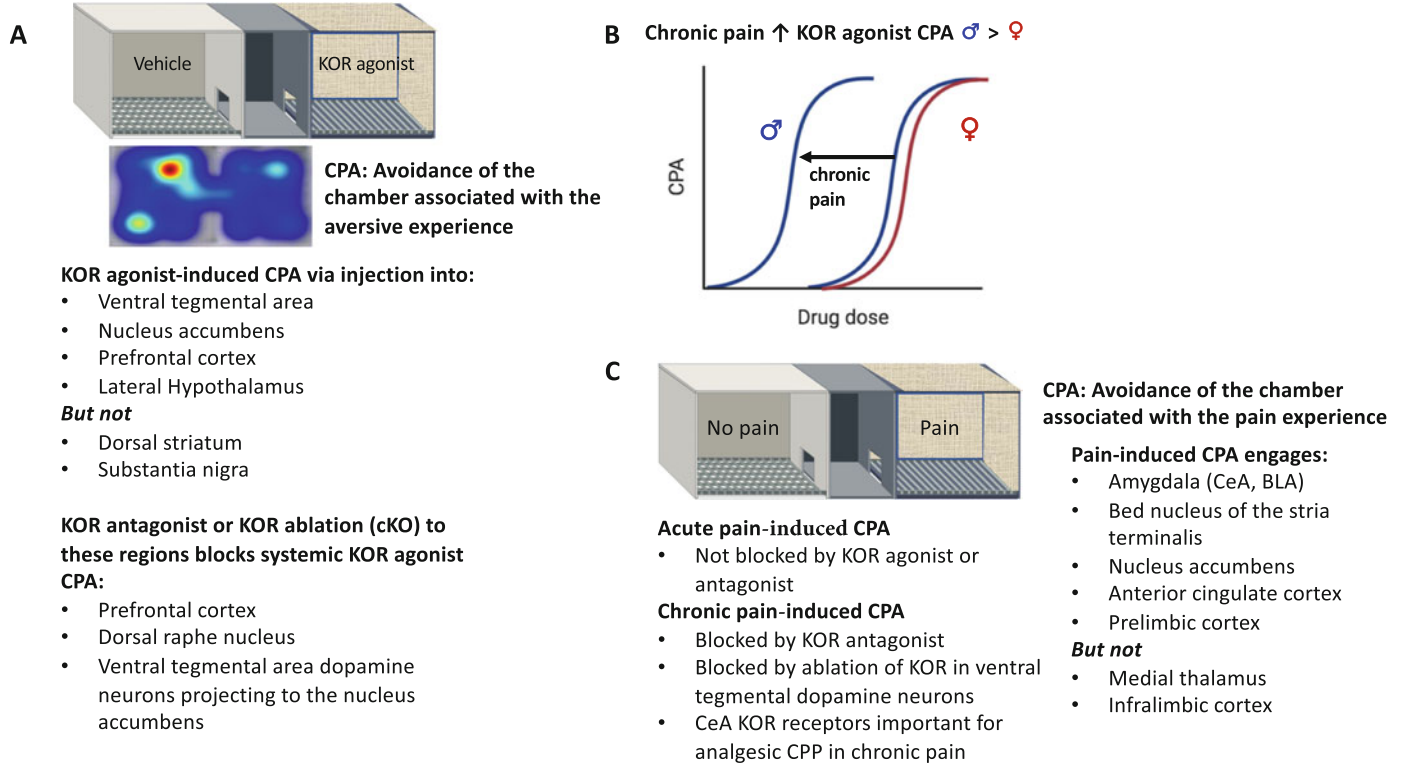

Fig. 2 Use of conditioned place preference protocols to measure kappa opioid receptor agonist mediated aversion and pain-induced aversion. (**a**) KOR agonist-induced place aversion (CPA) can be generated by intracerebral injection into specific brain regions, whereas genetic ablation of KORs from specific brain regions eliminates place aversion following systemic agonist administration. (**b**) Chronic pain enhances KOR agonist-induced place aversion in male, but not female, mice. (**c**) Pain-induced aversion where an acute pain stimulus is used for conditioning is not blocked by either KOR agonists or antagonists, however, KOR antagonists block CPA in chronic pain states. Brain regions important for the expression of pain-induced place aversion include the amygdala, bed nucleus of the stria terminalis, nucleus accumbens, anterior cingulate cortex, and prelimbic cortex

stress-induced anhedonia, as measured by intracranial self-stimulation (ICSS, an operant behavior sensitive to increases and decreases in the reinforcing efficacy of rewarding brain stimulation) of monopolar electrodes within the lateral hypothalamus. This study concluded that the KOR expression on dopamine neurons contributed to an increase in stress resilience. Nevertheless, the findings from this study argue that the dynorphin and KOR upregulation in chronic pain states is not generalizable across all types of chronic stress, but the dial up in function appears to be generalizable to withdrawal associated with chronic drug use (Koob 2020).

To determine the extent KOR aversion may be enhanced in chronic pain, we used the CPA assay. The KOR agonist-induced dose-dependent CPA was shifted to the left in male, but not female, chronic pain mice, suggesting a sex-dependent enhancement of KOR-mediated effects in chronic pain states (Fig. 2b) (Liu et al. 2019). This sex difference is consistent with human imaging data showing that KOR binding is greater in men than women in multiple brain regions, including the anterior cingulate cortex, which is involved in the emotional affective dimension of pain (Vijay et al. 2016). Furthermore, females were shown to be less sensitive than males to the KOR agonist-induced deficits in motivation as measured by ICSS of the medial forebrain bundle and less effective in suppressing evoked NAc dopamine release measured via fast scanning cyclic voltammetry (Conway et al. 2019). Nevertheless, there are conflicting reports of the effects of ongoing pain on KOR-mediated aversion, where the place aversion to a KOR agonist was absent in male rats with persistent ongoing pain induced by complete Freund's adjuvant (Shippenberg et al. 1988). Although it is unclear if the pain may have interfered with the essential cue learning required to elicit a CPA as a positive control was not included in the study. Taken together, chronic pain causes a sex-dependent enhancement of KOR-mediated aversion, although it is unknown if the same circuits are engaged as those in the absence of pain.

3 Kappa OR Modulation of the Affective, Emotional Dimension of Pain

Similar to the use of a CPA protocol to detect a drug-induced aversive state, pairing a painful event (rather than a drug) with contextual cues allows animals to learn the association of an environment with a painful stimulus. Painful stimuli such as intraplantar formalin or carrageenan, or intraperitoneal injection of acetic acid can produce a pain evoked CPA (Fig. 2c). Optogenetic activation or inhibition of nociceptive neurons using real-time place conditioning paradigms also shows place conditioning to painful stimuli (Daou et al. 2013; Iyer et al. 2014; Park et al. 2015; Beaudry et al. 2017). The aversive component of pain has been captured by other place avoidance paradigms to try to capture pain affect in chronic pain states. LaBuda and Fuchs (2000) reported a place avoidance escape assay whereby the rodent has a conflict between staying in a dark compartment associated with a mechanical stimulus or entering a light compartment with no, or non-painful,

sensory stimuli. A CPA can also be produced by combining experimenter-provoked mechanical allodynia during the place conditioning, whereby von Frey filaments are applied to the injured hind paw through a grid floor in the place conditioning apparatus (Hummel et al. 2008). The CPA induced by sensory stimulation in either neuropathic or inflammatory chronic pain models was maintained for at least one month in the absence of further conditioning and non-rewarding doses of morphine given during the pain-paired conditioning sessions attenuated the CPA. But what, if any role does the KOR system have in modifying the affective emotional aversive component of this pain experience?

The role of KORs in mediating the affective dimension of pain states most likely depends on the duration of pain (acute vs. chronic) or the conditioning protocol used to assess the aversive painful state or more likely the co-occurrence of a negative affective state associated with chronic pain. The CPA produced by intraperitoneal injection of acetic acid was *not* blocked by pretreatment with a KOR antagonist (or KOR agonist) (Bagdas et al. 2016). Electroacupuncture prevented the expression of a CPA to intraplantar injection of complete Freund's adjuvant and this effect was absent in rodents pretreated with a MOR, but not KOR, antagonist injected into the rostral anterior cingulate cortex (Zhang et al. 2012). Indeed, in rats with postsurgical or neuropathic pain, endogenous MOR signaling in the anterior cingulate cortex was necessary for the expression of a conditioned place preference (CPP) to non-opioid pain relieving treatments such as a peripheral nerve block or spinal clonidine (an alpha2-adrenergic agonist) and this receptor activation was also associated with dopamine release in the NAc (Navratilova et al. 2015).

3.1 Kappa OR Ligands Do Not Alter Acute Pain-Induced Aversion or Depression of ICSS

The affective dimension of pain can be captured using ICSS, an operant procedure in which subjects emit a learned response such as a lever press to earn pulses of electrical stimulation to brain reward areas and is reliant on dopamine release in the ventral striatum. Studies have shown that acute visceral pain (Leitl et al. 2014a) and different models of inflammatory pain (formalin or complete Freund's adjuvant) (Leitl et al. 2014b) depressed ICSS. Pain-induced (complete Freund's adjuvant, formalin or lactic acid) depression of ICSS was not prevented by pretreatment with a KOR antagonist (Leitl et al. 2014a, b). Despite the lack of involvement in KOR systems for the expression of acute pain-induced aversion, it does not rule out the possibility that activation of KORs contributes to negative affective states associated with chronic pain states. Indeed, chronic pain is highly co-morbid with mood disorders in clinical populations. KOR agonists activate the HPA axis, and produce pro-depressive and anxiogenic-like effects, whereas KOR antagonists cause opposing effects in producing anxiolytic-like and anti-depressant effects (Van't Veer and Carlezon 2013). Ablation of KORs from dopamine neurons (Van't Veer and

Carlezon 2013) or KORs on BLA glutamatergic neurons that project to the medial prefrontal cortex (Tejeda et al. 2015) results in an anxiolytic phenotype, suggesting that KOR modulation of these circuits is critical to the expression of negative affect. KOR activation within the BLA was necessary for the anxiogenic effect of corticotropin releasing factor (Bruchas et al. 2009) and eliminating KOR from BLA glutamatergic neurons projecting to the BNST also has an anxiolytic phenotype (Crowley et al. 2016). Considerable evidence suggests that the KOR system within the NAc also underlies negative affective states and heightens stress reactivity in various psychiatric disorders. For example, dynorphin expression is increased in the ventral striatum of suicidal individuals (Hurd et al. 1997) and in animal models of depression (Shirayama et al. 2004; Carlezon and Krystal 2016; Tejeda and Bonci 2018).

3.2 Kappa OR Systems Contribute to Ongoing Persistent Pain States

The lack of KOR involvement in acute pain aversion or ICSS depression may be due to the temporal relationship between the onset of pain and development of negative affective-like behaviors. Anxiety and depressive behaviors that accompany chronic pain states in rodents do not typically begin to manifest until weeks 4–8 following injury (Yalcin et al. 2011). Thus, the KOR system may only be engaged following tissue or nerve injury that induces the negative affective-like behaviors (anxiety and depression) versus the initial associated with an acute painful event. This idea is supported by the finding that, in a model of joint pain, KOR knockout mice showed reduced anxiety (elevated plus maze) (Negrete et al. 2016), where global KOR knockout does not produce an anxiolytic phenotype in the absence of ongoing pain (pain-naïve animals) (Kieffer 1999). Thus, there are contradictory findings reported for the involvement of KOR in pain-induced negative affect and pain aversion, but if one considers the temporal relationship with development of co-morbid affective states, the delayed KOR system involvement more closely correlates with negative affective states.

While the CPP paradigm is useful for studying reward and aversion, the interpretation of data in chronic pain states can be somewhat difficult; the effect size of this CPP may be exaggerated if the rodent was experiencing an aversive state in the non-drug paired chamber. To circumvent this confound, we used a one-sided conditioning protocol (Bechara and van der Kooy 1992) to determine whether chronic pain could evoke a CPA in the absence of stimulating the dermatome affected by an injury. Both mice and rats, independent of sex, produced a CPA to ongoing persistent inflammatory and neuropathic pain, whereas sham control animals had no preference to either chamber (Liu et al. 2019). We interpreted these data to suggest that one can capture the ongoing aversive component of chronic pain. In separate cohorts of mice, KOR antagonism with JDTic prevented place

aversion in male, but not female, chronic neuropathic pain mice, demonstrating a sex-specific engagement of KORs in pain aversive states (Fig. 2c), which was generalizable to chronic inflammatory pain. It is noteworthy that KOR blockade had no effect on mechanical withdrawal thresholds in sham or chronic pain mice demonstrating that KOR involvement in pain circuitry is restricted to the affective, but not sensory, dimension of chronic pain. This study demonstrated that KOR expression on dopamine neurons was sufficient to produce the chronic pain-induced CPA. In addition to capturing this ongoing pain response, other studies have shown that the negative reinforcement produced by gabapentin and morphine in chronic pain models was absent in rodents that received KOR antagonists (Liu et al. 2019; Navratilova et al. 2019). One interpretation of these data is that blocking the negative affect by KOR antagonism eliminated the motivation and drive for pain relief produced by gabapentin and morphine. One could even suggest that the alleviation of the negative affective state was the only motivation to seek morphine or gabapentin.

In addition to the contribution of KORs within mesolimbic dopamine neurons to the tonic aversive component of ongoing chronic pain, KORs within the amygdala (CeA and BLA) have also been implicated in modulating the pain experience including pain-induced aversion (Corder et al. 2019; Navratilova et al. 2019; Phelps et al. 2019). In un-injured pain-naïve animals, stress produced allodynia (painful response to something not normally painful) via a KOR-dependent mechanism in the CeA (Xie et al. 2017). The CeA receives both nociceptive sensory input from the spinal cord via the parabrachial nucleus (Chiang et al. 2020) and noradrenergic innervation from the locus coeruleus (also implicated in reinstatement of drug-seeking behavior) (España et al. 2016), making it a central brain region for integrating pain and stress. The lateral CeA has been termed the "nociceptive amygdala" given that neurons in this brain region respond exclusively to noxious stimulation (Neugebauer et al. 2020). Intra-CeA administration of a KOR antagonist had no effect on mechanical hypersensitivity in a rat model of neuropathic pain, but prevented the CPP to intravenous gabapentin, suggesting that KOR blockade in this brain region eliminated the aversiveness of ongoing pain (Navratilova et al. 2019). This latter study also demonstrated that KOR blockade also reduced synaptically evoked CeA neuronal spiking in chronic pain, but not sham control, animals, leading the authors to hypothesize that increased KOR activity produces a tonic disinhibition of CeA output neurons that promotes ongoing aversive aspects of neuropathic pain. While KORs in the CeA do not modulate pain hypersensitivity, they may be involved in the loss of diffuse noxious inhibitor control (descending circuits such as the PAG, locus coeruleus and dorsal raphe nucleus that regulate nociceptive transmission) that occurs in various chronic pain states (Phelps et al. 2019). Another recent study reported that optogenetic activation of CeA neurons suppressed both pain-elicited reflexive and self-recuperating behaviors across sensory modalities and abolished neuropathic pain hypersensitivities, whereas inhibiting CeA neuronal activity exacerbated pain and produced a strong aversion (Hua et al. 2020). Future studies are needed to understand to what extent KOR systems in the extended

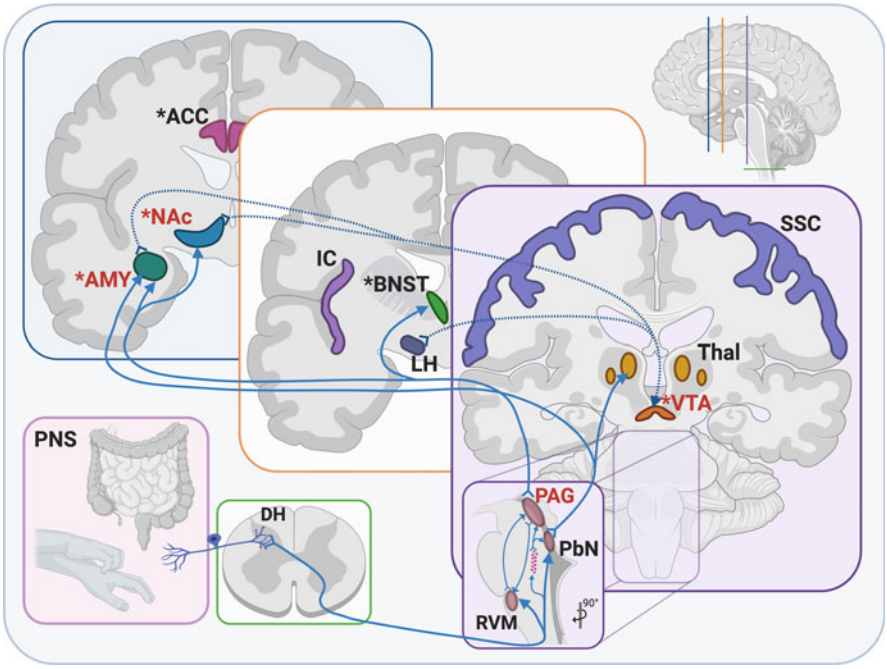

Fig. 3 Anatomical expression and contribution of the dynorphin-KOR system in the sensory and affective components of chronic, persistent pain. KORs are present on peripheral sensory inputs from skin and visceral targets, where they synapse onto the DH of the spinal cord. Within the spinal cord, KORs are concentrated in the lamina II of the superficial DH. From here, sensory information is sent via ascending connections into numerous brain stem nuclei, including the RVM, PbN, and a cluster of LJA5 cells. From there, sensory information is relayed into midbrain regions, including PAG and VTA, where KORs are concentrated and shown to be upregulated following chronic pain. Connections from the brain stem and midbrain are further relayed to numerous forebrain areas, including PbN to Thal and AMY, as well as PAG to AMY, BNST, and NAc, all regions shown to contribute to the affective dimension of pain. KOR is also increased in NAc and AMY following chronic pain. Other regions involved in the affective and/or sensory dimensions of pain are the ACC, IC, and SSC, though the connectivity and specific contribution of KOR is not yet fully understood. *areas known to be involved in affective pain; red text: areas where KOR activity is increased following chronic pain; dotted arrows: known dynorphin connections; pink stars: LJA5 cells. *ACC* anterior cingulate cortex, *AMY* amygdala, *BNST* bed nucleus of the stria terminalis, *DH* dorsal horn, *IC* insular cortex, *LH* lateral hypothalamus, *NAc* nucleus accumbens, *PAG* periaqueductal gray, *PbN* parabrachial nucleus, *PNS* peripheral nervous system (sensory components), *RVM* rostral ventromedial medulla, *SSC* somatosensory cortex, *Thal* thalamic nuclei, *VTA* ventral tegmental area

amygdala also contribute to the pain experience. Figure 3 highlights the anatomical expression and contribution of the dynorphin-KOR system in the sensory and affective components of chronic, persistent pain.

4 Is There Potential for KOR Antagonism as a Management Strategy for Chronic Pain-Associated Negative Affect?

The dysphoric and negative affective states induced by KOR activation generated promise for KOR antagonists as a novel treatment strategy for mood disorders and pharmacotherapy for substance use disorders. A review is available summarizing the completed clinical trials that evaluated safety and effectiveness of KOR antagonists for treatments of substance use and mood disorders (Carlezon and Krystal 2016). Pharmacokinetic studies and potential drug interactions with ethanol were evaluated for the KOR antagonist LY2456302 in healthy subjects (Lowe et al. 2014) as was safety, tolerability, and pharmacokinetics of oral doses of JDTic, which was prematurely stopped due to cardiac adverse events (Buda et al. 2015). One mechanism implicated in KOR-mediated aversion and negative effects on mood is the modulation of mesolimbic dopamine circuitry (Van't Veer and Carlezon 2013). This circuitry plays a central role in motivational processes and incorporates dopaminergic neurons in the VTA that project to the NAc (Berridge and Kringelbach 2013, 2015; Zarrindast and Khakpai 2015). KORs are known to modulate this circuitry by inhibiting dopamine release at dopaminergic nerve terminals within the NAc (Spanagel et al. 1992; Ebner et al. 2010; Cahill et al. 2014; Ehrich et al. 2015b; Chartoff et al. 2016). Considering that systems involved in affective aspects of pain processing and other affective and motivational systems interact extensively (Jarcho et al. 2012; Elman et al. 2013), there is potential that KOR blockade may prove to be a novel pain management strategy.

Epidemiological evidence shows that chronic pain is second only to bipolar disorder as the major cause of suicide among all medical illnesses (Asmundson and Katz 2009; Elman et al. 2013) and that mood disorders are highly co-morbid in chronic pain patients, where the prevalence of depression ranges between 30 and 80%, depending on the pain etiology (Bair et al. 2003; Howe and Sullivan 2014). It is consistently reported that co-morbid psychopathology in patients with chronic pain exhibit increased pain intensity and increased pain-related disability (Jamison and Edwards 2013; Martel et al. 2014), thus managing negative affect holds promise in reducing pain ratings in patients with chronic pain. We, and others, identified that mesolimbic circuitry dysfunction (including dopamine neurotransmission) precipitates mood disorders, impairs motivated behavior, and likely contributes to chronic pain (Narita et al. 2005; Cahill et al. 2014; Hipólito et al. 2015; Taylor et al. 2015; Borsook et al. 2016; Evans and Cahill 2016; Cahill and Taylor 2017; Liu et al. 2019; Massaly et al. 2019). There is compelling evidence that KORs within the ventral striatum underlies negative affective states and heightens stress reactivity in psychiatric diseases (Ebner et al. 2010; Knoll and Carlezon 2010). Supporting this thesis is the report that prodynorphin mRNA was increased in the ventral striatum of victims of suicide (Hurd et al. 1997), although no difference in dynorphin levels in cerebral spinal fluid was found in patients with non-suicide self-injurious behavior, yet this group did have lower levels of other endogenous opioids (Stanley et al. 2010).

Preclinical and human subject research have identified strong correlations between dysfunction of this mesolimbic circuitry and chronic pain (Jarcho et al. 2012; Elman et al. 2013; Borsook et al. 2016; Evans and Cahill 2016; Taylor et al. 2016; Cahill and Taylor 2017). The increase in extracellular dopamine following intra-VTA or systemic administration of MOR agonist is absent in chronic neuropathic pain (Ozaki et al. 2002; Taylor et al. 2015) and inflammatory pain states (Narita et al. 2005; Hipólito et al. 2015), which correlates with the loss of opioid reward as measured by CPP following intra-VTA or systemic administration of MOR agonists (Ozaki et al. 2002; Taylor et al. 2015). Using *in vivo* microdialysis in awake, freely moving animals, we demonstrated that blocking KORs recovered the loss of opioid-induced dopamine release in the NAc associated with neuropathic pain (Liu et al. 2019). This finding is consistent with previous reports that blocking KORs recovered opioid-evoked dopamine release in the formalin model of inflammatory pain (Narita et al. 2005). Interestingly, the morphine-evoked increases in NAc extracellular dopamine can be blocked by administration of a KOR agonist into the NAc but not the VTA (Spanagel et al. 1992), implying that KORs within the ventral striatum are important in the negative modulation of mesolimbic dopamine dependent motivation produced by MOR agonists such as morphine. Given that hypo-dopaminergic states contribute to chronic pain (Borsook et al. 2016; Taylor et al. 2016) and mood disorders co-morbid with chronic pain (Elman et al. 2013), recovering rewarding effects is an important component of alleviating chronic pain. While KOR antagonism had no effect on opioid reward in pain-naive cohorts, it restored DAMGO-induced CPP in chronic pain animals (Liu et al. 2019). This study further highlights the involvement of NAc KORs in chronic pain-induced negative affective states. Using an inflammatory model of chronic pain induced by complete Freund's adjuvant, Massaly and colleagues showed that the reduced motivation for sucrose self-administration (via progressive ratio testing) required the engaged dynorphin neurons in the NAc and was prevented by KOR antagonism (Massaly et al. 2019). Taken together, KORs contribute to the hypo-dopaminergic tone and reward-related behaviors in chronic pain without altering sensory pain hypersensitivities, suggesting that KOR antagonists may be an effective treatment in pain management for chronic pain patients.

5 Evidence That KOR Systems May Contribute to Drug-Seeking Behavior in Chronic Pain States

As noted above, mood disorders are highly co-morbid in chronic pain patients (Bair et al. 2003; Howe and Sullivan 2014) and are a risk factor for the development of opioid use disorder (OUD) (Evans and Cahill 2016). A systematic review reported that chronic pain patients have high rates (13-38%) of opioid misuse (defined as opioid use contrary to the directed or prescribed pattern of use, regardless of the presence or absence of harm or adverse effects) (Vowles et al. 2015). This is consistent with reports where electronic health records of >5,000 patients with opioid use disorder revealed the majority of patients reported chronic pain preceded

their opioid use disorder, and 85% of this cohort had a co-morbid mental disorder (Hser et al. 2017), a finding recently duplicated by another study (Higgins et al. 2020). The high abuse rates of therapeutic opioids have fueled a strong debate on the treatment practices of using prescription opioids and whether pain is a major factor in addiction susceptibility. With the overwhelming evidence that negative affective states drive opioid use disorder (Evans and Cahill 2016), including in chronic pain patients, it is expected that chronic pain states lead to activation of KOR systems that are involved in stress-induced reinstatement of opioid drug-seeking behavior. However, how KOR systems modulate drug-seeking behavior in chronic pain states has not been explored, but there are various clinical trials that have reported the effectiveness of KOR partial agonism or antagonism in modulating substance use disorders (Table 1).

As noted above, there is overlapping expression of the KOR and its endogenous ligands dynorphins within reward and stress pathways, which contributes to the ability of this system to alter stress- and reward-related signaling in the brain (Bruchas et al. 2010; Wee and Koob 2010; Crowley and Kash 2015). Drug relapse can be produced by a stressful event, presentations of cues associated with drug taking, as well as the drug itself (drug-priming). A number of studies have implicated KORs in stress-induced relapse/reinstatement of drug-seeking behavior (Table 2). KOR antagonists block stress-induced reinstatement, while KOR agonists, such as U50,488, induce reinstatement of cocaine (Beardsley et al. 2005; McLaughlin et al. 2006; Redila and Chavkin 2008; Polter et al. 2014; Heinsbroek et al. 2018), nicotine (Jackson et al. 2013; Nygard et al. 2016), and alcohol (Funk et al. 2014, 2019a, b; Lê et al. 2018) seeking behavior. Stress caused by injection of yohimbine (Zhou et al. 2013) or food deprivation (Sedki et al. 2015) precipitated reinstatement of heroin drug-seeking, is also blocked by pretreatment with the KOR antagonist nor-BNI.

While there is significant promise in the potential for KOR antagonists to attenuate drug relapse, distinct mechanisms have been shown to underlie reinstatement due to either cue, drug-priming or stress. For example, context-induced reinstatement of oxycodone was blocked by MOR, but not KOR antagonists (Bossert et al. 2019), albeit low dose naloxone (0.03 mg/kg) increased heroin drug intake and elevated ICSS thresholds (above already elevated baseline levels) when stimuli was paired with the naloxone treatment (Kenny et al. 2006). Additionally, social defeat-induced stress produced KOR-mediated anhedonia, as measured by ICSS (Donahue et al. 2015). Other studies show that KOR antagonism *did not affect* morphine (Glick et al. 1995) or heroin (Negus et al. 1993) self-administration or context-induced reinstatement of oxycodone self-administration (Bossert et al. 2019). Furthermore, KOR agonists decreased cocaine intake and cocaine drug-seeking (Glick et al. 1995; Schenk et al. 1999; Morani et al. 2009), as well as cocaine or amphetamine-induced reinstatement in mice (Schenk and Partridge 2001) and non-human primate (Negus et al. 1997; Mello and Negus 2000; Rüedi-Bettschen et al. 2010). Similarly, KOR agonists reduced oxycodone self-administration in non-human primates (Zamarripa et al. 2020a), decreased morphine self-administration in mice (Glick et al. 1995) and blocked oxycodone CPP in male rats (Zamarripa et al. 2020b). Considering ongoing pain is a stressor in itself, it is unknown to what extent KOR systems contribute to

Table 1 Clinical trials that KOR partial agonism or antagonism improves substance use disorder (pain is noted). Nalmefene (Selincro®), a mu opioid receptor inverse agonist with weak partial KOR agonist properties, is used in the treatment of alcohol use disorder. Buprenorphine (partial mu opioid agonist, ORL-1 agonist, KOR antagonist/partial KOR agonist, and delta opioid antagonist) and naloxone (Zubsolv®, Suboxone®) combination has been used for treating opioid use disorder (Heo and Scott 2018) and this combination was reported to reduce pain. Buprenorphine in combination with samidorphan (MOR antagonist) showed beneficial effects over placebo to reduce symptoms in major depressive disorder patients who had inadequate responses to antidepressants, although this drug combination was recently rejected by the US Food and Drug Administration. Polymorphisms in the KOR gene are associated with opioid dependence. Previous reviews have discussed the safety and efficacy of buprenorphine for the treatment for chronic pain (Davis et al. 2018; Pergolizzi and Raffa 2019) or opioid use disorder (Parida et al. 2019)

Species	Sex	Protocol	KOR ligand	Brain region	Model	Outcome	Ref
Human	M/F	Phase IV clinical trial	Nalmefene	na	EtOH-dependent outpatients	↓ Heavy drinking days ↓ Total EtOH consumption	(Barrio et al. 2018)
Human	M/F	Double -blind Multicenter Randomized	Nalmefene	na	AUD (>60 g/day M >40 g/day F)	↓ Heavy drinking days ↓ Total EtOH consumption	(Miyata et al. 2019)
Human	M/F	FMRI Placebo controlled, double- blind	Nalmefene	Putamen, angular gyrus, supramarginal gyrus, (↔amygdala)	AUD	↑ BOLD to emotional faces in areas responsible for empathy and social cognition, attentional shift happy > fearful	(Vollstädt-Klein et al. 2019)
Human	M/F	Double-blind, placebo control trial	Nalmefene	na	AUD	↓ Heavy drinking days	(Mason et al. 1999)
Human	Male	FMRI with i.v. EtOH (6% v/v to achieve 80 mg/dL)	Nalmefene	Striatum	Heavy drinkers	↓ BOLD during reward anticipation	(Quelch et al. 2017)
Human			ORPK1 gene polymorphism (rs10958350-rs7016778-rs12675595)	na	Methadone maintenance for OUD	Polymorphism was associated with opioid withdrawal	(Wang et al. 2014)

(continued)

Table 1 (continued)

Species	Sex	Protocol	KOR ligand	Brain region	Model	Outcome	Ref
Human			ORPK1 gene polymorphism (rs997917, rs6985606)	na	Methadone maintenance for OUD	Polymorphism was associated with opioid dependence	(Albonaim et al. 2017)
Human			ORPK1 gene polymorphism (KOR 36G > T SNP, rs6473797, rs16918842, rs3802279)	na	Heroin-dependence	Polymorphism was associated with heroin dependence	(Yuferov et al. 2004; Gerra et al. 2007; Levran et al. 2008; Yuanyuan et al. 2018)
Human	Male	Suicide ideation for inpatients	Buprenorphine (sublingual)	na	OUD and major depressive disorder	↓ Suicide ideation	(Ahmadi et al. 2018)
Human	M/F	Multicenter double- blind placebo controlled trial	Buprenorphine/ samidorphan (not approved by FDA)	na	Major depressive disorder	↓ Depression (HAM-D, 17-item scale, Montgomery-Åsberg depression rating scale and the clinical global impressions severity scale)	(Ehrich et al. 2015a; Fava et al. 2016)
Human	M/F	OUD	Buprenorphine-naloxone	na	OUD	↓ Pain intensity (in non-chronic pain cohorts)	(Becker et al. 2015)
Human	M/F	In patient oxycodone self-administration	Buprenorphine-naloxone	na	OUD/chronic pain transitioning from opioid to Bup/Nx	↓ Pain ratings when switched to Bup/Nx ↔ between placebo and oxycodone preference, those that showed oxycodone preference had lower Bup/Nx dose, more withdrawal and more pain	(Roux et al. 2013)

Human	M/F	Pilot clinical trial	Buprenorphine-naloxone	na	OUD/chronic pain transitioning from opioid to Bup/Nx	↓ Average and worse pain after switching to Bup/Nx	(Rosenblum et al. 2012)
Human	M/F	Post-secondary analysis of randomized trials	Buprenorphine-naloxone	na	OUD	>50% reported pain at baseline Improvement in pain correlated with increased retention of treatment	(Shulman et al. 2020)
Human	M/F	Open-label randomized	Buprenorphine-naloxone	na	OUD	↔Pain intensity, affective pain or sensory pain but women had greater affective pain than men	(Latif et al. 2019)
Human	M/F	Post-secondary analysis of randomized trials	Buprenorphine-naloxone	na	OUD	Patients with flare-up pain were at higher risk of relapse	(Griffin et al. 2016)
Human	M/F	Post-secondary analysis of randomized trial	Buprenorphine-naloxone	na	OUD with chronic pain	↓ Pain over course of 12 week treatment, those with high pail volatility were more likely to relapse	(Worley et al. 2015, 2017)
Human	Male	Case study (40 year+ with chronic pain)	Buprenorphine-naloxone	na	Chronic pain with long-term opioid use	↓ Pain, improved function and quality of life	
Human	Male	Randomized trial	Buprenorphine-naloxone vs. methadone	na	Chronic pain and OUD	↓ Pain at 6 months	(Neumann et al. 2013)

Table 2 Evidence that KOR activation contributes to stress-induced reinstatement of opioid seeking behavior in preclinical models

KOR ligand	Species	Sex	Drug of abuse/ protocol	Stressor/pain	Main outcome	Reference	Pain component
Nor-BNI	Sprague Dawley rats	Male	Heroin IVSA and stress-induced reinstatement	Yohimbine (YOH)	KOR blockade reduced reinstatement	(Zhou et al. 2013)	No
Nor-BNI	Sprague Dawley rats	Male	Heroin IVSA and stress-induced reinstatement	Food deprivation	KOR blockade reduced reinstatement	(Sedki et al. 2015)	No
Nor-BNI	Mouse (C57Bl/ 6)	Male	Morphine CPP and drug-prime reinstatement	Incisional postsurgical pain model	KOR blockade enhanced drug-primed reinstatement	(Nwaneshiudu et al. 2020)	Yes
LY2456302	Sprague Dawley rats	Male	Oxycodone IVSA and cue-induced reinstatement	None	KOR blockade had no effect on reinstatement	(Bossert et al. 2019)	No
CJ-15208 (Cyclo[Pro-Sar-Phe-D-Phe]) or Nor-BNI	Mouse (C57Bl/ 6)	Male	Morphine CPP and stress-induced reinstatement	Forced swim stress	Both drug interventions prevented reinstatement	(Brice-Tutt et al. 2020; Ferracane et al. 2020)	No
Buprenorphine + naltrexone	Sprague Dawley rats	Male	Morphine CPP and drug-prime reinstatement	None	The combination blocked drug-primed reinstatement	(Cordery et al. 2014)	No
Dezocine (partial MOR agonist, KOR antagonist) or buprenorphine	Sprague Dawley rats	Male	Morphine CPP and drug-prime reinstatement	None	Both drug interventions prevented reinstatement	(Wu et al. 2019)	No
Nor-BNI	Sprague Dawley rats	Male	Morphine CPP and drug-prime reinstatement	None	KOR blockade did not alter drug-primed reinstatement	(He et al. 2019)	No

stress- and drug-priming-induced reinstatement under conditions of chronic pain. However, one study reported that post-operative pain (incisional model) suppressed morphine-primed reinstatement in self-administration studies and KOR antagonism reversed this inhibition (Table 2) (Nwaneshiudu et al. 2020).

6 Conclusions

The dynorphin-KOR system is a near ubiquitously expressed receptor system found throughout the brain and body that has been implicated in a wide array of physiology and behaviors, including nociceptive processing, chronic pain states, and emotional regulation/dysregulation. It remains a prime target for the potential development of novel therapeutics for the treatment of acute and chronic pain, in the absence of abuse liability. However, likely due to the widespread expression of both the endogenous dynorphin peptides and KOR, designing appropriate pharmacotherapies has proved challenging, due to many unwanted physical and affective side effects. Over the past few decades, researchers have developed a deeper understanding of dynamic signaling capabilities involved with both dynorphin and KOR under various physiological conditions, as well as within and between various tissues and brain regions. This knowledge has resulted in the potential development of both biased agonists and peripherally restricted compounds targeting KOR for the alleviation of pain. Furthermore, while KOR agonists have long been considered the ultimate goal for drug development, due to the upregulation of KOR observed in the chronic pain states, researchers have found some success in using KOR antagonists to decrease the affective dimension of pain. This has opened another avenue of research into the role of dynorphin and KOR in emotional dysregulation following chronic pain and/or opioid use.

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KOR Control over Addiction Processing: An Exploration of the Mesolimbic Dopamine Pathway

Paige M. Estave, Mary B. Spodnick, and Anushree N. Karkhanis

Contents

Abstract

Drug addiction is a complex, persistent, and chronically relapsing neurological disorder exacerbated by acute and chronic stress. It is well known that the dynorphin/kappa opioid receptor (KOR) system regulates stress perception and responsivity, while the mesolimbic dopamine system plays a role in reward and reinforcement associated with alcohol and substance use disorders. Interestingly,

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the dopamine and dynorphin/KOR systems are highly integrated in mesolimbic areas, with KOR activation leading to inhibition of dopamine release, further altering the perception of reinforcing and aversive stimuli. Chronic or repeated exposure to stress or drugs potentiates KOR function ultimately contributing to a hypodopaminergic state. This hypodopaminergic state is one of the hallmarks of hyperkatifeia, defined as the hypersensitivity to emotional distress that is exacerbated during drug withdrawal and abstinence. The relationship between stress and drug addiction is bidirectional; repeated/chronic stress promotes pro-addictive behaviors, and repeated cycles of drug exposure and withdrawal, across various drug classes, produces stress. Neuroadaptations driven by this bidirectional relationship ultimately influence the perception of the reinforcing value of rewarding stimuli. In this chapter, we address the involvement of the dopamine and dynorphin/KOR systems and their interactions in shaping reinforcement value processing after drug and stress exposure, as well as a combinatorial impact of both drugs and stress.

Keywords

Alcohol use disorder · Dopamine · Dynorphins · Kappa opioid receptor · Nucleus accumbens · Substance use disorder

1 Introduction

The dynorphin/kappa opioid receptor (KOR) system is a main player involved in regulating stress responsivity, mood, and reinforcement learning. Previous literature suggests that KOR and its interaction with dopamine modulates motivated behaviors as well as aversion and negative affective states – all of which contribute to the role of KOR in drug addiction (Margolis and Karkhanis 2019). Modulation of the KOR system shapes drug-seeking behaviors at various stages of the drug addiction cycle (Wee and Koob 2010; Chavkin and Koob 2016). Because the KOR is coupled to inhibitory G-proteins and potassium channels, KOR activation ultimately inhibits neuronal function, thereby reducing neurotransmission of various neurotransmitters such as dopamine, serotonin, glutamate, and GABA (Heijna et al. 1990; Hill and Brotchie 1995, 1999; Tao and Auerbach 2002; Grilli et al. 2009; Li et al. 2012; Karkhanis et al. 2016a). Although KOR activation inhibits neurotransmitter release, the ultimate behavioral outcome of reduced neuronal activity in a single location depends on the circuit in which KOR is involved. In this review, we focus on dynorphin/KOR system-mediated regulation of dopamine in the mesolimbic pathway and the resulting behavioral impact on stress responsivity, drug self-administration, and stress-induced development of drug addiction. We extend the discussion to how increased function of the dynorphin/KOR system and its interactions with the dopamine system alter the hedonic state of the animal. This results in hyperkatifeia, defined as the hypersensitivity to emotional distress leading to negative affective symptoms seen during drug withdrawal and abstinence.

2 KOR Interactions with the Dopamine System

The modulatory control of the dynorphin/KOR system over dopamine in mesolimbic areas has several behavioral implications including development of a negative affective state and changes in drug self-administration. Using electron microscopy immunohistochemistry, Svingos et al. (2001) showed that among neuronal synapses in the nucleus accumbens (NAc), KOR and dopamine transporter (DAT) are colocalized in presynaptic structures, as well as on the plasma membrane of vesicles within terminals. These data suggest that KOR is located on dopamine neuron terminals and potentially regulate dopamine transmission directly. In this section, we will discuss the interaction of KOR with various dopamine receptors and transporter proteins.

2.1 KOR-Mediated Regulation of Dopamine Release

The activation of KOR leads to inhibition of dopamine release in the NAc core and shell (Heijna et al. 1990; Karkhanis et al. 2016a; Rose et al. 2016a; Siciliano et al. 2016; Melchior and Jones 2017). This KOR activation reduces overall extracellular dopamine levels (Di Chiara and Imperato 1988; Spanagel et al. 1992; Zhang et al. 2004; Chefer et al. 2006; Fuentealba et al. 2006; Gehrke et al. 2008; Karkhanis et al. 2016b) as well as decreases salient stimulus evoked tonic and phasic dopamine release (Karkhanis et al. 2016b; Rose et al. 2016a; Siciliano et al. 2016; Melchior and Jones 2017). A recent study showed that the inhibitory effect of KOR activation on dopamine release is potentiated in the caudal compared to rostral NAc shell (Pirino et al. 2020). These changes in release ultimately occur as a result of augmented K^+ and attenuated Ca^{2+} conductance, resulting in cell hyperpolarization, decreased cell firing, and blockade of neurotransmitter release (Dhawan et al. 1996).

Interestingly, there are sex differences in KOR regulation of dopamine release. Particularly, KOR activation-mediated inhibition of dopamine release is blunted in females compared to males (Abraham et al. 2018; Conway et al. 2019). This sex difference is likely driven by two mechanisms: first, dopamine synthesis is greater in female compared to male rats (Conway et al. 2019); second, estradiol increases phosphorylation of G-protein receptor protein kinase 2, thereby reducing G-protein signaling and ultimately attenuating KOR control over dopamine release in mice (Abraham et al. 2018). Notably, mRNA of dynorphin-precursor (*Pdyn*) and KOR genes (Oprk1) were equivalent in male and female rats (Conway et al. 2019), suggesting that the availability of KOR and dynorphins may not be different between the two sexes but rather dopamine availability and gonadal hormones may mediate differential intracellular signaling further, promoting the observed differences in magnitude of dopamine release. For further review on sex differences in KOR function, see Chartoff and Mavrikaki (2015).

2.2 KOR-Mediated Regulation of Dopamine Uptake

The DAT is predominantly involved in regulating the reuptake of dopamine from the synapse. Interestingly, co-immunoprecipitation, bioluminescence resonance energy transfer (BRET), and Fröster resonance energy transfer (FRET) data suggest that the DAT and KOR may physically interact, with KOR activation increasing the number of DAT-KOR complexes (Kivell et al. 2014). Because KOR and DAT are colocalized on dopamine terminals, potentially forming physical complexes, it is likely that KOR activation alters DAT function thereby influencing dopamine uptake. Indeed, DAT and KOR have been shown to functionally interact; salvinorin A, a selective KOR agonist, increases DAT function resulting in enhanced rate of dopamine reuptake in an ERK 1/2 dependent manner (Kivell et al. 2014). However, this increase in DAT function may be transient since repeated agonist administration resulted in decreased rate of dopamine reuptake in the NAc (Thompson et al. 2000). This decrease in dopamine uptake rate has also been observed using ex vivo fast scan cyclic voltammetry with the partial KOR agonist nalmefene (Rose et al. 2016b), but not with full KOR agonists (Ebner et al. 2010; Ehrich et al. 2015; Hoffman et al. 2016). These differences could be due to differential intracellular mechanisms of action of the partial agonist nalmefene and full agonists such as U50,488. Furthermore, nalmefene also acts as an antagonist at the mu and delta opioid receptors (Bart et al. 2005). Thus far, there have been no studies showing mu or delta opioid receptors on dopamine terminals, making it unlikely that inhibition or activation of these receptors would result in any changes in DAT function (rate of uptake). Conversely, inhibition of KOR using the antagonist norbinaltorphimine (norBNI) augmented dopamine uptake rate measured by quantifying extraction fraction (Ed, an indirect measure of dopamine uptake) with no-net flux microdialysis (Chefer et al. 2006; Azocar et al. 2019). Overall, these studies suggest that KOR activation modulates DAT function, although this occurs in a manner that is sensitive to acute versus repeated KOR activation, or rather the homeostatic state of the animal.

2.3 KOR-Mediated Regulation of D2/D3 Receptors

Behavioral studies examining interactions between the KOR and membrane bound dopamine receptors have shown synergistic effects of coactivating these receptors. Specifically, acute coadministration of U69,593, a KOR agonist, and quinpirole, a D2R/D3R agonist, resulted in increased dynorphin mRNA expression in the dorsal striatum (Perreault et al. 2007), while no changes were seen with quinpirole alone (Jiang and Wang 1991; Engber et al. 1992; You et al. 1994; Perreault et al. 2007) or U69,593 alone (Tzaferis and McGinty 2001; Perreault et al. 2007). Two days after repeated U69,593 injections not only increased dynorphin mRNA levels (Perreault et al. 2007) but also reduced D2R density in the dorsal striatum. In contrast, 10 days after repeated U69,593 administration, D2R density in the NAc increased (Izenwasser et al. 1998). Repeated KOR activation has also attenuated D2R function

on presynaptic dopamine terminals (Acri et al. 2001; Fuentealba et al. 2006), likely due to a decrease in D2R density as observed by Izenwasser et al. (1998), ultimately resulting in elevated extracellular levels of dopamine (Fuentealba et al. 2006). However, when D2R and KOR are repeatedly activated concomitantly, there is an increase in D2R density (Perreault et al. 2007), which may subsequently reduce dopamine levels. Studies have also shown differential changes in locomotor behavior following combinatorial KOR and D2R/D3R activation and activation of each receptor alone. For example, administration of U69,593 or low dose of quinpirole (0.05 mg/kg, considered to target presynaptic D2/D3 autoreceptors) decreased locomotor activity, whereas high dose quinpirole (0.5 mg/kg, considered to target postsynaptic D2/D3 receptors) increased locomotor activity (Perreault et al. 2006). Coadministration of high dose quinpirole and U69,593 further enhanced locomotor activity (Perreault et al. 2006; Escobar et al. 2017) suggesting that postsynaptic D2R and D3R functionally interact with KOR. Together these studies suggest that KOR activation alone versus KOR and D2R/D3R combined activation results in distinct neurochemical and neurobiological changes ultimately affecting behavior. Overall, the interaction between KOR and D2Rs is complex, and more research is necessary to pinpoint the mechanism driving these interactions.

3 Behavioral Effects of KOR Activation in Humans and Animal Models

In humans, KOR activation leads to analgesia, psychotomimesis, sedation, and dysphoria (MacLean et al. 2013; Pande et al. 1996; Pfeiffer et al. 1986; for review see Wadenberg 2003), and similar effects are seen in animals (Shippenberg and Herz 1986; Katoh et al. 1990; Todtenkopf et al. 2004; McLaughlin et al. 2006a). Direct infusions of KOR agonists into the NAc (Bals-Kubik et al. 1989) and systemic KOR agonist administration (Land et al. 2008) have been shown to promote conditioned place aversion or block conditioned place preference (CPP). Furthermore, the aversive effects of exposure to forced swim or foot-shock stress, as reflected by the development of conditioned aversions to odors or places paired with stress, were blocked by KOR antagonist treatment and were absent in *Pdyn*−/− mice (Land et al. 2008), suggesting that dynorphins and KOR are crucial in forming context-stress associations. Interestingly, however, recent studies have shown that KOR activation results in opposing behavioral outcomes – both negative and positive affective responses. Particularly, activation of KOR in the rostral NAc shell stimulated orofacial hedonic responses to sucrose and CPP; in contrast, activation of KOR in the caudal NAc shell inhibited these orofacial hedonic responses and promoted conditioned place aversion (Castro and Berridge 2014). These differences are likely driven by differential effects of KOR activation on dopamine release as mentioned above (Pirino et al. 2020). Topographical differences were also reflected in operant optical self-stimulation of dynorphinergic neurons; mice exhibited enhanced self-stimulation of dynorphinergic neurons in the dorsal NAc shell but not the ventral shell. This effect in the dorsal NAc shell was reversed in the presence of norBNI

(Al-Hasani et al. 2015). Furthermore, selective photostimulation of dynorphinergic neurons in the dorsal NAc shell promoted place preference while stimulation of the neurons in the ventral shell promoted place avoidance (Al-Hasani et al. 2015). Such topographically dependent variations in behavioral outcomes are likely driven by differential and cell-specific KOR expression along the rostro-caudal or dorso-ventral axis.

In addition to topographical effects of KOR activation, direction of study outcomes is also dose- and time-dependent. For example, relatively low doses of U50,488 (0.3-3.0 mg/kg) administered daily within 10 min of conditioning blocked ethanol-induced CPP (Matsuzawa et al. 1999; Logrip et al. 2009); conversely, daily administration of U50,488 (10 mg/kg) 90 min before conditioning potentiated ethanol-induced CPP (Sperling et al. 2010). Similarly, when KOR agonists were acutely administered before intracranial self-stimulation (ICSS) testing, ICSS thresholds dose dependently increased, suggesting a KOR-induced aversive and anhedonic-like state (Todtenkopf et al. 2004; Carlezon et al. 2006; Ebner et al. 2010). This anhedonic/dysphoric state enhances reward sensitivity; when salvinorin A is administered 15 to 60 min before cocaine, ICSS thresholds decreased. In contrast, 24 h after salvinorin A administration ICSS thresholds increased in the presence of cocaine, suggesting decreased sensitivity to rewarding stimuli (Chartoff et al. 2016). The temporal effects of KOR activation may be a result of differential temporal dynamics of KOR interaction with various proteins that regulate dopamine transmission, potentially affecting perceived reward value.

KOR agonists are also known to produce pro-anxiety and pro-depressive effects while antagonists produce anxiolytic and antidepressive effects (for review, see Van't Veer and Carlezon 2013). For example, in the forced swim test, a classic test used to screen potential antidepressants (Porsolt et al. 1977; Detke et al. 1995), systemic administration of U69,593 in rats promotes immobility (a sign the animal is no longer trying to escape), whereas intracerebroventricular administration of norBNI diminishes immobility behavior. These results suggest that KOR agonists increase depressive-like behaviors while antagonists have antidepressant-like effects (Mague et al. 2003; Carr et al. 2010). Similarly, in the learned helplessness model of behavioral depression both intracerebroventricular and intra-NAc administration of norBNI decreased escape failures, suggesting that norBNI had an anti-depressant-like effect (Newton et al. 2002; Shirayama et al. 2004). Likewise, KOR antagonists produced anxiolytic-like effects in multiple assays, including the elevated plus maze (Knoll et al. 2007; Huang et al. 2016), fear-potentiated startle (Knoll et al. 2007), novelty induced hypophagia (Knoll et al. 2007; Carr and Lucki 2010), and defensive burying tests (Carr and Lucki 2010). For further review, see Hang et al. (2015).

Behavioral effects of KOR activation, including analgesia, anxiety, depression, and anhedonia are sex-dependent (for review see Chartoff and Mavrikaki 2015). While the KOR agonist U50,488 dose-dependently augmented ICSS responding in both male and female rats, female rats, independent of estrous cycle phase, were significantly less sensitive to its reward-decreasing effects (Russell et al. 2014). Another study showed that this effect in females was not due to circulating gonadal hormones (Conway et al. 2019). Although rodent studies have shown no differences

in gene expression of the KOR and *Pdyn* genes in the NAc or VTA (Conway et al. 2019), human PET imaging studies of the whole brain revealed less KOR availability in females compared to males (Vijay et al. 2016). It is possible that active KOR availability is in fact different even though gene expression is comparable between the two sexes in preclinical models; similar PET imaging studies in preclinical models have not been completed. Ultimately, further research is needed in order to understand the mechanisms that drive these sex differences and how they may affect clinical translation of KOR-targeted medications.

4 Involvement of KOR in Addiction Processing and Stress

The dynorphin/KOR system has been shown to interact bidirectionally with stress and drug exposure. Some studies even suggest that acute and chronic exposure to stress drive opposing changes in levels of dynorphins. Moreover, numerous studies have shown a direct link between chronic drug exposure and KOR function in the NAc. In general, repeated exposure to various drug classes such as alcohol, opioids, and stimulants produces a hyperfunctioning KOR system (Wee and Koob 2010). In this section, we will discuss the interaction between the dynorphin/KOR system and exposure to (1) alcohol; (2) other substances of misuse; and (3) stress. Lastly, we tie these three areas of research together and explain the role of the dynorphin/KOR system in stress-induced development of addiction.

4.1 KOR and Alcohol Use Disorder

Based on preclinical evidence, the United States Food and Drug administration approved naltrexone, a nonselective opioid receptor antagonist, as a treatment option for alcohol use disorder (Fairbanks et al. 2020). In humans with moderate to heavy drinking experience, PET imaging with the selective KOR ligand [^{11}C]-LY2795050 showed that occupancy of the KOR by naltrexone is associated with naltrexone-mediated reduction in alcohol drinking and craving, suggesting that alcohol use changes dynorphin/KOR system function, which potentially is involved in the clinical response of reduced consumption (de Laat et al. 2019, 2020). Particularly, augmented craving before naltrexone administration was correlated with higher KOR occupancy by naltrexone, which suggests greater KOR availability (de Laat et al. 2020). Given the interaction between the dynorphin/KOR and dopamine systems, it is possible that both are involved in the progression of alcohol use disorder. Indeed, preclinically, acute systemic administration of ethanol elevates extracellular levels of dynorphins (Marinelli et al. 2006) and dopamine (Di Chiara and Imperato 1988; Yim and Gonzales 2000). Interestingly, however, dopamine levels peak roughly 20 min post injection (Di Chiara and Imperato 1988; Yim and Gonzales 2000), while levels of dynorphins peak at roughly 30–40 min (Marinelli et al. 2006) when dopamine levels are descending. These studies indicate that ethanol may cause both acute and persistent changes in the dynorphin/KOR and

dopamine systems. Indeed, changes in KOR function have been observed using various ethanol exposure paradigms across species (Karkhanis et al. 2016a; Siciliano et al. 2016). Using a chronic intermittent ethanol (CIE) exposure model in adult male mice, KOR function was potentiated, which was demonstrated by greater inhibition of tonic and phasic dopamine release in CIE-exposed mice compared to air-exposed controls (Karkhanis et al. 2016a; Siciliano et al. 2016; Melchior and Jones 2017). Monkeys with a history of excessive ethanol consumption also exhibited augmented KOR-mediated inhibition of dopamine release (Siciliano et al. 2016). Interestingly, levels of dynorphins were observed to be diminished following CIE exposure in mice (Rose et al. 2016a). These studies collectively suggest that repeated ethanol exposure and withdrawal cycles may result in prolonged activation of the dynorphin/KOR system even though these cycles of exposure acutely increase levels of dynorphins. The repeated elevations in levels of dynorphins may eventually lead to overall reduction in availability of dynorphins, which may further enhance KOR function at the receptor. More research is necessary to understand this relationship between availability of dynorphins and KOR function.

As mentioned before, an acute ethanol challenge in naïve mice and rats elevates extracellular levels of dopamine (Yim and Gonzales 2000; Karkhanis et al. 2014, 2016a). In contrast, an acute ethanol challenge during acute withdrawal following CIE exposure decreases extracellular levels of dopamine, an effect reversed by pretreatment with a KOR antagonist (Karkhanis et al. 2016a). These data suggest that CIE-mediated increase in KOR function ultimately alters the reinforcing value of ethanol. Together, the augmented control of KOR over dopamine at baseline and the blunting effects of ethanol-induced elevation in dopamine suggest that the dynorphin/KOR system may also contribute to the CIE exposure-dependent enhancement in ethanol consumption and anxiety-like/compulsive behaviors (Anderson et al. 2016; Rose et al. 2016b). Indeed, the CIE-associated augmentation in ethanol consumption and marble burying (compulsive) behavior in mice was reversed with KOR blockade (Rose et al. 2016b).

Ethanol-induced changes in the dynorphin/KOR system extend to other mesolimbic regions such as the bed nucleus of the stria terminalis (BNST). For example, KOR blockade via direct infusion of the KOR antagonist norBNI into the BNST decreased both binge drinking and sucrose consumption in mice exposed to drinking-in-the-dark (DID), a model of binge-like ethanol consumption (Haun et al. 2020). Moreover, systemic administration of norBNI, as well as DREADD-based inhibition of dynorphinergic neurons into the central amygdala, also reduced binge drinking in the DID model (Anderson et al. 2019). Together, these studies show that heavy and moderate ethanol exposure enhances ethanol consumption due to, at least in part, augmented KOR activity in the mesolimbic pathway. Neurochemical analysis in the NAc suggests that these behavioral changes are likely driven by enhanced KOR-mediated inhibitory control over dopamine transmission. The switch from dopamine-exciting effects of ethanol after acute exposure to the dopamine-depleting effects of ethanol after chronic exposure is likely a result of this augmented KOR control over dopamine. Ultimately, these neuroadaptations, summarized in Fig. 1,

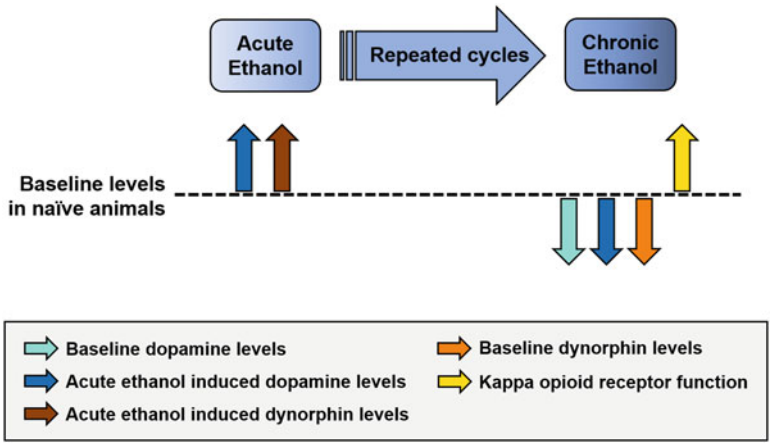

Fig. 1 Impact of acute and chronic ethanol exposure on dopamine and dynorphin/KOR systems. In naïve animals, acute ethanol results in elevations in extracellular levels of dopamine (blue arrow) and dynorphins (brown arrow). Repeated cycles of ethanol exposure and withdrawal result in attenuated baseline dopamine (aqua arrow) and dynorphins (orange arrow) levels and augmented KOR function (yellow arrow). Subsequent acute ethanol challenge following chronic ethanol exposure during acute withdrawal results in a reduction in extracellular levels of dopamine (dark blue arrow). The reduced baseline dopamine levels and reduction in dopamine in response to acute ethanol challenge suggest that repeated cycles of ethanol exposure and withdrawal produce a hypodopaminergic state and ethanol tolerance, respectively. These effects may be driven by augmented KOR function or diminished levels of dynorphins. Current literature does not identify the order of changes in dopamine, dynorphins, and KOR. However, it is important to recognize that both the dopamine and dynorphin/KOR systems are integrated, leading to neuroadaptations that may bidirectionally affect each system

not only result in temporary negative affect but also promote prolonged hyperkatifeia, maintaining the animal in a negative emotion state.

The observed ethanol-induced changes in dopamine and KOR function are both sex-specific and dependent on age of ethanol exposure; findings reported thus far have been in male adult rodents. In this subsection, we will discuss the impact of ethanol exposure during adolescence on the dynorphin/KOR system. Adolescent intermittent ethanol (AIE) exposure during early adolescence (PD 25–45) followed by prolonged forced abstinence potentiated KOR function as measured by its inhibitory control over dopamine transmission in adult female rats (Spodnick et al. 2020). Interestingly, this effect was not observed in male rats. On the contrary, KOR function was attenuated following prolonged forced abstinence in male rats exposed to AIE during late adolescence (PD 45–65), an effect not observed in female rats exposed during late adolescents (Spodnick et al. 2020). These sex differences suggest that KOR function in males and females is profoundly distinct and dependent on the age of ethanol exposure. While measurements of KOR mRNA levels in male animals across perinatal development indicate that KOR mRNA reaches adult levels by early adolescence (Georges et al. 1998), hardly any studies exist examining the ontogeny of the female KOR system. From a developmental perspective, it is

important to note that the inhibitory control of KOR over dopamine release increases between embryonic day 17 through PD 7 and then decreases between PD 7 and 21 in male rats to the adult levels, suggesting that the KOR system is matured at the beginning of adolescence (De Vries et al. 1990). However, the relationship between the dynorphin/KOR and dopamine systems is complex, especially during the developmental adolescence stage since both systems mature at distinct ages. While the dynorphin/KOR system appears to mature by PD 21, the dopamine system continues to mature into young adulthood, albeit in a sex-dependent manner. For instance, studies of dopaminergic neural markers suggest that the female dopaminergic system steadily progresses toward levels typical of adulthood throughout adolescence, whereas studies of dopaminergic firing in males follow an inverted-U-shaped trajectory of development in which firing peaks at PD 42-48 and then declines toward adult levels (Prakash and Wurst 2006; Alavian et al. 2008; Katunar et al. 2009; McCutcheon and Marinelli 2009; Marinelli and McCutcheon 2014). Thus, it is possible that the impact of ethanol on the dopamine and KOR systems is highly dependent on age. KOR regulation of dopamine transmission may also differ between sexes since maturation of the dopamine system is delayed in males. These differences likely drive the differential effects of adolescent intermittent ethanol exposure on KOR function following forced abstinence. More systematic research is necessary to pinpoint exact mechanisms that drive these age- and sex-specific neuroadaptations.

Based on the preclinical literature, at least in adulthood, KOR has emerged as a promising therapeutic target to treat alcohol use disorder. Naltrexone, an antagonist of the opioid receptors with higher affinity for the mu opioid receptor over delta and kappa opioid receptors, is currently approved by the Food and Drug Administration for treating alcohol use disorder. It functions by blunting the positive reinforcing effects of alcohol; however, its effectiveness is moderate in the clinic (Ashenhurst et al. 2012). Most preclinical studies used specific KOR antagonists that were long-acting; the complex mechanisms of action and interactions of these drugs deem them problematic in humans. In fact, phase I clinical trial of JDTic failed due to cardiotoxic effects (Buda et al. 2015). JDTic activates the cJun N-terminal kinase (JNK), similar to norBNI, ultimately inactivating the KOR (Melief et al. 2010, 2011; Buda et al. 2015; Schattauer et al. 2017). Though norBNI can no longer be detected in plasma levels after 1 day, the behavioral effects of norBNI are persistent for at least 21 days (Horan et al. 1992; Bruchas et al. 2007b; Munro et al. 2012), with one study showing effects for at least 85 days (Potter et al. 2011). KOR antagonists that are not long-lasting and do not target the JNK pathway may be safer therapeutic alternatives. Thus, recent clinical studies have begun examining the effects of short-acting KOR antagonists such as aticaprant (developmental codes JNJ-67953964, CERC-501, and LY-2456302) (Page et al. 2019). CERC-501 (now aticaprant) has been shown to reverse anxiety-like behaviors resulting from chronic ethanol exposure as well as block stress-induced ethanol reinstatement (Domi et al. 2018). LY2444296, an analog of aticaprant, has also been effective in reducing ethanol consumption in rodent studies (Anderson et al. 2019). Positive results from preclinical studies using

short-acting KOR antagonists have prompted human studies and clinical trials of aticaprant (NCT02800928, NCT02218736, NCT01913535, NCT02641028).

4.2 KOR and Substance Use Disorder

Similar to alcohol, exposure to other drugs such as stimulants alters the dynorphin/KOR system, making the KOR a prime cellular target for therapeutics to treat substance use disorder. For instance, cocaine exposure elevates levels of dynorphins (Sivam 1989; Smiley et al. 1990), with repeated injections of cocaine (5 injections, 30 mg/kg, i.p., 6 h apart) increasing immunoreactivity to content of dynorphins to approximately 270% of the control in the NAc 8 h after the last cocaine injection (Smiley et al. 1990). In a similar study, repeated injections of cocaine (20 mg/kg, i.p.) once per day for 4 days resulted in augmented levels of dynorphins in the striatum, which were persistent 4 days post-cocaine administration (Sivam 1989). Likewise, *Pdyn* mRNA and dynorphin expression levels in the striatum were elevated in rodents after non-contingent cocaine exposure (Daunais et al. 1993; Spangler et al. 1993, 1996; Schlussman et al. 2003, 2005; Sun et al. 2020). Repeated cocaine administration reduced synthesis of dynorphins due, at least in part, to augmented ΔFosB expression and attenuated CREBS (Nestler et al. 2001). With respect to KOR, in situ U69,593-induced [^{35}S]GTPγS binding in VTA of rats increased 24 h into withdrawal after chronic cocaine exposure, suggesting greater KOR function in an acute withdrawal state (Piras et al. 2010). It is possible that the augmented KOR function is a resulting neuroadaptation due to attenuated availability of dynorphins observed during drug withdrawal. In humans with a recent history of cocaine use, as well as cocaine-overdose victims, dynorphin mRNA and KOR receptor binding was increased in postmortem striatal tissue, also suggesting an upregulation of the KOR system (Hurd and Herkenham 1993; Staley et al. 1997; Mash and Staley 1999). After a 3-day cocaine binge (average 1,500 mg of smoked cocaine), PET imaging revealed that cocaine-dependent individuals had reduced KOR agonist binding by approximately 20%, suggesting an elevation in levels (Martinez et al. 2019). Together, these studies suggest that cocaine exposure results in an upregulation of the dynorphin/KOR system. The contingency factor of cocaine self-administration, timing of the last cocaine exposure, and withdrawal state seem to be critical in driving changes in the dynorphin/KOR system, particularly modulating levels of dynorphins. Alterations in levels of dynorphins likely promote upregulation in KOR function.

Interestingly, both KOR agonists and antagonists are efficacious at decreasing drug-taking behaviors in preclinical models of substance use (for review see Karkhanis et al. 2017), with both agonists and antagonists showing similar effects. It is very important to understand the link between KOR activation and drug-taking/seeking behaviors and how the behavioral output of KOR activation is time-dependent. KOR agonists may modulate drug-seeking behaviors through diminishing the rewarding effects of cocaine by KOR activation (Shippenberg et al. 2007). This would make the effects of KOR agonists most beneficial early in

cocaine use since KOR activation averts the dopamine-elevating properties of drugs. In several preclinical studies, the beneficial effects of KOR activation are time-dependent. For example, administering cocaine 15 or 60 min after systemic KOR agonist administration led to profound or slight decreases, respectively, in ICSS threshold, suggesting an increased reward valuation of cocaine; however, when cocaine was administered 24 h after KOR activation, ICSS threshold increased, suggesting reduced reward valuation (Chartoff et al. 2016). Interestingly, McLaughlin et al. (2006a) observed that activating KOR 15 min before conditioning blocked cocaine CPP in mice, whereas activation 60 min prior to conditioning potentiated cocaine CPP, confirmed by Ehrich et al. (2014). Although the ICSS and CPP data suggest contrasting findings at the 15-min timepoint, but similar results at 60-min timepoint, these differences could be due to the differential aspects of behavioral responses of the two paradigms. It is possible that this contrasting result is due to KOR activation and cocaine administration occurring in close temporal proximity (15 min), thus the cocaine injection would have a negative association, further leading to blockade of CPP. Ultimately, however, given these time-dependent differences, timing of KOR activation with respect to cocaine administration likely have differential effects on the perceived reward value of cocaine.

On the other hand, preclinical behavioral studies have consistently shown that KOR antagonists do not block the rewarding effects of drugs as KOR agonists do; instead, KOR antagonists block the stress-induced potentiation of drug reward, stress-induced reinstatement, and escalation of drug consumption (for review see Bruchas et al. 2010). After animals self-administered cocaine on a long access paradigm (6 h/day with unlimited infusions, 0.5 mg/kg/inf), administration of norBNI does not alter the amount of cocaine consumed on a fixed ratio one schedule of reinforcement. However, when conditions were switched to a progressive ratio schedule, norBNI treated animals exhibited lower breakpoints, suggesting reduced motivation to self-administer cocaine (Wee et al. 2009). These data suggest that KOR blockade selectively reduces cocaine seeking (appetitive behavior) but not cocaine consumption. Interestingly, only animals with a history of extended cocaine exposure were sensitive to norBNI effects, suggesting an importance of drug history; animals that self-administered on a short access procedure (1 h/day with unlimited infusions, 0.5 mg/kg/inf) were unaffected by norBNI (Wee et al. 2009). These data indicate that longer sessions with cocaine promote KOR function, while shorter access sessions either have no effect or the opposite effect on KOR function. Indeed, long access (6 h/day) and short access (1–2 h/day) to cocaine have differing effects of cocaine potency at the dopamine transporter (DAT). Long access leads to reduced inhibition of dopamine uptake at the DAT by cocaine, while short access increases the effect of cocaine on the DAT (Calipari et al. 2013). As discussed earlier, KOR and DAT interact with each other (Kivell et al. 2014); therefore, cocaine may result in complex changes in the two proteins and how these proteins interact, which could be dependent on the amount of cocaine exposure. Interestingly, cocaine potency at the DAT tracks with motivational state of the animal measured by cocaine seeking/motivation to perform even at greater response requirements to maintain blood

cocaine levels, but it does not track or predict consumption (Siciliano and Jones 2017). Given the interaction between KOR and DAT, KOR potentially contributes to this effect on motivated behavior. This motivational state of the animal is likely crucial for the antagonist to be effective in reducing drug-seeking behaviors.

Research thus far has shown that KOR antagonists reduce drug-seeking behaviors (for review see Karkhanis et al. 2017). However, as mentioned earlier, most rodent studies to date have used long-acting antagonists that have complex actions, which have increased interest in developing short-acting KOR antagonists to study more discrete changes with KOR blockade. The short-acting KOR antagonist LY244296 was recently shown to reduce escalation of cocaine intake on an extended access paradigm (fixed ratio one, 18 h/day) in rats (Valenza et al. 2020). Additionally, responding decreased when re-exposed to cocaine after 2 days of abstinence in animals treated with LY2444296 compared to vehicle-treated animals (Valenza et al. 2020). Animals in these studies may be sensitive to the behavioral effects of KOR antagonists due to an upregulation of KOR after repeated cocaine exposure (Spangler et al. 1993; Unterwald et al. 1994). Clinical studies have shown that the KOR antagonist LY2456302 is safe to administer in early-abstinent cocaine-dependent individuals (Reed et al. 2018). This study examined the effects of only one dose of LY2456302 (10 mg) for 4 consecutive days, which did not alter subjective cocaine craving (Reed et al. 2018). Overall, illicit substances affect multiple brain mechanisms leading to various neuroadaptations; the ones related to the dynorphin/KOR and dopamine systems are summarized in Fig. 2. For example, as discussed in this section, although cocaine primarily targets the DAT, repeated exposure to cocaine affects both dynorphin/KOR and dopamine systems, perhaps due to repeated cycles of withdrawal, which likely promotes stress. Preclinical studies have shown promising results for the use of KOR antagonists for substance use disorder, with clinical studies showing that short-acting KOR antagonists are safe in cocaine-dependent individuals. However, further clinical research is necessary to fully understand the effects of KOR antagonism on cocaine craving and taking throughout various phases of the addiction cycle.

4.3 Acute and Chronic Stress Impact on Dynorphin/KOR System

Both acute and chronic stress exposure affect dopamine transmission; acute stressors such as foot shock, tail pinch, and single social defeat stress exposure elevate dopamine levels in the NAc (Rougé-Pont et al. 1993; Kalivas and Duffy 1995; Lapiz et al. 2003; Holly et al. 2015), while chronic or repeated stress exposure attenuates tonic dopamine levels in the NAc (Scheggi et al. 2002; Karkhanis et al. 2016b). The initial dopamine response to acute stress is likely driven by arousal, and the later subsequent decrease in dopamine may be related to neural adaptations or coping failure at a behavioral level (Cabib and Puglisi-Allegra 1991; Pani et al. 2000). Given the interaction between dopamine and dynorphin/KOR systems, the neuroadaptive changes may be associated with changes in the dynorphin/KOR system (see Margolis and Karkhanis 2019 for review). In this section, we discuss

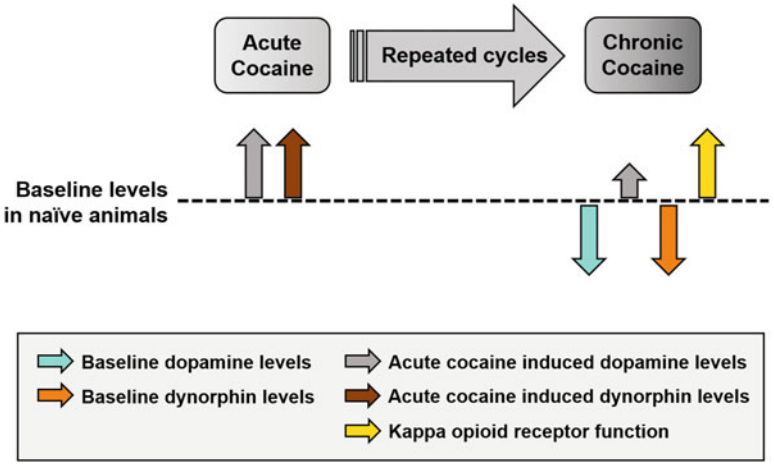

Fig. 2 Impact of acute and chronic cocaine exposure on dopamine and dynorphin/KOR systems. In naïve animals, acute cocaine results in elevations in extracellular levels of dopamine (gray arrow) and dynorphins (brown arrow). Repeated cycles of contingent cocaine exposure and withdrawal result in attenuated baseline dopamine (aqua arrow) and dynorphins (orange arrow) levels and augmented KOR function (yellow arrow). Subsequent acute cocaine challenge following chronic cocaine exposure results in an increase in dopamine levels (gray arrow), albeit the difference from baseline is smaller than in cocaine naïve animals. The reduced baseline dopamine levels and a reduction in dopamine in response to acute cocaine challenge suggest that repeated cycles of cocaine exposure and withdrawal produce a hypodopaminergic state and cocaine tolerance, respectively. It is important to note that the chronic cocaine effects summarized here are based on long daily sessions of operant self-administration. It is possible that cocaine-induced changes in the dopamine system produce profound neuroadaptive alterations in the dynorphin/KOR system as a result of the interactions between the two neuromodulatory systems

the interaction between stress and KOR, particularly examining the impact of stress on the dynorphin/KOR system and ultimately exploring the impact that KOR may have on stress-related drug-seeking behaviors. Both acute and chronic/repeated stress exposure induce changes in the dynorphin/KOR system, affecting ligand levels as well as receptor function. Acute stress, such as acute psychological stress (Takahashi et al. 1990), electric tail or foot shock (Watkins et al. 1992; Menendez et al. 1993; Land et al. 2008), forced swim stress (McLaughlin et al. 2003; Shirayama et al. 2004), immobilization (Shirayama et al. 2004), and social defeat stress (McLaughlin et al. 2006a; Donahue et al. 2015) elevate levels of dynorphins. On the other hand, the impact of prolonged or repeated stress on levels of dynorphins is inconsistent; for example, we and others have shown that prolonged adolescent social isolation and repeated exposure to social defeat stress attenuated tissue levels of dynorphins in the NAc (Donahue et al. 2015; Karkhanis et al. 2016b). In contrast, Bérubé et al. (2013) show augmented accumbal levels of dynorphins in a subgroup of animals found to be vulnerable to social defeat (animals quick to assume a subordinate position) with no change in resilient, or less subordinate, rats. Despite discrepancies in the literature regarding ligand levels, KOR expression has been

shown to increase after chronic stress exposure. For example, KOR immunoreactivity was augmented after repeated swim stress (Bruchas et al. 2007a), and KOR activity in the NAc was enhanced following prolonged adolescent social isolation (Karkhanis et al. 2016b). In the subsequent text, we will discuss the impact of various types of chronic stress exposure on KOR-mediated regulation of behavioral outcomes and dopamine transmission.

Repeated social defeat stress episodes increase ICSS thresholds, indicating anhedonic-like behavior. Interestingly, this effect is dampened in mice lacking KOR specifically on dopamine neuron terminals (Donahue et al. 2015), suggesting that KOR-mediated changes in dopamine transmission are essential for stress-induced anhedonia. Furthermore, blockade of the KOR on second and third exposure to social defeat stress reduced stress-induced immobility and defeat posture (McLaughlin et al. 2006b). Similarly, KOR blockade in mice before social defeat stress exposure prevented the development of anhedonia and social avoidance behaviors (Williams et al. 2018). In rats, repeated restraint stress attenuated social investigation and preference behaviors in a KOR-dependent manner (Varlinskaya et al. 2018). Particularly, this stress paradigm also decreased palatable tastant intake in both adult and adolescent rats (Anderson et al. 2013). Interestingly, however, while acute pharmacological KOR activation promoted social avoidance and taste aversion to a palatable substance in stress-naïve adult rats, stress-exposed rats showed a dampened taste aversion and no change in social avoidance compared to controls (Anderson et al. 2013; Varlinskaya et al. 2018). Moreover, KOR antagonists blocked ethanol-induced conditioned taste aversion in stress-exposed but not stress-naïve adult rats (Anderson et al. 2013). Interestingly, conditioned taste aversion is not blocked by KOR inhibition in restraint stress-exposed adolescents as it is in restraint stress-exposed adults (Anderson et al. 2013), suggesting that stress-associated conditioned taste aversion is mediated via a mechanism other than KOR in adolescents. Together, these studies show that repeated KOR activation due to stress is imperative to induce anhedonic-like behaviors and stress responsivity. However, it is important to note that the effects of KOR modulation are largely dependent on age and homeostatic state of the animal.

Overall, these studies show that while acute activation of KOR induces stress, acute and chronic stress differentially affect the dynorphin/KOR system, with acute stress exposure elevating levels of dynorphins and chronic/repeated stress attenuating levels of dynorphins but augmenting functional responsivity of the dynorphin/KOR system (Fig. 3). This biphasic response of KOR may be driven by presynaptic and postsynaptic neuroadaptations occurring from repeated stress exposure. Effects of stress on the dynorphin/KOR system are also dependent on age of exposure to stress.

4.4 Stress-Induced Development of Drug Addiction

The dynorphin/KOR system plays an integral role in stress responsivity and resultant drug-related behaviors. Phenotypes of depression and anxiety can be induced by

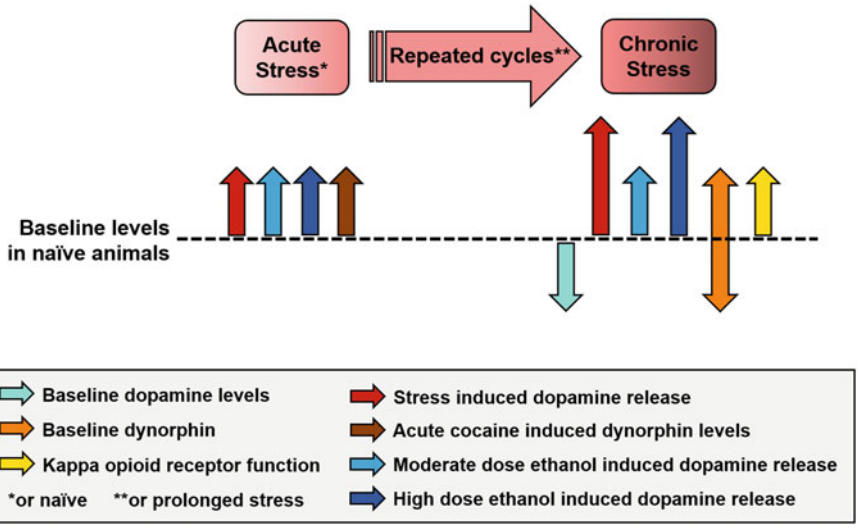

Fig. 3 Impact of acute and chronic stress exposure on dopamine and dynorphin/KOR systems. In naïve animals, acute stress results in elevations in extracellular levels of dopamine (red arrow) and dynorphins (brown arrow). Acute systemic ethanol administration at moderate (1 g/kg; light blue arrow) and high doses (2 g/kg; dark blue arrow) result in comparable elevations in extracellular levels of dopamine. Repeated cycles of stress exposure or prolonged stress exposure attenuates baseline dopamine levels (aqua arrow); however, reports on levels of dynorphins (orange arrow) are mixed with some studies showing an elevation and others showing a reduction. Interestingly, all studies report an elevation in KOR function (yellow arrow). Dopamine response to subsequent stress (red arrow) and high dose of ethanol (2 g/kg; dark blue arrow) exposure is exacerbated in chronic stress-exposed animals. A subsequent moderate ethanol dose (1 g/kg; light blue) elicits a dopamine response comparable to stress naïve animals, but this response is augmented with KOR blockade (see text). Because stress affects both the dopamine and dynorphin/KOR systems, the resulting interactions are complex. Ultimately, however, these neuroadaptations promote pro-addictive behaviors

stressors in humans (Kessler 1997), with stress exposure in animal models resulting in similar depression- and anxiety-like behaviors (for review see Chavkin and Koob 2016). These effects can be attributed, at least in part, to KOR decreasing dopamine system function in mesocorticolimbic areas involved in reinforcement circuitry (Margolis and Karkhanis 2019 for review). Substance use after stress exposure, as well as stress related to drug withdrawal, plays a role in drug craving and relapse (Sinha 2007). One theoretical framework proposed that the effects of KOR activation on drug self-administration is dependent on the hedonic state of the animal, which changes based on stress history (see Bruchas et al. 2010 for review). A reward such as cocaine would increase the hedonic state to a smaller degree in non-stressed animals compared to stress-exposed animals since a stressed animal is likely in a dysphoric-like state resulting from KOR hyperfunction. This would contribute to a drug reward having a larger positive valence in stress-exposed animals, increasing drug reinforcement and subsequent taking.

One connection between stress and addiction identified in humans thus far includes a human variant on the KOR gene (*OPRK1*, rs6989250 C>G), which is associated with increased stress-induced cocaine craving and risk of relapse (Xu et al. 2013). Functional MRI results also showed that individuals with this *OPRK1* variant had increased activity in the limbic and midbrain regions during stress and drug-related cues (Xu et al. 2013). *OPRK1* variants have also been associated with increased risk of opioid addiction (SNP G36 T, Gerra et al. 2007) and alcoholism (multiple SNPs, Xuei et al. 2006). Findings from Martinez et al. (2019) support this relationship between stress and increased cocaine-taking behaviors in humans. Subjects with cocaine use disorder were subject to a cold pressor task, which induces a stress response, prior to self-administering cocaine in a choice procedure. Results showed that subjects who made more stress-induced cocaine choices had greater KOR availability in the striatum as determined by PET scanning (Martinez et al. 2019).

Several preclinical stress models exhibit pro-addictive behaviors. In the following section it will be important to consider (1) the type of stressor, (2) the stage of addiction (acquisition, maintenance, relapse), as well as (3) the outcome measure when trying to understand the association between stress, KOR system activation, and development or exacerbation of an addiction-like phenotype.

Repeated forced swim stress and repeated social defeat stress potentiate cocaine CPP and nicotine CPP, both blocked by pretreating animals with norBNI (Jackson et al. 2013; McLaughlin et al. 2003; Nygard et al. 2016). Repeated forced swim stress has also been shown to elevate motivation to self-administer cocaine on a threshold procedure, which was attenuated after KOR inhibition via norBNI (Groblewski et al. 2015). Genetic disruption of KOR or dynorphin (KOR −/− or *Pdyn* −/−) has been shown to block cocaine and nicotine CPP (McLaughlin et al. 2003; Nygard et al. 2016). Stress-induced reinstatement, a self-administration model of relapse-like behavior, is another way the role of the KOR system in modulating stress-induced drug and alcohol seeking can be examined. For example, norBNI prevented stress-induced cocaine reinstatement in rats exposed to cold-water forced swim stressor (Polter et al. 2014, 2017). Another KOR antagonist JDTic reduced foot shock-induced reinstatement (Beardsley et al. 2005). These studies indicate that KOR antagonists are capable of reducing drug-seeking behavior after stress exposure. KOR antagonists have been shown to prevent not only stress-induced reinstatement of cocaine (Beardsley et al. 2005; Carey et al. 2007; Redila and Chavkin 2008) but also stress-induced reinstatement of heroin (Sedki et al. 2015) and ethanol (Sperling et al. 2010) without any effects on drug-primed reinstatement. These results suggest a conserved role of the KOR in inducing stress-dependent reinstatement of various drugs of abuse.

Prolonged stress exposure during adolescence, such as the adolescent social isolation model discussed earlier, promotes anxiety-like behavior, ethanol drinking, cocaine consumption, cocaine seeking, and a hypodopaminergic state (Yajie Ding et al. 2005; Karkhanis et al. 2014; Skelly et al. 2015; Rose et al. 2016a; Fosnocht et al. 2019). These studies have linked augmented ethanol consumption and changes in the dopamine system to an increase in KOR system function (Karkhanis et al.

2016b). For example, adolescent socially isolated (aSI) rats exhibited greater ethanol intake and ethanol preference compared to adolescent group housed (aGH) rats. This augmented ethanol intake and preference was reversed by norBNI administration selectively in aSI animals, suggesting a greater inhibitory control of KOR over dopamine (Karkhanis et al. 2016b). Indeed, direct examination of KOR function using ex vivo fast scan cyclic voltammetry showed hyper-responsivity of KOR to the agonist U50,488, as the inhibitory effects of KOR activation on dopamine release were observed to be significantly greater in aSI compared to aGH rats (Karkhanis et al. 2016b). Rats exposed to aSI also showed lower baseline extracellular dopamine levels in the NAc compared to aGH rats; KOR blockade with norBNI resulted in an increase in the extracellular dopamine levels, matching those in aGH rats, suggesting that augmented KOR function contributes to the low-dopamine state (Karkhanis et al. 2016b). Furthermore, dopamine response to ethanol (1 g/kg, i.p.) was greater in the presence of KOR blockade selectively in aSI rats, indicating that the KOR function was increased following aSI exposure and dampened the dopamine-elevating effects of ethanol at moderate doses (Karkhanis et al. 2016b). In summary, stress-mediated changes in KOR function not only promote anxiety-like and depressive-like behaviors but also alter reward value and motivation, thus increasing vulnerability to develop addictive behaviors, particularly drug consumption.

5 Conclusion

The literature reviewed here support that the dynorphin/KOR system plays a primary role in stress responsivity and shapes drug-seeking behaviors at various stages of the drug addiction cycle, partially through modulation of the mesolimbic dopamine system. Exposure to stress is a well-recognized risk factor for exacerbation of drug use and relapse during abstinence. Repeated cycles of drug exposure and withdrawal also induce stress, further affecting KOR signaling and dopamine transmission. The interaction between the two systems shapes the overall behavioral outcome and reward perception. For example, prior stress exposure promotes a hypodopaminergic state, characterized by low tonic levels of dopamine; KOR hyperfunctioning influences this state, as KOR activation leads to inhibition of dopamine release and KOR inhibition mediated reversal of low extracellular levels of dopamine. The augmented KOR function also affects perception of reward value by its influence on the mesolimbic dopamine signaling. For example, chronic stress and drug exposure produce an anhedonic state, driven by high KOR function in combination with low synaptic levels of dopamine; subsequent exposure to drugs during this state exacerbates the reinforcing value of the drug mostly due to larger changes in dopamine concentrations. Blockade of KOR signaling reverses anhedonia by elevating synaptic levels of dopamine and thus changing perceived reinforcing value of drugs. ln summary, the development of addictive behaviors and negative affect occur as a result of an orchestrated interaction between the dynorphin/KOR and dopamine systems.

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The Kappa Opioid Receptor System in Temporal Lobe Epilepsy

Luca Zangrandi and Christoph Schwarzer

Contents

Content: This chapter deals with the role of dynorphins (Dyns) and kappa opioid receptors (KOR) in temporal lobe epilepsy (TLE), seizure control and potential therapeutic prospects. A short introduction to epilepsy and the medical needs related to TLE is followed by a description of the hippocampal formation, the key area for seizure generation in TLE, or more specific mesial temporal lobe epilepsy (mTLE). Subsequently, we discuss the Dyns/KOR system in the hippocampal formation and how it regulates neuronal excitability. Moreover, we discuss seizure-induced alterations of the Dyns/KOR system in animal models of TLE and human patients. The chapter concludes with a paragraph on therapeutic potentials of the Dyns/KOR system for the treatment of TLE.

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Abstract

Temporal lobe epilepsy is considered to be one of the most common and severe forms of focal epilepsies. Patients frequently develop cognitive deficits and emotional blunting along progression of the disease. The high incidence of refractoriness to antiepileptic drugs and a frequent lack of admissibility to surgery pose an unmet medical challenge. In the urgent quest for novel treatment strategies, neuropeptides and their receptors are interesting candidates. However, their therapeutic potential has not yet been fully exploited. This chapter focuses on the functional role of the dynorphins (Dyns) and the kappa opioid receptor (KOR) system in temporal lobe epilepsy and the hippocampus.

Genetic polymorphisms in the prepro-dynorphin (pDyn) gene causing lower levels of Dyns in humans and pDyn gene knockout in mice increase the risk to develop epilepsy. This suggests a role of Dyns and KOR as modulators of neuronal excitability. Indeed, KOR agonists induce inhibition of presynaptic neurotransmitter release, as well as postsynaptic hyperpolarization in glutamatergic neurons, both producing anticonvulsant effects.

The development of new approaches to modulate the complex KOR signalling cascade (e.g. biased agonism and gene therapy) opens up new exciting therapeutic opportunities with regard to seizure control and epilepsy. Potential adverse side effects of KOR agonists may be minimized through functional selectivity or locally restricted treatment. Preclinical data suggest a high potential of such approaches to control seizures.

Keywords

Excitability · Hippocampus · Seizures · Temporal lobe epilepsy

Abbreviations

AAV	Adeno-associated virus
AEDs	Antiepileptic drugs
CA1	Cornu ammonis regio superior
CA3	Cornu ammonis regio inferior
CRE	cAMP response element
CREB	cAMP response element-binding protein
DG	Dentate gyrus
DRE	Downstream regulatory element
DREAM	Downstream regulatory element antagonizing modulator
Dyn A	Dynorphin A
Dyn B	Dynorphin B
Dyns	Dynorphins

EC	Entorhinal cortex
ILAE	International league against epilepsy
KOR	Kappa opioid receptor
LTD	Long-term depression
LTP	Long-term potentiation
mTLE	Mesial temporal lobe epilepsy
mTOR	Mechanistic target of rapamycin
pDyn	Prepro-dynorphin
TLE	Temporal lobe epilepsy

1 Introduction

With a prevalence of 0.5–1% worldwide, epilepsy is one of the most frequent neurological disorders affecting people of all ages (Fiest et al. 2017). In the United States about three million adults and 500,000 children suffer from epilepsy, while the European area counts over five million patients. Epilepsy has a major impact on both social and psychological well-being, including social isolation, stigmatization, or disability, thus resulting in lower educational achievement and worse employment outcomes (WHO 2019). In line with this, the International League Against Epilepsy (ILAE) defined epilepsy as "a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures and by the neurobiological, cognitive, psychological and social consequences of this condition" (Fisher et al. 2005, 2014).

The term epilepsy (or epilepsy disorder) is not referring to a unique defined condition, but rather to a group of chronic neurological diseases characterized by epileptic seizures, as a result of abnormal excessive or synchronous neuronal activity in the brain (Fisher et al. 2005, 2014). Epileptic seizures are episodes that vary from brief and nearly undetectable to prolonged events, and may involve a part of the brain (partial seizure), multiple brain centres or the entire brain (generalized seizure), and are sometimes accompanied with a loss of consciousness and control of bowel or bladder functions.

The aetiology of epilepsy is unknown in about 50% of patients, although a history of brain injury, stroke, brain tumour and substance use disorders have been associated with increased probability of developing epilepsy. Genetic anomalies like congenital gene mutations (single or multiple), as well as developmental defects, are responsible for several forms of epilepsy, especially among younger people (Berkovic et al. 2006; Thurman et al. 2011).

In about 70% of all epileptic patients, the temporal lobe is the brain area where seizures originate (i.e. the epileptogenic focus). This type of epilepsy is called temporal lobe epilepsy (mTLE) and involves all the limbic system structures with the hippocampus as one of the main epileptogenic foci (Blümcke et al. 2012; Goldberg and Coulter 2013). During seizures, the limbic network of mTLE patients is exposed to high concentrations of glutamate creating an excitotoxic environment

that leads to loss of interneurons as well as principal neurons (Meldrum 2002). The disruption of the physiological network generates an imbalance between inhibition and excitation that may favour a hyperexcitable state and exacerbate the propensity for seizure generation and epileptogenesis. In addition, mTLE is often associated with hippocampal sclerosis and granular cell dispersion, resulting in one of the most refractory forms of human epilepsy (Asadi-Pooya et al. 2017).

The mainstay treatment of epilepsy relies on antiepileptic drugs (AEDs), mostly for the person's entire life. On the average about 30% of patients do not become seizure-free with the currently available pharmacotherapies (Pohlen et al. 2017), however in case of mTLE with hippocampal sclerosis, this number raises to 80% (Asadi-Pooya et al. 2017). Moreover, the current pharmacotherapies cause a number of side effects (e.g., sedation, nausea, depression, headache, ataxia) in 10–90% of patients (Eadie 2012; Perucca and Gilliam 2012). In 2008, the FDA issued a black-box warning for several AEDs due to increased risk of suicidal thoughts and behaviours among users (Mula and Sander 2013). Furthermore, the contribution of AEDs to the neuronal loss observed in epilepsy patients is a matter of discussion. Enhanced neuronal cell death and loss of white matter have been observed in the rodent developing brain after a single acute treatment with several AEDs (Forcelli et al. 2011; French and Staley 2012; Kaushal et al. 2016; Kim et al. 2007). For some patients whose seizures cannot be efficiently controlled by AEDs or neuro-stimulation, surgical resection of the epileptogenic focus remains as an ultimate solution (Bergey 2013; Duncan 2007). Even with surgery, only about 50–80% become seizure-free for at least 1 year (Spencer and Huh 2008).

In the quest for alternative treatment options, neuropeptides and their receptors have received increasing attention. Neuropeptide systems have been demonstrated to play crucial roles in the modulation of neuronal excitability. Several neuropeptides, such as neuropeptide Y, galanin, somatostatin, ghrelin and Dyns, have been reported to elicit direct antiepileptic and antiepileptogenic effects, and their receptors represent promising potential drug targets (Kovac and Walker 2013). The aim of this chapter is to summarize the role of the Dyns/KOR system in epileptogenesis and mTLE, as well as reflecting upon their therapeutic potentials.

2 The Hippocampal Formation and Temporal Lobe Epilepsy

The hippocampal formation is a subcortical area embedded in a larger network of structures called the limbic system, which is involved in motivation, emotion, learning and memory. Beside the hippocampal formation, the limbic system includes the entorhinal, perirhinal and piriform cortices and the amygdala. The neurons within this circuitry have both intrinsic properties and local connections which, when sufficiently provoked, can induce strong recurrent excitation leading to seizure activity. Furthermore, the strong communication among these areas could amplify the seizures and recruit neurons with efferent connections that distribute widely throughout the brain, subsequently provoking a generalized seizure.

2.1 The Hippocampal Formation

In temporal lobe epilepsy (TLE), the hippocampus is, without doubt, the most affected limbic system region and the one most extensively investigated. The hippocampal formation is usually divided into four main subregions: the dentate gyrus (DG), the cornu ammonis regio superior (CA1), the cornu ammonis regio inferior (CA3) and the subiculum. Each of these regions is characterized by its unique pattern of afferents and efferents as well as a distinct neuronal population.

These regions are interconnected through a robust glutamatergic circuitry, which plays a crucial role in spatial orientation and learning. This excitatory pathway involves the granule cells of the DG, which receive their inputs from entorhinal cortex (EC) through the perforant pathway and send projections ("mossy fibres") to CA3 pyramidal cell dendrites in the stratum lucidum. The CA3 pyramidal cells extend their axons (termed Schaffer collaterals) to CA1. CA1 cells project to the subiculum (the main output station of the hippocampus), which closes the circuitry by projecting back to EC (Fig. 1). However, this view of the hippocampal circuitry is

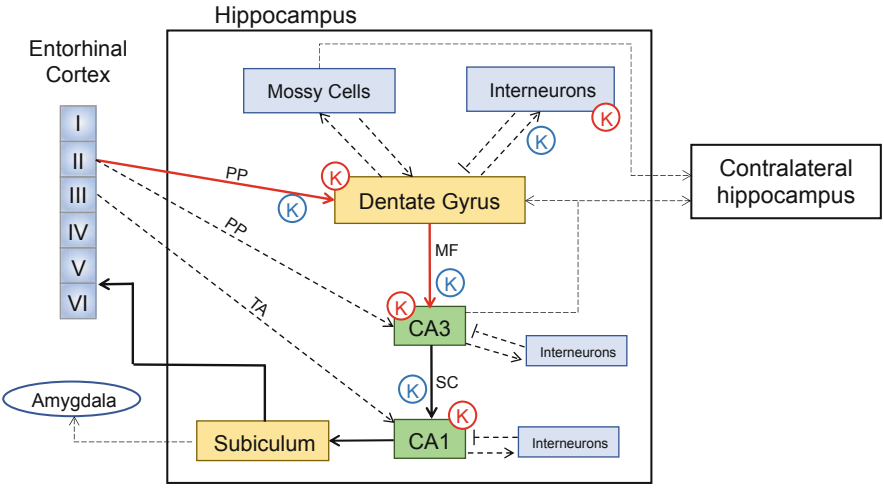

Fig. 1 Diagram of the hippocampal neural network and KOR expression. Layer II neurons in the entorhinal cortex (EC) project to the dentate gyrus through the perforant pathway (PP). The dentate gyrus sends projections to the pyramidal cells in CA3 through mossy fibres (MF). CA3 pyramidal neurons relay the information to CA1 pyramidal neurons through Schaffer collaterals (SC). CA1 pyramidal neurons send projections to the main hippocampal output structure, the subiculum, which targets deep-layer neurons of the EC. CA3 also receives direct projections from EC layer II neurons through the PP. CA1 receives direct input from EC layer III neurons through the temporoammonic pathway (TA). The dentate granule cells also project to the mossy cells in the hilus and hilar interneurons, which send excitatory and inhibitory projections back to the granule cells. The classic excitatory pathway is indicated by solid arrows. The arrowhead lines indicate excitatory glutamatergic projections, while the blunt end lines indicate inhibitory GABAergic projections. The blue and red "K" indicate pre- and post-synaptic KOR expression, respectively. Red arrows indicate projections containing Dyn. However, PP axons express Dyn only in human tissue, but not in rodents

oversimplified. CA3 neurons project to the contralateral hippocampus, to other CA3 neurons ipsilaterally, as well as back to the ipsilateral DG, where they excite interneurons and mossy cells (Witter 2007). Furthermore, hilar neurons interconnect ipsilateral and contralateral dentate regions (Amaral et al. 2007; Witter and Amaral 2004). In the next sections, we will explore how the connectivity and the cellular heterogeneity of each of these regions may contribute to seizure generation and epilepsy development.

2.1.1 The Dentate Gyrus

The dentate gyrus consists of the granule cell region and the dentate hilus. The granule cells are densely packed in the stratum granulosum and their dendrites extend to the stratum moleculare. The hilus region is partially surrounded by the stratum granulosum and consists of a variety of polymorphic cells that include the excitatory mossy cells and inhibitory interneurons (Fig. 2; Amaral et al. 2007).

The DG is particularly relevant in mTLE because (a) granule cells are especially resistant to seizure-associated damage (Sloviter 1994), (b) their axons exhibit high degree of plasticity in epileptic brains (Cavazos et al. 1991; Danzer 2017) and (c) their maximal activation is associated with generalization of seizure activity through the limbic system and beyond (Lothman 1994).

The intrinsic and synaptic electrical properties of the granule cells have been extensively studied and they were found rather unexcitable because they maintain a very negative resting potential. Prolonged depolarization also failed to induce rapid, repetitive firing for long periods of time because these cells exhibit good spike firing

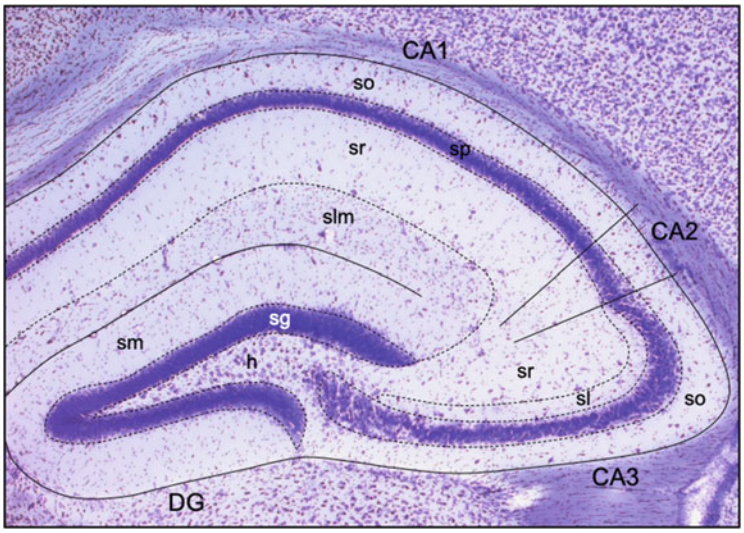

Fig. 2 Nissl staining of the hippocampus. Substructures and lamination are highlighted by solid and dotted lines, respectively. *CA* cornu ammonis, *DG* dentate gyrus, *h* hilus, *sg* stratum granulosum, *sm* stratum moleculare, *so* stratum oriens, *sp* stratum pyramidale, *sr* stratum radiatum, *slm* stratum lacunosum-moleculare, *sl* stratum lucidum

adaptation, and action potentials are followed by a large after-hyperpolarization (Mody et al. 1988). In addition, EC inputs activate local interneurons with a much lower threshold compared to granule cells (Buckmaster and Schwartzkroin 1995a, b). The hippocampus contains more than 20 different types of interneurons (Freund and Buzsáki 1998), and each subpopulation has a specific dendritic and axonal arborization pattern (Han et al. 1993; Somogyi and Klausberger 2005). For example, somatostatin-containing interneurons in the hilus send their axons to the outer molecular layer where they synapse onto distal granule cell dendrites and with the incoming lateral perforant pathway from EC (Houser 2007; Jinno and Kosaka 2000). Chandelier cells, instead, interact with the granule cell axon hillock inducing strong inhibition (Buhl et al. 1994; Houser 2007; Soriano et al. 1993). In addition, other interneurons have been described sending axons to the middle and inner stratum moleculare (Han et al. 1993), and projecting to CA3 and CA1 (Buckmaster and Schwartzkroin 1995a, b). Activation of this local and powerful inhibitory circuit suppresses granule cell firing resulting in rather discrete and restricted dentate outputs to the hilus and CA3 (Lothman 1994).

However, different interneurons are differentially sensitive to excitotoxic damage. Parvalbumin-positive basket cells are the most resistant to damage (Figueredo-Cardenas et al. 1998; Sloviter 1989) and are still well preserved in epileptic hippocampi, whereas somatostatin-containing interneurons are more vulnerable and their loss may contribute to the hyperexcitability of the DG (Hofmann et al. 2016).

Beside granule cells, mossy cells are the other glutamatergic population in the DG. They send their axons to the inner stratum moleculare, both ipsi- and contralaterally, to form excitatory connections on granule cell dendrites. Mossy cells are among the most sensitive cells in the hippocampus, and their death is thought to trigger the sprouting of granule cell mossy fibres to the stratum moleculare, frequently observed in tissues from epileptic patients (Cavazos et al. 1991). Although mossy fibre sprouting is frequently observed in epilepsy, data on its functional role are ambiguous. This might be due to the multiplicity of pathologies contributing to epilepsy (Godale and Danzer 2018).

2.1.2 CA3

Large pyramidal cells and associated interneurons are the main components of the CA3 region and are the recipient of mossy fibre inputs from DG. It has been observed that CA3 is a generator of frequency oscillations and rhythmic activity, thus it is considered the pacemaker of the hippocampus (Csicsvari et al. 2003; Schlingloff et al. 2014). This is due to CA3 axon collaterals extensively innervating their neighbouring pyramidal cells and the coupling of interneurons via gap junctions (Mann and Paulsen 2005; Traub et al. 2000). Despite the fact that CA3 output is effective in driving postsynaptic targets, it also appears important for the inhibition of ictal activities (Barbarosie and Avoli 1997).

The propensity of CA3 pyramidal cell to generate rhythmic activity is probably due to the high density of calcium channels on their proximal dendrites (Fisher et al. 1990). The robust calcium influx causes a prolonged depolarization that in turn

generates additional action potential forming rhythmic burst discharges. However, the very mechanism of burst generation involves a self-limiting process, such as the after-hyperpolarization produced by calcium-dependent potassium conductance (de Sevilla et al. 2006). In addition, the same mossy fibre afferents that innervate CA3 principal cells also engage the local inhibitory circuit providing a strong and effective inhibitory control (Gulyás et al. 1993). These mechanisms make CA3 reluctant to participate in seizure-like activity, which usually require prolonged depolarization.

However, even a minor deficiency in GABAergic tone can lead to synchronized bursting spreading to CA1 ipsilaterally and to CA1 and CA3 contralaterally possibly contributing to the generation of ictal events.

2.1.3 CA1

The dense CA1 pyramidal cell layer receives its primary glutamatergic afferent from CA3 Schaffer collaterals on apical and basal dendrites in the stratum radiatum and oriens. Additionally, axons originating from EC reach CA1 distal apical dendrites in the stratum lacunosum-moleculare (van Groen et al. 2003). Like other hippocampal subregion CA1 pyramidal cells receive inhibitory inputs from various GABAergic interneurons (Milstein et al. 2015). However, the strength of inhibition on CA1 neurons seems less effective than that in the DG and CA3, therefore this area is more susceptible to recruitment into seizure-like episodes and excitotoxic damage.

The electrophysiological characteristic of CA1 pyramidal cells have been extensively investigated due to their propensity to develop long-term potentiation (LTP) and long-term depression (LTD; Malenka 1994), which are accompanied by spatially widespread changes in excitability (Narayanan and Johnston 2007). These forms of synaptic plasticity are frequently associated with a reduction in the inhibitory tone in CA1. This finding might be significant for epilepsy research since a similar reduction in inhibitory efficacy is associated with the generation of seizure-like activity.

CA1 cells are not capable of generating burst of activity or oscillatory rhythm like those in CA3, and the cell properties and circuits intrinsic to CA1 are not epileptogenic per se. However, if recruited or provoked, they lack the control mechanisms necessary to prevent seizure activity. In tissue sections from mTLE patients, CA1 is the area that shows the most severe damage (Wieser 2004), reflecting its poor capacity to reduce cell excitation. Although it is evident that CA1 can generate seizure-like activity, it is still unclear whether the pronounced neurodegeneration observed in mTLE patients is the cause or the consequence of the epileptogenic process affecting the hippocampus.

2.1.4 Subiculum

Despite its apparently central role, only recently the subiculum started to be carefully investigated in the context of epilepsy and epileptogenesis. The subiculum is the main output station of the hippocampal formation, receiving information mainly from CA1 and projecting it to cortical (EC) and subcortical areas (Witter 2012). The pyramidal cells present in this region are grouped in two categories. The first group

consists of cells capable of producing burst-like discharges similar to those in CA3 and the second group instead exhibits a "regular" firing pattern like most of the CA1 cells (Stafstrom 2005).

In tissue of epileptic patients with hippocampal sclerosis, even if CA1 region is heavily damaged and sometimes completely absent, still the subiculum of these patients is frequently intact (Babb et al. 1984; Sloviter 1994). One hypothesis in mTLE is that epileptic activity originates from the sclerotic hippocampus, given the fact that CA1 is typically damaged, and perhaps the subiculum is only responsible for spreading the aberrant neuronal activity to the rest of the brain, causing generalization of seizures. The intrinsic characteristic of the pyramidal cells present in the subiculum would theoretically allow the generation of burst-like discharges and a hyperexcitable state. Interestingly, silencing parvalbumin positive neurons in the subiculum is sufficient to trigger epilepsy (Drexel et al. 2017). However, our knowledge of the local subiculum circuit is still very limited and it remains unclear to what extent the subiculum participates in the epileptogenic process.

3 The Kappa Opioid Receptor and Dynorphin Expression in the Hippocampal Formation

KOR are widely expressed in the central nervous system of adult rats and mice with binding sites and mRNA expression observed in prefrontal cortex, olfactory tubercle, hypothalamus, periaqueductal gray area and throughout the limbic system (DePaoli et al. 1994; Gackenheimer et al. 2005). The KOR expression pattern in human brains has also widespread distribution (Peckys and Landwehrmeyer 1999), however some differences between rodents and humans have been observed. In particular, high KOR levels were detected in the human cerebellum (Simonin et al. 1995), whereas it is completely absent in rodents (Meng et al. 1993). Autoradiographic studies from rat hippocampi showed dense KOR expression in and adjacent to the pyramidal and granule cell layers. Specifically, KOR has been observed in the outer two thirds of the dentate molecular layer (McLean et al. 1987; Wagner et al. 1992) where EC inputs synapse onto granule cell dendrites, in CA3 stratum lucidum at the level of mossy fibres–CA3 dendrites interaction (McLean et al. 1987), as well as in the stratum oriens and pyramidal layer of CA1 (Halasy et al. 2000). This anatomical distribution suggests that KOR may be localized in the pre- or postsynaptic subcellular compartment of all the major hippocampal connections (Drake et al. 1996; Mathieu-Kia et al. 2001).

The distribution of the endogenous agonists (the Dyns) is mostly overlapping with KOR expression throughout the brain, with high levels of pDyn mRNA in the cortex, hypothalamus, premammillary nucleus and the limbic system in humans (Hurd 1996; Nikoshkov et al. 2005) and rodents (Lin et al. 2006; Merchenthaler et al. 1997; Morris et al. 1986). In the rodent and human hippocampus, Dyns are produced primarily by granule cells and its immunoreactivity is prominent in the mossy fibre pathway (Chavkin et al. 1985; Drake et al. 1994; McGinty et al. 1983; McLean et al. 1987). Electron microscopic analysis revealed that Dyns are also present in the

molecular layer of the DG, and that specifically 75% of the Dyns-containing dense core vesicles were found in granule cell dendrites (Drake et al. 1994). The same study demonstrated that the onset of endogenous Dyns action in the molecular layer is very rapid and that high concentrations of Dyn B locally applied in the hilus do not affect field potentials recorded in the molecular layer. These data suggest that Dyns could be released directly from granule cell dendrites via a mechanism requiring L-type and N-type calcium channels and exert physiologic effects (Simmons et al. 1995). Since the KOR is present at presynaptic level too, Dyns may act as a retrograde feedback messenger at perforant pathway afferents (Drake et al. 1994).

In the stratum lucidum Dyns are released by mossy fibres where they can act postsynaptically on CA3 dendrites, but also on presynaptically on mossy fibre commisurals, thus establishing an inhibitory feedback loop (Simmons et al. 1995; Weisskopf et al. 1993).

Interestingly, in humans but not in rodents, Dyns expression has been observed in the hippocampal afferents coming from EC (perforant pathway), extending the possible action range of Dyns to the CA1 pyramidal cells dendrites (Hurd 1996).

4 Kappa Opioid Receptors and the Control of Network Excitability

In the scenario we just described, KOR and its endogenous agonist are present at all major synapses in the hippocampal formation, thus making KOR a likely modulator of neurotransmission.

Early studies on the role of KOR on neuronal excitability revealed that KOR agonists inhibit depolarization-induced calcium uptake in cortical synaptosomes, by blocking N-type calcium channels (Xiang et al. 1990). In another study, administration of U69593 inhibited N-type, L-type and P/Q-type currents in nerve terminals in rats (Rusin et al. 1997). Reduction of calcium influx in presynaptic terminals would effectively reduce neurotransmitter release. Dyns present in the stratum moleculare of the DG, originating from perforant pathway fibres in humans or from granule cell dendrites in humans and rodents, take advantage of this mechanism to reduce glutamate release and thereby dampen the excitatory afferents from EC (Drake et al. 1994; Salin et al. 1995; Wagner et al. 1993). Similarly, Dyns released from mossy fibres in the CA3 stratum lucidum field mediate heterosynaptic inhibition of neighbouring mossy fibres by acting on KOR present on axo-axonal synapses (Weisskopf et al. 1993).

Modulation of calcium channels is not the only mechanism for KOR to regulate network excitability. Coupling of KOR with potassium channels has been demonstrated in the hippocampus. In rat CA3 pyramidal cells, low concentration of Dyn A and U50488H, a selective KOR agonist, enhanced the M-current, postsynaptic voltage-dependent potassium current (Moore et al. 1994). The KOR agonist U69593 induced hyperpolarization in 35% of substantia gelatinosa neurons. Changes in external potassium concentration shifted the magnitude of this effect, suggesting that the hyperpolarization was due to increased potassium conductance

(Grudt and Williams 1993). Furthermore, it has been demonstrated in Xenopus oocyte that KOR activation can open the G protein-activated inward rectifier potassium channel Kir 3.1 (Henry et al. 1995; Ma et al. 1995; Nagi and Pineyro 2014) through interaction with the Gβγ subunits (Sadja et al. 2003; Wickman and Clapham 1995). Therefore, when activated KOR enhances the outward potassium current, hyperpolarize the cell membrane and shorten the action potential duration, which would secondarily reduce voltage-dependent calcium influx and thereby reduce glutamate release.

The role of second messengers in the KOR-mediated modulation of ion conductance or inhibition of neurotransmitter release is not clear yet. Inhibition of adenylate cyclase via KOR is well established (Bruchas and Chavkin 2010), but the physiologic response of the reduction in cAMP levels is not well understood.

Taken together, the Dyns/KOR has an unequivocal role in the modulation of synaptic transmission at all, but not limited to, excitatory synapses of the hippocampus, making it a potential therapeutic target in epilepsy.

5 Alterations of Dynorphins and Kappa Opioid Receptor System in Epilepsy

Epilepsy is associated with many functional, morphological and neuropathological changes, which may impact the endogenous opioid system. These alterations may contribute or counteract to seizure susceptibility depending on their net effect on glutamatergic and GABAergic transmission (Table 1).

Dyns are highly expressed in the mossy fibres of rodents (McGinty et al. 1983) and humans (Houser et al. 1990; Houser 1992). In animal models of acute seizures or temporal lobe epilepsy, it has been observed that the long-lasting, high frequency stimulation induced by seizures triggers the release of Dyns stored in the mossy fibres. Thus, in the maximal electroshock seizure model, the Dyns pool was depleted for about 6 h when applied once, but up to 2 weeks upon repetitive treatment (Kanamatsu et al. 1986a; Xie et al. 1989a). In rodents, intrastriatal injection of kainic acid reduced Dyns levels for several hours (Kanamatsu et al. 1986b), whereas systemic injection depleted Dyns up to 4 weeks (Douglass et al. 1991; Gall 1988; Lasoń et al. 1992a; Rocha and Maidment 2003). A similar reduction was observed in several kindling models of epileptogenesis (Harrison et al. 1995; Iadarola et al. 1986; Lee et al. 1989; McGinty et al. 1986; Morris et al. 1987; Rosen et al. 1992; Xie et al. 1989b), and microdialysis data reported significantly reduced extracellular opioid peptide levels during interictal periods in fully-kindled rats (Rocha et al. 1997). Furthermore, Dyns immunoreactivity has been found reduced in surgically removed hippocampal tissue of mTLE patients who experienced seizures within 48 h before surgery (De Lanerolle et al. 1997). The changes in Dyns immunoreactivity described for humans and animal models are not observed in every epileptic patient. For example, Dyns immunoreactivity in tissue of TLE patients differs between those with or without mossy fibre sprouting (De Lanerolle et al. 1997, 2003; Houser et al. 1990). pDyn mRNA (de Lanerolle et al. 1992) and peptide (Gall 1988; De Lanerolle

Table 1 Alterations of the hippocampal endogenous KOR system in epilepsy (modified from Burtscher and Schwarzer 2017)

Alteration	Model	References
Strong Dyns release at seizure onset, followed by Dyns depletion	Rodent kainic acid model	Kanamatsu et al. (1986b), Gall (1988), Douglass et al. (1991), Lasoń et al. (1992a)
Dyns depletion after seizures	Electroconvulsive shocks in rodents	Kanamatsu et al. (1986a), Xie et al. (1989a)
Variable transient increase in Dyns mRNA expression after seizures	Various models	Xie et al. (1989a, b), Douglass et al. (1991), Lasoń et al. (1992a, b), Hong et al. (1993), Schwarzer and Sperk (1998)
Reduction in Dyns protein and mRNA levels	Rodent kindling models	Iadarola et al. (1986), McGinty et al. (1986), Morris et al. (1987), Lee et al. (1989), Xie et al. (1989a), Rosen et al. (1992), Harrison et al. (1995), Rocha et al. (1997)
Reduced KOR binding in CA1, reduced Dyns immunoreactivity, elevated Dyns mRNA levels	Hippocampal tissue of mesial temporal lobe epilepsy patients	de Lanerolle et al. (1997), Pirker et al. (2009)
Strong release of Enk and Dyns after status epilepticus, followed by reduction of peptide levels	Rodent kainic acid model	Rocha and Maidment (2003)
Brain-region specific upregulation of opioid receptor availability	PET studies in human mTLE patients	Hammers et al. (2007)

et al. 1997) were also reported from hilar interneurons and CA3 pyramidal cells in mTLE. However, this was observed neither in healthy brain nor in epilepsies without hippocampal sclerosis and mossy fibre sprouting, such as mass-associated or paradoxical temporal lobe epilepsy (Hurd 1996).

Pathological and morphological changes in the hippocampus may, at least in part, explain the alterations in Dyns/KOR system. Seizures provoke neuronal death due to excitotoxicity and at the same time the release of neuropeptides. As a consequence of recurrent seizures, Dyns immunoreactivity is reduced due to loss of Dyns positive neurons (De Lanerolle et al. 1997), as well as excessive release during seizures (Marksteiner et al. 1989; McDermott and Schrader 2011; Sperk et al. 1986). In contrast, mossy fibres sprout to the inner-molecular layer and innervate the basal dendrites of granule cells (for review see, Ben-Ari 2001), resulting in atypical Dyns immunoreactivity in this area (Houser et al. 1990).

Partial loss of KOR-expressing somatostatin-immunoreactive interneurons is also common for temporal lobe epilepsy (Rácz and Halasy 2002). Nonetheless like Dyns immunoreactivity, KOR levels in the hippocampi of patients suffering from mass-associated or paradoxical temporal lobe epilepsy are almost unaltered. These patients displayed similar [^{3}H]U69,593 binding as post mortem controls (De Lanerolle et al. 1997).

As a consequence of the decline in Dyns immunoreactivity, a transient increase in mRNA expression is observed, ranging from 200 to 1,300% in distinct models (Douglass et al. 1991; Hong et al. 1993; Lasoń et al. 1992a, b; Schwarzer and Sperk 1998; Xie et al. 1989a, b), probably as a compensatory response for the low peptide levels. Similarly, a dynamic upregulation of pDyn expression was observed in TLE patients (Pirker et al. 2009).

The loss of Dyns, probably resulting in a lack of inhibition of voltage-gated Ca^{2+} channels and less stimulation of K^+ channels, may be functionally important. Increased Ca^{2+} currents lead to augmented glutamate release, while postsynaptic hyperpolarization is lacking. Such a scenario thereby facilitates the generation of seizures. Of note is the fact that the loss of inhibition on voltage-gated Ca^{2+} channels was closely associated with mossy fibre sprouting and hippocampal sclerosis (Jeub et al. 1999).

The intracellular Ca^{2+} level of neurons within epileptic circuitries is altered by the sudden and repetitive hyperactivity induced by seizures, and this can play a dual role in the regulation of Dyns expression. The transcription factor CREB (cAMP response element-binding protein) is tightly regulated by intracellular Ca^{2+} levels, once activated it binds to CRE (cAMP response element) sites, thus increasing the activity of pDyn promoter. On the other hand, Ca^{2+} also enhances the expression of DREAM (downstream regulatory element antagonizing modulator), which counteracts CREB by binding to DRE (downstream regulatory element) sequence in the promoter (Cheng et al. 2002). Pronounced seizure-induced DREAM expression was shown in the mouse hippocampus (Matsu-Ura et al. 2002).

The importance of Dyns/KOR system in the regulation of synaptic excitability comes from studies on Dyns knockout animals. Deletion of the coding region of the pDyn gene in mice resulted in an increased seizure susceptibility and affected neurodegeneration during epileptogenesis (Loacker et al. 2007). In line with this, low pDyn levels, due to mutations in the promoter regions in humans result in an increased vulnerability towards epilepsy (Gambardella et al. 2003; Stögmann et al. 2002).

Overall, alterations in the Dyns/KOR system in epilepsy suggest a loss of inhibition on glutamatergic neurons. This may contribute to the progression of disease development and severity. The depletion of Dyns with concomitant conservation of KOR offers the opportunity for pharmacological intervention.

6 Kappa Opioid Receptor as a Potential Therapeutic Target

Considering the anticonvulsant properties of Dyns and the fact that Dyns levels are transiently reduced interictally, application of exogenous KOR agonists may have beneficial effects. The approach to activate KOR has indeed been shown to have a potential to suppress seizures (Loacker et al. 2007; Solbrig et al. 2006; Tortella 1988), and it increases the survival of neurons in the hippocampus and amygdala after unilateral injection of kainic acid into the hippocampus of mice (Schunk et al.

2011). In addition, Dyns successfully reduced epileptiform activity in human hippocampal slices obtained from epileptic patients (Agostinho et al. 2019).

However, in the 1990s clinical trials testing the potential of KOR agonists (spiradoline and enadoline) as antipruritic drugs were failed due to dysphoric side effects (Barber and Gottschlich 1997). As a consequence, industrial research on KOR agonists has been essentially discontinued.

Recently, it has been reported that, using KOR agonists biased towards the G-protein signalling cascade, it is possible to separate the KOR-mediated anticonvulsant activity from the undesired dysphoric response (Zangrandi et al. 2016). Furthermore, phosphoproteome analysis of mice treated with unbiased and G protein biased agonists revealed that the former group activates distinct pathways, such as the mTOR (mechanistic target of rapamycin) pathway. Pharmacological inhibition of mTOR pathway abolished aversion in mice, while maintaining the anticonvulsant effect (Liu et al. 2018, 2019), revitalizing the interest for KOR as therapeutic target for epileptic patients.

Another promising approach to target the opioid system and counteract seizures in a disease modifying way is adeno-associated virus (AAV)-based gene therapy. AAV gene therapy is a promising tool to target a broad array of neurological diseases (Weinberg et al. 2013). Several pre-clinical studies on AAV-mediated gene-delivery of neuropeptides are already available (for review see, Kovac and Walker 2013). Gene therapy with Dyns might be an interesting approach to activate KOR and achieve anticonvulsant effects through replenishment of Dyns in different phases of endogenous Dyns depletion. In addition, side effects known from systemic application of KOR agonists may be avoided, due to local and restricted application of the therapy.

Lately, an attempt to replenish Dyns expression in epileptic rodents has been made. The overexpression of Dyns in the epileptic focus of mice and rats reduced both number and duration of epileptic events up to 90 days after the gene therapy application. Furthermore, Dyns overexpression rescued the cognitive impairment typically observed in kainic acid injected animals (Agostinho et al. 2019).

The Dyns/KOR system bears great potential as a therapeutic target in epilepsy and epileptogenesis. Still further studies are needed in order to confirm the anticonvulsant effect and to assess the consequences of a permanent Dyns overexpression, in the case of the gene-therapy approach. Moreover, the possibility of excluding side effects through drug-design or the selection of relevant agonists may contribute to rehabilitate KOR as an attractive drug target for epilepsy patients, as well as other KOR-related disorders.

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Kappa Opioid Agonist-Induced Diuresis: Characteristics, Mechanisms, and Beyond

Saadet Inan

Contents

Abstract

Activation of the kappa opioid receptor (KOR) induces antinociception, antipruritic activity, diuresis, sedation, and dysphoria. KOR agonist-induced diuresis is characterized as water diuresis, in which water excretion with urine is increased without altering electrolyte excretion. Both centrally and peripherally acting KOR agonists promote diuresis. KOR antagonists block KOR agonist-evoked diuresis suggesting that the diuretic effect is through activation of the KOR. Studies in different experimental animal species and in humans indicate that KOR agonists decrease antidiuretic hormone (ADH) secretion and release from the hypothalamus and posterior pituitary; decrease response to ADH in kidneys; increase renal sympathetic nerve activity; and increase adrenaline, noradrenaline, and dopamine release from the adrenal medulla. The therapeutic potentials of KOR agonists as water diuretics have been studied in animal models of cerebral edema due to ischemia and intracranial mass, hypertension, and cirrhosis. This chapter reviews characteristics, possible mechanisms, as well as therapeutic potentials of KOR agonist-induced diuresis.

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Keywords

Aldosterone · Angiotensin II · Antidiuretic hormone · Diuresis · Kappa opioid agonist · Kappa opioid receptor

Abbreviations

ADH	Antidiuretic hormone
Ang I	Angiotensin I
Ang II	Angiotensin II
BBB	Blood brain barrier
KOR	Kappa opioid receptor
MOM-sal B	2-methoxymethyl-salvinorin B
PVN	Paraventricular nucleus
U_KV	Urine potassium
$U_{Na}V$	Urine sodium
Uosm	Urine osmolality

1 Introduction

Activation of kappa opioid receptors (KOR) by an agonist promotes analgesia, antipruritic activity, diuresis (increase in urine output), sedation, and dysphoria. This chapter describes KOR agonist-induced diuresis, a well-known effect that has been studied since the late 1970s. Diuretic activity of KOR agonists has been studied in different species such as the rat, mouse, dog, lamb, monkey, as well as in humans, to elucidate mechanism(s) and therapeutic potentials. Here, characteristics, possible mechanisms, and therapeutic value of diuresis induced by KOR agonists are reviewed.

2 KOR Agonist-Induced Diuresis

Miller (1975) first reported KOR-induced diuresis with butorphanol, a KOR agonist-mu opioid receptor antagonist. Studies with cyclazocine (Gavend et al. 1978), ethylketocyclazocine (EKC) (Slizgi and Ludens 1982), and U50,488 (von Voigtlander et al. 1983) in rats demonstrated that KOR agonist-induced diuresis is water diuresis. In water diuresis, there is an increase in water excretion without an increase in electrolyte secretion, resulting in a low urine osmolality. The list of KOR agonists that have been studied for their diuretic effect is shown in Table 1. As seen in Table 1, KOR agonists elicit diuresis in the mouse, rat, monkey, dog, lamb, and human.

Characteristics of KOR Agonist-Induced Diuresis Most of the characteristics of KOR agonist-induced diuresis are demonstrated here using our own study

Table 1 List of KOR agonists studied for diuresis effect

KOR agonist	Species	Diuresis	References
Asimadoline (EMD-61753)	Rat	Y	Barber et al. (1994b)
	Human	Y	Kramer et al. (2000)
Bremazocine	Rat	Y	Leander (1983), Leander et al. (1985, 1987), Bhargava and Gulati (1988), Takemori et al. (1988), Cook et al. (2000), Craft et al. (2000)
	Monkey	Y	Ko et al. (2003)
BRL 52656	Rat	Y	Brooks et al. (1993)
	Dog	Y	Brooks et al. (1993)
BRL 52974	Rat	Y	Brooks et al. (1993), Wang et al. (1994)
BRL 53114	Rat	Y	Brooks et al. (1993)
	Dog	Y	Brooks et al. (1993)
BRL 53117	Rat	Y	Wang et al. (1994)
Butorphanol	Rat	Y	Miller (1975), Leander (1983), Craft and McNiel (2003)
	Monkey	Y	Vivian et al. (1999)
Cyclazocine	Rat	Y	Gavend et al. (1978), Leander (1983)
EMD 60400	Rat	Y	Barber et al. (1994a)
Ethylketocylazocine	Rat	Y	Slizgi and Ludens (1982, 1986), Leander (1983), Leander et al. (1987), Bhargava and Gulati (1988), Takemori et al. (1988), Borkowski (1989)
GR89,696	Monkey	Y	Butelman et al. (2001)
ICI 197067	Rat	Y	Barber et al. (1994a, 1994b)
ICI 204448	Rat	Y	Barber et al. (1994a, 1994b)
JT09	Rat	Y	Beck et al. (2019)
Ketazocine	Rat	Y	Leander (1983)
MOM-Sal B	Rat	Y	Inan et al. (2009)
MR 2033	Human	Y	Pfeiffer et al. (1986)
MR 2034	Human	Y	Pfeiffer et al. (1986)
	Rat	Y	Leander et al. (1987)
Nalbuphine	Monkey	Y	Vivian et al. (1999)
	Rat	Y	Craft and McNiel (2003)
Nalfurafine	Rat	Y	Inan et al. (2009)
Nalorphine	Rat	*Y*	Leander (1983), Smith et al. (2003)
Niravoline (RU 51599)	Rat	Y	Bosch-Marcé et al. (1995)
	Dog	Y	Brooks et al. (1997)
	Human	Y	Bellissant et al. (1996), Gadano et al. (2000)
Pentazocine	Rat	Y	Craft et al. (2000), Craft and McNiel (2003)
Salvinorin A	Rat	N	Inan et al. (2009)
	Mouse	N	Inan and Cowan (unpublished data)
SB 215519	Dog	Y	Brooks et al. (1997)
SB 215520	Dog	Y	Brooks et al. (1997)

(continued)

Table 1 (continued)

KOR agonist	Species	Diuresis	References
Spiradolin (U62,066E)	Human	Y	Peters et al. (1986), Rimoy et al. (1991)
	Rat	Y	Yamada et al. (1989), Cook et al. (2000), Smith et al. (2003), Minervini et al. (2017)
Tifluadom	Rat	Y	Bhargava and Gulati (1988), Takemori et al. (1988), Borkowski (1989), Ashton et al. (1990)
U50,488	Rat	Y	Leander et al. (1985, 1987), Slizgi and Ludens (1986), Silvia et al. (1987), Bhargava and Gulati (1988), Oiso et al. (1988), Takemori et al. (1988), Bhargava et al. (1989), Borkowski (1989), Ashton et al. (1990), Kapusta and Obih (1993), Craft et al. (2000), Carroll et al. (2004), Gottlieb et al. (2005), Guo et al. (2007), Inan et al. (2009), Beardsley et al. (2010), Franklin et al. (2015), Owens et al. (2016)
	Mouse	Y	Inan and Cowan (unpublished data)
	Dog	Y	Slizgi et al. (1984)
	Monkey	Y	Ko et al. (2003)
	Lamb	Y	Qi et al. (2007)
U69,593	Rat	Y	Craft et al. (1998), Craft et al. (2000), Cook et al. (2000), Smith et al. (2003)
	Monkey	Y	Butelman et al. (2001)

Y yes, *N* no, *MOM-Sal B* 2-Methoxymethyl-salvinorin B

(Inan et al. 2009). We studied the diuretic effects of three non-arylacetamide KOR agonists in normally hydrated male rats: nalfurafine (Endoh et al. 1999; Togashi et al. 2002; Inan and Cowan 2004), salvinorin A, as well as 2-methoxymethyl-salvinorin B (MOM-sal B), a C-2 modified longer-acting analog of salvinorin A (Wang et al. 2008) with the arylacetamide kappa agonist U50,488H (Von Voigtlander et al. 1983) as the reference agonist. Urine was collected in metabolism cages (Fig. 1) for 5 h following subcutaneous (s.c.) administration. As seen in Fig. 2, nalfurafine, MOM-sal B, and U50,488, but not salvinorin A, provoked diuresis in a dose-dependent manner. All three KOR agonists increased urine volume and free water clearance (CH_2O) compared to saline (Fig. 2). Since sodium is not excreted in urine, urine sodium ($U_{Na}V$) levels and urine osmolality (U_{osm}) were found significantly lower in rats administered with a KOR agonist compared to rats injected with saline as seen in Table 2. Salvinorin A, which is an active (hallucinatory) ingredient of the psychoactive plant *Salvia divinorum* and the first non-nitrogenous, highly selective KOR agonist (Roth et al. 2002; Sheffler and Roth 2003), did not elicit diuresis in rats. The lack of an effect on diuresis by salvinorin A might be due to its brief duration of action. Schmidt et al. (2005) identified salvinorin A and its inactive metabolite, salvinorin B, ex vivo in extracted body fluids (from monkeys and humans) and found that salvinorin A is quickly metabolized to salvinorin B by blood esterases. In a study performed in rats, it was shown that following 10 mg/kg

Fig. 1 Urine output in 5 h in rats treated s.c. with nalfurafine, U50,488H, salvinorin A, MOM-sal B, and vehicle ($n = 6$–10). Nalfurafine, U50,488H, and MOM-sal B cause increases in urine output. Urine output for rats injected with saline was 1.37 ± 0.17 mL/5 h and for rats injected with the vehicle for both salvinorin A and MOM-sal B was 2.57 ± 0.46 mL/5 h (Reprinted permission by Spring. Naunyn-Schmiedeberg's Arch Pharmacol (2009) 379:263–270)

salvinorin A injection, plasma t_{max}, plasma elimination $t_{1/2}$ are 15 min and 75.4 min, respectively (Teksin et al. 2009).

Tolerance To understand whether tolerance develops to diuretic effect of KOR agonists, we (Inan et al. 2009) injected fixed doses of U50,488 (10 mg/kg), nalfurafine (as a KOR agonist of our interest, 0.02 mg/kg), and saline once a day for 7 days. There was no difference in urine volume between day 1 and day 7, indicating lack of tolerance in the diuretic effect of both U50,488H and nalfurafine. In rats, following repeated injection of KOR agonists, urine volume and free water clearance were significantly higher and urine osmolality as well as $U_{Na}V$ and potassium levels (U_KV) was significantly lower, compared to repeated saline injection. Following 7-day diuresis, serum sodium levels were in the normal range in rats given KOR agonists despite reduced excretion of sodium with urine (Tables 2 and 3). Tolerance also did not develop to bremazocine-induced diuresis following 5-day administration in rats (Leander 1983), nor did it develop to U50,488 in the study of Bhargava et al. (1989).

Sex Differences Craft et al. (2000) reported that U50,488-, bremazocine-, and pentazocine-induced diuresis were similar between females and males following a single administration. Plasma ADH levels were also found to be similar in both sexes. In addition, Craft et al. (1998) found that following chronic administration of U69,593 (two–four times injection every week for 12 months), urine volume was significantly less in females compared to males when urine output was calculated as mL/body weight. This suggests that following long-term administration of U69,593, tolerance to diuresis develops in females, but not in males. As the pharmacokinetic

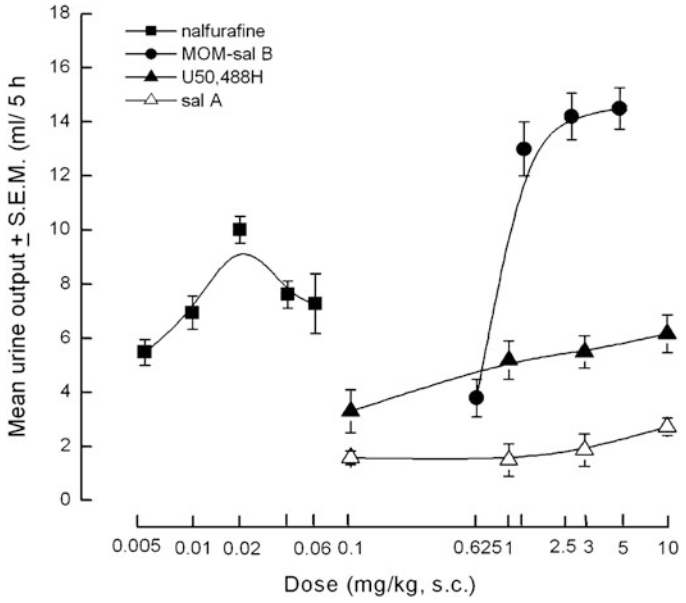

Fig. 2 A metabolism cage use for urine collection in rats

Table 2 Mean ± SD values of cumulative urine output, urine osmolality, free water clearance, and urinary excretion of sodium in rats ($n = 6$) treated (s.c.) with saline, U50,488H, nalfurafine, and MOM-sal B (Reprinted by permission from Spring. Naunyn-Schmiedeberg's Arch Pharmacol (2009) 379:263–270)

	Saline	U50,488H (10 mg/kg)	Nalfurafine (0.02 mg/kg)	MOM-sal B (2.5 mg/kg)
Urine output (mL/5 h)	2.1 ± 0.9	6.2 ± 0.9[a]	9.8 ± 1.2[a]	14.2 ± 1.99[a]
Uosm (mOsm/kg)[b]	473 ± 46.7	75.8 ± 23.7[a]	106 ± 32.1[a]	63.8 ± 18.2[a]
CH_2O (mL/5 h)[c]	−1.4 ± 0.9	4.4 ± 0.9[a]	6.2 ± 1.1[a]	11.1 ± 2.3[a]
$U_{Na}V$ (mEq/5 h)[d]	46.3 ± 21.2	2.7 ± 0.8[a]	2.9 ± 0.9[a]	1.3 ± 0.8[a]

[a] $p < 0.01$ (compared with saline)
[b] Uosm: urine osmolality
[c] CH_2O: free water clearance
[d] $U_{Na}V$: urinary excretion of sodium

properties of U69,593 were similar in both sexes, the differences are due to other factors. It was hypothesized that the different activity in females and males might be due to higher activity of the antidiuretic hormone (ADH, vasopressin), which plays an important role in water excretion in male rats compared to female rats.

Table 3 Mean ± SD values of urine output, urine osmolality, free water clearance, urinary excretion of sodium, and serum Na^+ levels on day 7 in rats treated (s.c.) once a day for 7 days with saline, U50,488H, and nalfurafine (Reprinted by permission from Spring. Naunyn-Schmiedeberg's Arch Pharmacol (2009) 379:263–270)

	Saline	U50,488H (10 mg/kg)	Nalfurafine (0.02 mg/kg)
Urine output (mL/5 h)	2.2 ± 0.34	8.0 ± 1.91[a]	10.8 ± 0.94[a]
Uosm (mosm/kg)[b]	290 ± 42.8	122 ± 63.9[a]	75 ± 25.9[a]
CH_2O (mL/5 h)[c]	−0.06 ± 0.31	4.7 ± 2.48[a]	7.9 ± 1.56[a]
$U_{Na}V$ (mEq/5 h)[d]	56.3 ± 21.7	7.7 ± 4.27[a]	4.2 ± 2.0[a]
U_KV (mEq/5 h)[e]	67.7 ± 22.4	13.7 ± 8.0[a]	6.1 ± 2.87[a]
Serum Na^+ (mmol/L)	134 ± 3.21	134 ± 7.1	132 ± 7.41

[a] $p < 0.01$ (compared to saline)
[b] Uosm: urine osmolality
[c] CH_2O: free water clearance
[d] $U_{Na}V$: urinary excretion of sodium
[e] U_KV: urinary excretion of potassium

Diuretic effects of the mixed agonist/antagonist compounds nalbuphine, butorphanol, and pentazocine were also studied in male and female rats (Craft and McNiel 2003). In normally hydrated rats, there was no sex difference in diuretic effects of all three compounds. In water-loaded rats, urine output was similar in both sexes following nalbuphine and pentazocine administration. Only in rats administered with butorphanol was urine output higher in female rats compared to male rats.

Hydration Levels Most of the diuresis studies were conducted in normally hydrated rats, while some were performed on water-loaded or water-deprived rats. Leander et al. (1987) reported that full KOR agonist-induced diuresis was independent of hydration (water loaded, normally hydrated, and water deprived), however partial KOR agonists did not induce diuresis in water-deprived rats. Bremazocine, ethylketazocine, and U50,488 provoked diuresis in rats that were water loaded, normally hydrated, and water deprived, however, the effect was more prominent in normally hydrated rats. Partial KOR agonists, butorphanol and nalorphine, induced diuresis in normally hydrated and water-loaded (less than full agonists) rats, but not in water-deprived rats. In the same study (Leander et al. 1987), researchers also studied plasma ADH levels in water-deprived rats injected with full and partial KOR agonist. During water deprivation ADH levels are increased to prevent water loss. While full KOR agonists significantly reduced ADH values, partial agonists had no effect on ADH levels (Leander et al. 1987). These authors concluded that studying diuretic effect of KOR agonists in water-deprived rats can differentiate full vs. partial agonists and that testing diuretic effects of KOR agonists gives better results under normal hydration.

Stereospecificity of KOR Agonists The (+) enantiomer of tifluadom, a benzodiazepine derivative with selective KOR agonist activity, was more potent than racemate and (-) enantiomer (100 times less potent than racemate) in producing diuresis in rats (Shearman and Tolcsvai 1986). While the benzomorphan class KOR agonist MR2033 and its (-) isomer, MR2034, induce diuresis in male healthy volunteers, (+) isomer, MR2035, did not increase urine output (Pfeiffer et al. 1986). (-)-(1S,2R)-U50,488 was also found more potent than U50,488 in promoting diuresis in rats (Erwin et al. 2019), similar to antinociception effect in monkeys (Rothman et al. 1989) and in rats (Erwin et al. 2019).

3 Possible Mechanisms for KOR Agonist-Induced Diuresis

Blockade by KOR Antagonists Studies have shown that both centrally and systemically administered KOR antagonists inhibit KOR agonist-induced diuresis, indicating that the effect is mediated by the KOR. A long-acting KOR antagonist, nor-binaltorphimine (nor-BNI) suppressed diuretic effects of ethylketazocine, tifluadom, bremazocine, and U50,488 in rats (Takemori et al. 1988) as well as in monkeys when diuresis was induced by U50,488 and bremazocine (Ko et al. 2003). In the Ko et al. study, intracisternal administration of nor-BNI blocked the diuretic effect for 20 weeks in monkeys. In another study, the KOR antagonist 5′-guanidinonaltrindole inhibited nalfurafine-, MOM-sal B-, and U50,488-induced diuresis in rats (Inan et al. 2009). The long-acting KOR antagonist JDTic and its analogs were also reported to suppress diuresis induced by U50,488 in rats (Carroll et al. 2004; Beardsley et al. 2010; Owens et al. 2016).

Central and Peripheral Actions Induction of diuretic effect not only by centrally acting, but also peripherally acting, KOR agonists suggests that the effect is both centrally and peripherally mediated. Peripherally restricted KOR agonists asimadoline (Barber et al. 1994b), ICI 204448 (Barber et al. 1994a, b), and JT09 (Beck et al. 2019) induce diuresis in rats. Asimadoline has also been shown to increase diuresis in humans (Kramer et al. 2000). Brooks et al. (1993) reported that BRL 52974, which has limited ability crossing blood brain barrier (BBB), was less potent than BRL 53114 and BRL 52656, which pass freely through the BBB, in producing a diuretic effect.

Central Actions Gottlieb et al. (2005) examined the role of the hypothalamic paraventricular nucleus (PVN), a site protected by BBB, in U50,488-induced diuresis in rats. In the PVN, magnocellular neurosecretory cells produce and secrete oxytocin and ADH and their nerve terminals end in the posterior pituitary gland, whereas parvocellular neurosecretory cells produce and release corticotropin-releasing hormone (CRH) and thyrotropin-releasing hormone (TRH) to anterior pituitary gland. Injection of U50,488 into the magnocellular division increased urine output without changing urinary sodium secretion and cardiovascular responses. On the other hand, injection of U50,488 into the parvocellular division

caused bradycardia, renal sympathoinhibition, natriuresis (sodium excretion with urine), and antidiuresis. Thus, centrally, KOR agonists act on the magnocellular part of the PVN of the hypothalamus, probably by inhibiting ADH. Rossi and Brooks (1996) used compartmentalized rat hypothalamo-neurohypophysial explants in culture to examine whether the main effect of the KOR agonist BRL 52656 on ADH secretion occurred in the hypothalamus or neurohypophysis. They found that while BRL 52656 inhibited osmotically stimulated ADH secretion in nM concentrations in the hypothalamus, higher concentrations were required to stimulate ADH secretion in the neurohypophysis. Also, nor-BNI blocked the effect in hypothalamus. In our study (Inan et al. 2009), chronic administration of nalfurafine caused a decrease in basal cAMP level in the rat hypothalamus. These results indicate that KOR agonists act primarily on the hypothalamus to induce diuretic effect.

Peripheral Actions To understand whether the diuretic effect of EKC also has a peripheral component, Slizgi and Ludens (1982) studied effects of EKC in the toad bladder, which is a model for renal distal tubule and collecting duct. EKC blocked ADH-stimulated water flow in the isolated toad bladder. They concluded that EKC induces diuresis by inhibiting ADH secretion centrally and attenuating of ADH response in the kidney. Bremazocine and U50,488 also suppressed ADH levels in water-deprived rats and the diuretic effect of bremazocine was abolished when desmopressin, a synthetic analog of vasopressin, was given (Leander et al. 1985). EKC (Slizgi and Ludens 1986), spiradoline (Yamada et al. 1989), and U50,488 (Ashton et al. 1990) showed a lack of diuresis in Brattleboro rats, a strain that lacks endogenous ADH. However, when ADH was given exogenously, EKC increased urine outflow suggesting that KOR modulates both ADH release and effects of ADH on renal responses (Slizgi and Ludens 1986). Ashton et al. (1990) found that while there was no diuresis, sodium excretion with urine was decreased and glomerular filtration rate was increased with a steady fractional fluid reabsorption in Brattleboro rats given U50,488. Researchers suggested that KOR agonists also alter renal management of water and electrolytes. On the other hand, intravenously administered U50,488 in anesthetized dogs did not change either renal plasma flow or glomerular filtration rate (Slizgi et al. 1984).

Renal Sympathetic Nerves Removal of the medullary zone of adrenal gland in rats attenuated diuretic effects of U50,488, EKC, and tifluadom in rats (Borkowski 1989). The adrenal medulla has cells that secrete adrenaline, noradrenaline, and dopamine in response to sympathetic stimulation. Kapusta and Obih (1993) showed that intracerebroventricular administration of U50,488 increased renal sympathetic nerve activity and that nor-BNI blocked U50,488-induced increase in urine flow, decrease in urine sodium excretion, as well as increased renal sympathetic nerve activity in rats. These investigators also showed that the decrease in sodium excretion in urine produced by U50,488 was abolished in rats with chronic bilateral renal denervation. These results suggest that renal sympathetic nerve activity is required for both increase in urine output and decrease in sodium excretion in urine induced by KOR agonists. Yohimbine, an alpha-2 adrenoreceptor antagonist, abolished both

BRL 53117- (crosses the BBB) and BRL 52974-(has a limited ability to cross BBB) induced diuresis in rats. Gottlieb and Kapusta (2005) studied role of endogenous kappa opioid system on diuresis, urinary sodium excretion, and augmentation of renal sympathetic nerve activity by giving nor-BNI centrally in rats during an acute stress response to hypotonic saline volume expansion. In nor-BNI treated animals renal sympathetic nerve activity was decreased and urinary sodium excretion was increased. In rats with chronic bilateral renal denervation, nor-BNI-induced effects were abolished. Franklin et al. (2015) studied two lamina terminalis sites, the median preoptic area (MPA) and bed nuclei of the stria terminalis (BST) to understand the sites for the cardiovascular and renal effects of central activation of KOR in rats. Microinjection of U50,488 into BST increased urine volume without changing blood pressure, heart rate, urine sodium excretion, and renal sympathetic nerve activity. However, microinjection of U50,488 into MPA increased renal sympathetic nerve activity without changing blood pressure, heart rate, and urine sodium excretion. This study showed that in the brain U50,488 also acts on BST and PMA, in addition to PVN.

Human Studies Diuretic effects of KOR agonists in humans also have been examined. MR 2033 and MR 2034 elicited an increase in urine output, a decrease in urine osmolality, a decrease in plasma cortisol and ACTH levels, and no change in plasma ADH levels (Pfeiffer et al. 1986). Spiradoline also increased urine output, decreased urine osmolality, did not change urine electrolyte excretion, and had no effect on plasma ADH levels or renal blood velocity (Pfeiffer et al. 1986; Rimoy et al. 1991). Peripherally restricted asimadoline was found to increase urine volume without affecting blood pressure, heart rate, glomerular filtration rate, and urine electrolyte excretion, atrial natriuretic peptide (ANP), and endothelin (Kramer et al. 2000). Another KOR agonist studied in healthy volunteers was niravoline (Bellissant et al. 1996). In addition to diuretic effect, it decreased urine osmolality and sodium and potassium excretion in urine, and a temporary increase in blood pressure as well as slight decreases in plasma and urine levels of ADH. Niravoline significantly increased plasma levels of norepinephrine, renin, aldosterone, and atrial natriuretic peptide (ANP).

Summary Renin–angiotensin–aldosterone axis, ADH, and sympathetic nerve activity play major roles in regulating plasma and urine osmolality and electrolytes. In summary, results from animal and human studies indicate that activation of KORs by an agonist affects a) ADH secretion and release, b) sympathetic nerve activity, c) secretion and release of adrenaline, noradrenaline, and dopamine from adrenal medulla, and possibly d) angiotensin II and aldosterone secretion and release. Possible sites of effects of KOR agonists have been summarized in Fig. 3.

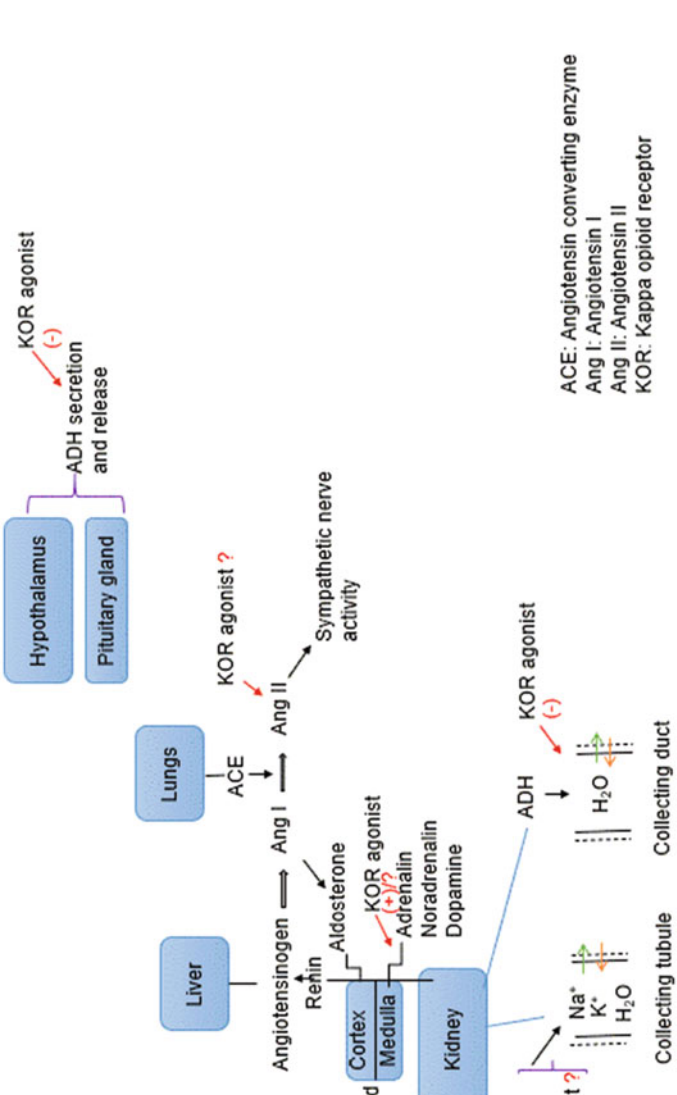

Fig. 3 A diagram summarizes possible activity sites of KOR agonists for diuresis effect. Renin–angiotensin–aldosterone axis, ADH, sympathetic nerve activity play major role in regulating plasma and urine osmolality and electrolytes. In summary, activation of KORs affects (**a**) ADH secretion and release, (**b**) sympathetic nerve activity, (**c**) secretion and release of adrenaline, noradrenaline, and dopamine from adrenal medulla, and possibly (**d**) angiotensin II and aldosterone secretion and release

4 Therapeutic Value of KOR Agonist-Induced Water Diuresis

The water diuretic effect of KOR agonists has been studied for its potential therapeutic value in animal models for cerebral edema, hypertension, and cirrhosis. Protective and therapeutic effects of KOR agonists against cerebral edema induced by ischemia were studied in rodents. Pretreatment with U50,488 prevented the development of cerebral edema induced by ischemia in rats (Silvia et al. 1987). Niravoline and U50,488 given 20 h following ischemia significantly reduced brain water content compared to the vehicle group in mice. Also, both KOR agonists increased serum osmolality as much as mannitol in ischemic and non-ischemic groups (Guénia and Oberlander 1997). Effect of niravoline on intracranial hypertension induced by epidural brain compression in cats was studied as well (Nagao et al. 1997; Bemana and Nagao 1998). Niravoline significantly reduced elevated intracranial pressure, increased lowered cerebral perfusion pressure, and decreased brain water content without changing serum electrolytes and osmolality. It was suggested that the reduction in intracranial pressure was the result of decrease in water content of brain. These results indicate that KOR agonists may be an alternative to hyperosmotic agents in the treatment of cerebral edema due to focal ischemia and intracranial mass.

Bhargava and Das (1986) reported for the first time that there is an increase in KOR density in hypothalamus and cortex membranes of spontaneously hypertensive rats compared to normotensive rats by [^{3}H]EKC binding. Analgesic and diuretic effects of tifluadom, U50,488, and bremazocine were found to be greater in hypertensive rats than normotensive rats (Bhargava and Gulati 1988). Effects of these three KOR agonists administered intravenously on heart rate and blood pressure were examined in normotensive rats with or without bilateral removal of adrenal medulla (Gulati and Bhargava 1988). It was found that KOR agonists depressed the cardiovascular system and that those effects were mediated via adrenal medulla and peripheral KORs. In addition, U50,488 had more profound effects on lowering mean arterial blood pressure, inducing diuresis, and decreasing plasma ADH and angiotensin in hypertensive rats than normotensive rats. Plasma atrial natriuretic peptide levels remained the same in both groups (Guo et al. 2007). Furthermore, U50,488 induces vasodilation on isolated renal artery segments from hypertensive rats.

U50,488 perfused just before the beginning of and 30 min after reperfusion following experimentally induced myocardial infarction in isolated rat hearts significantly reduced infarct size, increased contractility, and reduced heart rate via KORs (Lee et al. 2008; Chun et al. 2010). An in vitro study on cardiomyocytes exposed to infarct/reperfusion injury showed that U50,488 increased viability of cells via KORs. Activation of KOR causes cardioprotection via AMPK/Akt/eNOS signaling (Zhang et al. 2018). AMPK (AMP-activated protein kinase), a serine/threonine protein kinase that is activated when AMP: ATP and ADP: ATP ratios change, plays an important role in energy regulation. Moreover, AMPK activation by KORs was also reported by Tian et al. (2019). They showed that treatment with U50,488 reduced apoptosis and significantly improved mitochondrial morphology and function in cardiomyocytes exposed to infarct/reperfusion injury. These effects were

blocked by nor-BNI, Compound C (an AMPK inhibitor), and AR-A014418 (a GSK3β inhibitor). Further, in cardiomyocytes, treatment with U50,488 significantly increased the expression of phosphorylation of AMPK and the phosphorylation of GSK3β, suggesting that U50,488 exerted cardioprotective effects by improving mitochondrial morphology and function through activation of the KOR-mediated AMPK/GSK3β pathway. Anti-hypertensive and cardioprotective effects, in addition to diuretic effect, of KOR agonists are advantageous for treatment of cardiovascular diseases. Post- mortem study on two human hearts showed the presence of immunoreactivity for KORs in myocardial cells as well as in intrinsic cardiac adrenergic cell-like structures (Sobanski et al. 2014). Therefore, KOR agonists may be studied in clinical trials not only for diuretic effect but also for possible cardioprotective effect following myocardial infarction/reperfusion injury as well as treatment of hypertension. In a recent study (Beck et al. 2019), the peripherally restrictive and orally active JT09 was shown to have a diuretic activity in rats. Researchers aimed to study JT09 in a heart failure model in rats in future experiments.

Since KOR agonist-induced diuresis is a water diuresis without excretion of sodium, niravoline was studied in rats and man with cirrhosis in which there is water retention and dilutional hyponatremia. In cirrhotic rats, niravoline induced diuresis without changing heart rate and mean arterial blood pressure (Bosch-Marcé et al. 1995). Niravoline increased urine output and plasma sodium concentration without altering heart rate and blood pressure in cirrhotic patients (Gadano et al. 2000).

Diuretic effect of KOR agonists has potential therapeutic value for diseases states in which water diuresis is needed, such as congestive heart failure, cirrhosis, cerebral edema, and hypertension. Further preclinical and clinical studies are required using KOR agonists that have less central effects.

5 Conclusions

Interest in the potential therapeutic activity of KOR agonists has increased substantially in recent years. Nalfurafine (a KOR agonist, Remitch 2.5 microgram, oral) has been approved to treat pruritus in chronic renal failure in Japan. A one-year follow-up study reported that nalfurafine is safe and efficacious without adverse reactions associated with typical KOR agonists, including psychotomimetic effect and anhedonia (Kozono et al. 2018). Insomnia was the most encountered side effect (4% of patients). In addition, KOR agonists have been studied for their analgesic and antipruritic activity in Phase II/III in humans (http://ir.caratherapeutics.com/; https://www.trevitherapeutics.com/nalbuphine).

To avoid central side effects (sedation, dysphoria, hallucinations, and dissociation) of KOR agonists, the development of peripherally restrictive KOR agonist for the purpose of water diuresis (without electrolyte excretion) in diseases like cirrhosis and congestive heart failure would be beneficial. The KOR agonist JT09 was shown to be as effective as Tolvaptan, a vasopressin 2 antagonist used for diuretic effect in

congestive heart failure, in rats (Beck et al. 2019). A peripherally acting KOR agonist (without central side effect) that reduces peripherally vascular resistance and increases heart muscle contractility together with diuresis effect would have been ideal and more beneficial than diuretics that are used in humans today.

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Kappa Opioid Receptor Expression and Function in Cells of the Immune System

Thomas J. Rogers

Contents

Abstract

The kappa opioid receptor (KOR) is expressed on a number of hematopoietic cell populations, based on both protein binding analysis and the detection of kappa opioid receptor gene (Oprk1) transcripts. There are prominent Oprk1 splice variants that are expressed in the mouse and human brain cells and leukocytes. The activation of KOR results in reduced antibody production, an inhibition of phagocytic cell activity, an inhibition of T cell development, alterations in the production of various pro-inflammatory cytokines, chemokines, and the receptors for these mediators. Finally, the activation of KOR also leads to the regulation of receptor functional activity of chemokine receptors through the process of

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heterologous desensitization. The functional activity of KOR is important for the regulation of inflammatory responses and may provide opportunities for the development of therapeutics for the treatment of inflammatory disease states.

Keywords

Analgesia · Chemokine · Cytokine · Dynorphin · Heterologous desensitization · HIV-1 · Inflammation · Kappa opioid receptor · Norbinaltorphimine · Protein kinase C · Splice variant · T cell development · U50,488H

Abbreviations

ChIP	Chromatin immunoprecipitation
DOR	Delta opioid receptor
GPCR	G protein-coupled receptor
HIV-1	Human immunodeficiency virus 1
IFN	Interferon
IL	Interleukin
IRF	Interferon regulatory factor
JAK	Janus kinase
KOR	Kappa opioid receptor
MOR	Mu opioid receptor
mRNA	Messenger RNA
norBNI	Norbinaltorphimine
Oprk1	Kappa opioid receptor gene
PAG	Periaqueductal gray
PBMC	Peripheral blood mononuclear cell
PKC	Protein kinase C
RT-PCR	Real-time polymerase chain reaction
siRNA	Small interfering RNA
STAT	Signal transducers and activators of transcription
TNF	Tumor necrosis factor
U50,488H	Trans-(±)3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide
U69,593	(+)-(5_,7_,8_)-N-Methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide

1 Introduction

This chapter will review the characteristics and functional activity of kappa opioid receptors (KOR) expressed by cells of the immune system. Early evidence that hematopoietic cells express opioid receptors was reported by Wybran in 1979 in a report which showed that both morphine and methionine enkephalin modulate the expression of the CD2 receptor on T cells (Wybran et al. 1979). Additional studies

which indicated the presence of opioid binding sites on leukocytes were published, although the presence of classical opioid receptors remained uncertain for some time (Bidlack et al. 2006; Machelska and Celik 2020). It is now clear that classical opioid receptors of the mu, kappa, and delta types are expressed by cells of the immune system, and the activation of these receptors results in substantial changes in immunological responses. While the consequences of mu opioid receptor (MOR) activation have received greater attention, it is clear that KOR activation may result in modulation of the functional activity of several of the hematopoietic cell types.

2 Expression of the KOR

Early evidence for the expression of KOR by leukocytes was reported based on work conducted with various stable cell lines. Some of this early work suggested the presence of non-classical kappa opioid binding activity, based on either the lack of stereospecificity or the high concentrations of either the kappa agonist U50,488H or the opioid antagonist naloxone required to displace either [^{3}H]bremazocine or [^{3}H] U69,593 agonist binding to the murine EL-4T cell line (Fiorica and Spector 1988). Additional studies carried out with the P388D1 murine macrophage cell line showed the presence of high-affinity U69,593 binding, but the binding activity was not completely blocked by either dynorphin or naltrexone (Carr et al. 1989). However, subsequent studies reported from the Bidlack laboratory demonstrated classical KOR binding activity for the R1.1 thymoma cell line, based on stereoselectivity and other binding characteristics (Bidlack et al. 1992). The KOR in R1.1 cells, along with the receptor expressed by two R1.1 derivative lines (R1.G1 and R1EGO), was found to be negatively coupled to adenylyl cyclase through a pertussis toxin sensitive G protein (Lawrence and Bidlack 1993; Lawrence et al. 1995b). Moreover, the activation of the R1.1 KOR decreases the level of cyclic AMP in the cell line, and it has been suggested that the reduction in cyclic AMP levels may be involved in the process of KOR signaling in cells of the immune system (Bidlack et al. 2006).

Classical binding studies have not been reported for primary leukocytes, due in large part to the fact that the expression of the KOR on these cells is at a low level. In addition, techniques which allow for a characterization of this receptor are challenging due to the absence of adequate specific antibodies. Nevertheless, there has been success in detecting the expression of the KOR expressed by leukocytes using a sensitive indirect immunofluorescence flow cytometric technique (Lawrence et al. 1995a). This assay was based on the use of fluorescein-conjugated arylacetamide (a high-affinity KOR agonist), and the assay was further amplified using biotinylated anti-fluorescein IgG and avidin-coupled phycoerythrin. The fluorescein-arylacetamide binding was decreased by about 50% (Ignatowski, Bidlack 1998) in the presence of the KOR antagonist norbinaltorphimine (norBNI) (Ignatowski and Bidlack 1999; Lawrence et al. 1995a). This technique was used successfully to detect the expression of the receptor by various murine T cell lines, primary T cells, and macrophages, and established the presence of kappa-selective binding to primary leukocytes.

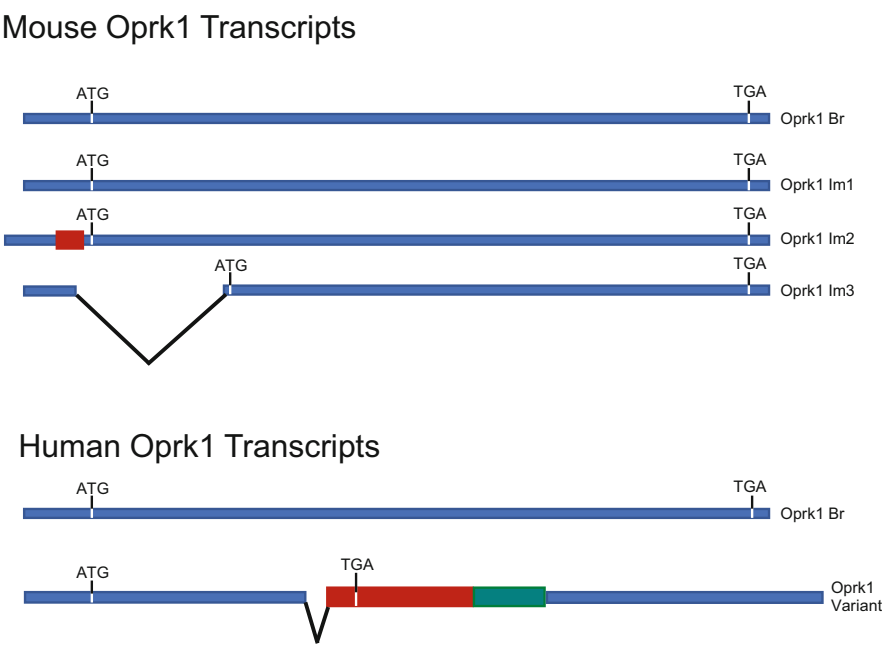

Fig. 1 Schematic diagram of the mouse and human Oprk1 transcripts. *Top*. The diagram shows the full-length transcript which has been reported for the brain (Oprk1 Br). This is the same sequence for the Oprk1 Im1 detected in leukocytes. The splice variants Oprk1 Im2 and Oprk1 Im3 are presented. *Bottom*. The diagram shows the full-length transcript reported for human brain (Oprk1 Br). The human splice variant is reported (Oprk1 variant) showing the insertion of an intron sequence (red) and a region of exon 4 (green)

The molecular expression of KOR by hematopoietic cells was first reported by the Rogers laboratory, initially using the R1.1 thymoma cell line, and subsequently for primary murine leukocytes (Belkowski et al. 1995b, c; Alicea et al. 1998). The cloning of the KOR gene (Oprk1) from each of these cell types revealed the presence of three distinct transcripts in cells of the immune system, and sequence analysis suggests that these transcripts are the result of exon–exon splice variation (Belkowski et al. 1995c; Alicea et al. 1998). The Oprk1 transcripts from the R1.1 cell line were reverse-transcribed, and amplified by polymerase chain reaction (PCR), cloned, and sequenced. A transcript which matches the reported sequence of neuronal KOR was identified in these immature T cells. A transcript variant was identified that contained a 30 bp insert, 15 bp upstream of the 5′ end of the translation initiation site, which corresponds to the junction between the first and second exons (Fig. 1). Both of these transcripts contain the same expected translation initiation site (ATG), which is flanked by a Kozak translation initiation consensus sequence, and both transcripts would be expected to result in the translation of the expected (full-length) KOR. Finally, it should be pointed out that studies of the transcription of Oprk1 in hematopoietic cells have been limited to studies based on RT-PCR. Based

on northern blot analysis, the transcription of Oprk1 in brain tissue results in the expression of mRNA species which vary between 5.2 and 6 kb in size (Yasuda et al. 1993; Zhu et al. 1995), with long 3′ untranslated regions.

The molecular analysis of the mRNA in the R1.1 cell line also led to the detection of an unexpected shorter transcript. This transcript is missing 301 bp, does not include the entire second exon, but the upstream and downstream sequence is intact (Alicea et al. 1998). The typical translation initiation site is deleted in this transcript (Oprk1 Im3), but there is a potential translation initiation site (ATG) that is in-frame near the 5′ end of the third exon. If a truncated protein is translated from this site, the protein would be missing about 100 amino acids, and would be missing the aminoterminal region, the first transmembrane domain, and a portion of the first intracellular loop. The balance of the receptor would remain intact. It is not known at this time whether this truncated transcript is translated and expressed on the cell membrane.

Analysis of KOR transcripts from murine brain tissue reveals the expected expression of the full-length transcripts (Fig. 1, designated Oprk1 Im1, and Oprk1 Im2) (Alicea et al. 1998). In addition, the truncated transcript was also detected in the brain tissue, but the expression of the full-length transcripts was about 10-fold more abundant than the truncated transcript. The expression of both full-length and truncated KOR transcripts was also detected in the DPK cell line, a second immature T cell line (Alicea et al. 1998). In addition, macrophage-like cell lines were also analyzed for KOR transcript expression, and both full-length and truncated transcripts were detected in the $P388D_1$ and WEHI-3 cell lines, but the KOR transcripts were undetected in the RAW.264 line (Alicea et al. 1998). These results are consistent with studies which showed that the functional activity (cytokine expression) of $P388D_1$, but not the RAW.264 cell lines, is modulated following kappa agonist treatment (Belkowski et al. 1995a). Finally, attempts have been made to detect KOR transcripts in primary non-elicited (non-activated) macrophages, and the truncated transcript was readily identified (Alicea et al. 1998). Notably, while the full-length transcripts were not easily detected in non-activated macrophages, these cells respond to treatment with kappa opioid agonists, which results in an inhibition of IL-1, IL-6, and TNFα (Alicea et al. 1996).

An alternative splice variant of human Oprk1 has also been reported following analysis of various immune and non-immune cell populations (Gaveriaux-Ruff et al. 1997). A splice variant has been identified that is composed of the insertion of additional intron sequences to form alternative second and third exon sequences. This gives rise to a variant transcript that is longer than the conventional Oprk1 transcript (Gaveriaux-Ruff et al. 1997). However, the expected translation product would be substantially truncated. In this case, the translation product would be expected to yield a four-transmembrane protein, which would include the amino terminal domain, the first and second intracellular loops, and only part of the second extracellular loop. This splice variant transcript is expressed in the MOLT-4 and HSB2 human T cell lines, and the human U937 macrophage-like cell lines, but not in the THP1 monocyte line. Furthermore, the conventional Oprk1 transcript is not observed in either the MOLT-4 or HSB2 T cell lines, or the U937 macrophage line,

while the variant transcript is not observed in human brain, peripheral blood leukocytes, or isolated monocytes. Here again, it is not clear whether the human Oprk1 splice variant is translated, and if translated whether it is expressed in the cell membrane. However, efforts to express the human variant in the outer membrane of COS cells have not been successful (Gaveriaux-Ruff et al. 1997).

3 Functional Activity of the KOR Expressed by Hematopoietic Cells

3.1 Antibody Response

Studies carried out in vitro have shown that treatment with U50,488H results in a significant and substantial reduction in the production of antibody from murine splenocyte cultures (Taub et al. 1991). The results showed that the inhibitory activity was dose-dependent, active at nanomolar concentrations, and blocked by pre-treatment with the antagonist norBNI. These results are consistent with studies of KOR-knockout mice, which showed an elevated level of total immunoglobulin, and specifically an increase in the levels of circulating IgM, IgG1, and IgG2 (Gaveriaux-Ruff et al. 2003). This suggests that the KOR may function to directly, or indirectly, inhibit the production of antibody through an unidentified process. An analysis of the mechanism of the kappa agonist-mediated suppression of antibody production is complicated since the antibody response is the result of the cooperation of several distinct populations of leukocytes, including accessory cells, and both T and B lymphocytes. Additional studies have been carried out by treating isolated populations of accessory cells, B cells, and T cells with U50,488H, followed by recombination of these cells. The results showed that both accessory cells and T cells are involved in the inhibition of antibody responses, and the inhibitory activity was blocked by pre-treatment with naloxone (Guan et al. 1994).

3.2 Phagocytic Activity

Phagocytic cells are among the more important accessory cells, and it is clear that these cells are responsive to the effects of U50,488H. Studies have shown that the phagocytic activity of murine peritoneal macrophages is inhibited by treatment with U50,488H, and the inhibition is blocked in a dose-dependent manner by pre-treatment with norBNI (Szabo et al. 1993). The reduction in phagocytic activity may contribute to the KOR-mediated inhibition of antibody production. On the other hand, the generation of superoxide production by neutrophils and macrophages (Sharp et al. 1985), the oxidative burst from macrophages (Tosk et al. 1993), and macrophage-mediated tumoricidal activity (Foster and Moore 1987; Hagi et al. 1994) were found to be increased following treatment with dynorphin A (1-13). Moreover, dynorphin A (1-13) is capable of stimulating monocyte chemotaxis in a dose-dependent manner, and this response is blocked by (−) but not (+) naloxone

(Ruff et al. 1985). Based on these studies it would appear that the KOR mediates a combination of both positive and negative effects on accessory cells.

3.3 Cytokine and Cytokine Receptor Expression

3.3.1 Accessory Cell Cytokine and Cytokine Receptor Expression

Several studies have been reported which characterize the impact of kappa opioids on the macrophage expression of various cytokines. Treatment of the P388D_1 murine macrophage cell line with U50,488H induces a dose-dependent reduction of IL-1 and TNFα expression, and the inhibitory activity was blocked by pre-treatment with either naloxone or norBNI (Belkowski et al. 1995a). The RAW 264.7 murine macrophage cell line did not exhibit any change following U50,488H treatment, which may suggest that this cell line does not express the Oprk1 mRNA. Subsequent studies with the P388D_1 showed that treatment with U50,488H also inhibits the expression of IL-6 in liposaccharide-stimulated cells (Parkhill and Bidlack 2006). The administration of U50,488H to non-elicited and lipopolysaccharide-activated murine peritoneal macrophages resulted in a reduction in the expression of the pro-inflammatory cytokines IL-1, IL6, and TNFα (Alicea et al. 1996). The inhibition of cytokine expression was blocked by naloxone or norBNI, and was apparent at the transcriptional level. However, the mechanism of these effects remains undefined. It should be pointed out that earlier work showed that treatment of bone marrow macrophages with dynorphin A (1-6) results in an increase in the expression of IL-1 from murine bone marrow macrophages (Apte et al. 1990). An explanation for the conflicting results in the latter studies remains to be determined.

3.3.2 T Cell Cytokine and Cytokine Receptor Expression

The capacity of kappa opioid agonists to modulate the production of cytokines from T cells has not been extensively analyzed. However, studies have been reported using a co-culture system in which murine thymocytes (immature T cells) were stimulated with Staphylococcal enterotoxin B (SEB) were combined with activated macrophages, and the production of the T cell cytokine IL-2 was measured. In these studies, treatment with U50,488H resulted in a significant dose-dependent, naloxone- and norBNI-sensitive, reduction in IL-2 expression (Guan et al. 1997). These results are consistent with the literature, reviewed above, which showed that developing T cells appear to express relatively abundant KOR (Belkowski et al. 1995b; Alicea et al. 1998). Indeed, the expression of KOR by immature T cells has been demonstrated using both KOR ligand binding analysis and RT-PCR (Bidlack et al. 1992; Belkowski et al. 1995b, c; Guan et al. 1998).

Superantigen stimulation of immature T cells, in the absence of activated accessory cells, results in an up-regulation of the expression of several cytokine and chemokine genes, including IL-2, IL-4, IL-13, interferon-γ (IFN-γ), lymphotactin (XCL1) (Zhang and Rogers 2000). For all of these genes, the addition of U50,488H over a dose range of 1 μM to 10 pM failed to alter gene expression. Superantigen

treatment also stimulates an increase in the expression of the several cytokine and chemokine receptor genes, including IL-2Rα, IL-2Rβ, IL-4Rα, IL-15Rα, TNF-Rp55, TNF-Rp75, and CCR5. Here again, the administration of U50,488H failed to modulate the expression of all of these genes. However, treatment of the T cells with U50,488H induced a significant reduction in the expression of IL-7Rα at doses of 1 nM and above. In contrast, the U50,488H administration induced an increase in the expression of CCR2 expression at doses of 10 nM and above. The production of IL-7 in the thymus is essential for normal differentiation, maturation, and survival of T cells (Kim et al. 1998; Hong et al. 2012). In contrast, CCR2 is reported to play a significant role in the egress of T cells from the thymus into the circulation (Aili et al. 2018). It has been suggested that the inhibition of IL-7, combined with the increase in CCR2 expression, in response to U50,488H may inhibit IL-7-dependent T cell maturation in order to promote T cell emigration (Zhang and Rogers 2000).

3.3.3 T Cell Development

These results suggest the possibility that KOR may play a role in T cell development or differentiation. However, little work has been done to directly assess the involvement of KOR in T cell development. Pluripotent lymphocytes immigrate to the thymus, and after some time, the cells express both of the cell surface markers CD4 and CD8. These cells compose approximately 80% of the thymic lymphocytes. It is at the CD4 + CD8+ stage that various "selective" processes take place, and the T cells which survive have been "educated" to distinguish between "self" and "non-self" (Klein et al. 2014). Once additional maturation has taken place the cells lose expression of either CD4 or CD8 (and are about 15% of the T cells in the thymus), and the single-positive T cells become responsive to extrathymic signals and emigrate into the peripheral tissues. The maturation of T cells at a basic level can be tracked by following the progression in the thymus of T cells from the CD4 and CD8 double negative stage, to the CD4 and CD8 double-positive stage, to the CD4 or CD8 single-positive stage.

Analysis of thymic T cells in Oprk1-knockout mice has shown that in the absence of KOR, there is a significant reduction in thymic cellularity, an increase in the percentage of T cells at the CD4 and CD8 double-positive stage, and a decrease in the percentage of CD4 single-positive cells (Gaveriaux-Ruff et al. 2003). This suggests that the normal progression of T cell maturation to the CD4 single-positive stage may be partially arrested in the absence of KOR expression. These results contrast with the work which was carried out with the DPK immature T cell line. As described above, these cells express the Oprk1 gene and appear to be representative of the CD4 and CD8 double-positive stage of maturation. Treatment of this cell line with superantigen drives these cells to the CD4 single-positive stage of development (Guan et al. 1998). Results from experiments with this cell line show that treatment with U50,488H inhibits the maturation of the DPK cells to the CD4 single-positive stage. The nature of the disagreement between the results from the KOR-knockout mice and the DPK cell line studies is not apparent. Nevertheless, further analysis of the precise role of KOR in T cell development requires greater attention.

3.3.4 Molecular Basis for Regulation of Chemokine Receptor Expression

The molecular mechanism responsible for the KOR-mediated regulation of chemokine receptor expression has been evaluated most extensively for CXCR4. The chemokine receptor CXCR4 is critical for the development of several organ systems, including both the immune and nervous systems (Rostene et al. 2007; Nash and Meucci 2014). In addition, CXCR4 is the major Human Immunodeficiency Virus-1 (HIV-1) co-receptor for strains of the virus that infect T cells. Treatment of either human monocytes or T cells with U50,488H, at doses as low as 0.1 nM, results in reduction in the expression of CXCR4 (Finley et al. 2011). The inhibition of CXCR4 expression is blocked by pre-treatment with norBNI, is U50,488H dose-dependent, and results in a substantial (but not complete) internalization of CXCR4 over a period of 48–96 h.

The inhibition of CXCR4 expression is apparent at both the protein and mRNA level, suggesting that the activation of KOR by U50,488H acts at by altering CXCR4 transcription (Finley et al. 2011). An assessment of the capacity of U50,488H to alter transcription factor activity showed that several factors are up-regulated, including Interferon Regulatory Factor (IRF) and Signal Transducers and Activators of Transcription (STAT) 1, 3, 4, 5, and 6. The STAT and IRF transcriptional regulators are involved in the expression of several interleukins and chemokines (Tamura et al. 2008; Oshima et al. 2004; Jaruga et al. 2004), and both the delta opioid receptor (DOR) and MOR have been shown to activate STAT3 and STAT5 (Lo and Wong 2004; Mazarakou and Georgoussi 2005). The activation of STAT factors is typically mediated by the initial activation of Janus Kinases (JAK), and results show that U50,488H induces the activation of JAK2, and activated JAK2 directs the activation of STAT3 (Finley et al. 2011). Furthermore, inhibitors of JAK2 and STAT3 block the U50,488H-induced inhibition of CXCR4 expression.

Treatment with U50,488H also induces the expression of IRF1 and IRF2, factors which are important regulators of the expression of a number of immunological mediators. Moreover, IRF transcriptional activators have been documented to be regulated by JAK and STAT kinases (Paun and Pitha 2007), and inhibitors of both JAK2 and STAT3 block the induction of IRF1 and IRF2 expression (Finley et al. 2011). Using an siRNA approach to knockdown IRF1 or IRF2 expression, additional results showed that IRF2 knockdown blocks the U50,488H-mediated inhibition of CXCR4. Finally, through the use of ChIP analysis, U50,488H treatment was shown to result in IRF2 binding to the CXCR4 promoter. These data reveal a molecular mechanism for the KOR-mediated inhibition of CXCR4 expression which is composed of the initial activation of JAK2, followed by activation of STAT3, and this leads to the up-regulation of IRF2 expression and inhibition of CXCR4 transcription. It is not clear at this time whether KOR regulation of other cytokine or chemokine genes involves the same or a similar signaling pathway.

The KOR-mediated inhibition of CXCR4 expression has been shown to have significant consequences for the resistance to infection with HIV-1. Peterson and his colleagues have shown that the activation of KOR leads to a significant reduction in the capacity of human T cells to be infected with HIV (Peterson et al. 2001). In

addition, U50,488H, dynorphin A (1-13) and dynorphin A (1-17) have been documented to attenuate the replication of HIV-1 in cultures of human microglial cells (Chao et al. 1996). Studies with human peripheral blood leukocyte cultures have demonstrated that the U50,488H-induced inhibition of HIV-1 infection significantly correlates with the inhibition of CXCR4 expression (Finley et al. 2011).

4 Cross-Talk Between KOR and CXCR4

The process of heterologous desensitization was first reported by the Snyderman laboratory and involves the activation of one G protein-coupled receptor (GPCR) to initiate a signaling process which leads to reduced response of a second (and unrelated) GPCR (Didsbury et al. 1991; Tomhave et al. 1994). The reduced response, or desensitization, of the second GPCR occurs in the absence of the agonist for this target receptor and is distinct from homologous desensitization. Heterologous desensitization has now been reported for a number of GPCRs, and typically involves phosphorylation of the target receptor mediated by second messenger-dependent kinases (Steele et al. 2002). Analysis of the cross-talk between several GPCRs suggests that there is a hierarchy which exists with respect to the ability of a GPCR to induce heterologous desensitization (Ali et al. 1999). Furthermore, the strength of a GPCR to induce cross-desensitization appears to be inversely related to the susceptibility of a target GPCR to be desensitized. Studies on the MOR and DOR have shown that these GPCRs are capable of cross-desensitization of several chemokine receptors, including CCR1, CCR2, CCR5, CXCR1, and CXCR2 (Grimm et al. 1998; Finley et al. 2008). However, these opioid receptors are not capable of cross-desensitizing CXCR4, suggesting that this chemokine receptor is relatively insensitive to heterologous desensitization (Szabo et al. 2003). On the other hand, activation of KOR with U50,488H induces significant cross-desensitization of CXCR4, which indicates that KOR is a strong cross-desensitizer (Finley et al. 2008). Conversely, the activation of CXCR4 is able to cross-desensitize KOR, and this bidirectional heterologous desensitization is apparent at nanomolar doses of the respective receptor agonists.

The heterologous desensitization between GPCRs may involve the internalization of the target receptor, but this is not always the case. For example, the activation of KOR results in the loss of approximately 40% of the CXCR4 from the cell surface, while the activation of CXCR4 does not lead to a significant loss of cell surface KOR (Finley et al. 2008). In general, heterologous desensitization is a process which results in uncoupling of the target receptor from downstream effectors, rather than a loss of receptor activity due to receptor internalization (Steele et al. 2002; Finley et al. 2008; Song et al. 2011). The mechanism for the heterologous desensitization between opioid and chemokine receptors has not been thoroughly investigated, but in almost all cases the desensitization of the target receptor is dependent on receptor phosphorylation (Steele et al. 2002). In contrast to homologous desensitization, cross-desensitization does not involve G protein receptor kinase-mediated target receptor phosphorylation. While the mechanism of the bidirectional

cross-desensitization of KOR and CXCR4 has not been examined, the cross-talk between the MOR and CCR5 is highly dependent on the MOR-induced activation of protein kinase C-ζ (PKCζ). In this case, the activation of the MOR leads to the formation of a complex of PKCζ and phosphorylated CCR5 (Song et al. 2011). KOR activation has been demonstrated to activate PKC (Bohn et al., 2000; Chiu et al., 2017). PKC may be also involved in KOR-induced heterologous desensitization of CXCR4.

Studies have been performed to examine the relevance of chemokine-driven cross-desensitization of opioid receptors in the brain. For example, the activation of CCR2, CCR5, or CXCR4 following administration of the respective chemokine agonists into the periaqueductal gray (PAG) results in a loss of the analgesic response to DAMGO (Szabo et al. 2002). Similar studies have shown that administration of the CXCR4 agonist CXCL12 into the PAG results in a loss of the analgesic response to dynorphin A (1-17) injected into the PAG (Finley et al. 2008). The reduced analgesic activity of KOR in the PAG is apparent even when both dynorphin A (1-17) and CXCL12 are administered simultaneously, and pre-treatment with the CXCR4 antagonist AMD3100 blocks the cross-desensitization of KOR. These results suggest that in situations where there is an elevation of pro-inflammatory chemokines, such as in neuroinflammatory disease states, there would be an opportunity for the cross-desensitization of KOR (along with MOR and DOR) in the brain and an associated increase in pain sensitivity. It is clear that hyperalgesia is an integral part of the stress response to inflammation (Junger and Sorkin 2000). The process of inflammatory chemokine-mediated cross-desensitization of opioid receptors, including KOR, may have implications for systemic inflammatory responses and chronic inflammatory disease states.

5 Summary

In general, KOR mediates a number of immunosuppressive effects that include a reduction in the production of antibodies, suppressed cytokine and chemokine expression, and an inhibition of both chemokine receptor expression and receptor functional activity. The mechanisms responsible for many of these immunomodulatory effects have not been investigated, and this is largely due to the much greater attention that has been given to MOR. This is not surprising given the much greater clinical relevance of MOR. Nevertheless, there is a great deal of potential for translational research for KOR, given the capacity of this opioid receptor to mediate beneficial immunological effects. For example, in certain inflammatory diseases, the reduction in chemokine and/or chemokine receptor expression and functional activity would seem very useful. Moreover, the ability of KOR agonists to inhibit the expression of CXCR4, a critical HIV-1 co-receptor, may have therapeutic potential for infected patients. Hopefully, it will be possible to investigate these various avenues of translational research in the immediate future.

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Conflict of Interest Dr. Rogers declares that he has no conflicts of interest related to the subject matter in this chapter.

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Pleiotropic Effects of Kappa Opioid Receptor-Related Ligands in Non-human Primates

Mei-Chuan Ko and Stephen M. Husbands

Contents

Abstract

The kappa opioid receptor (KOR)-related ligands have been demonstrated in preclinical studies for several therapeutic potentials. This chapter highlights (1) how non-human primates (NHP) studies facilitate the research and development of ligands targeting the KOR, (2) effects of the endogenous opioid peptide, dynorphin A-(1-17), and its analogs in NHP, and (3) pleiotropic effects and therapeutic applications of KOR-related ligands. In particular, synthetic ligands

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targeting the KOR have been extensively studied in NHP in three therapeutic areas, i.e., the treatment for itch, pain, and substance use disorders. As the KORs are widely expressed in the peripheral and central nervous systems, pleiotropic effects of KOR-related ligands, such as discriminative stimulus effects, neuroendocrine effects (e.g., prolactin release and stimulation of hypothalamic-pituitary-adrenal axis), and diuresis, in NHP are discussed. Centrally acting KOR agonists are known to produce adverse effects including dysphoria, hallucination, and sedation. Nonetheless, with strategic advances in medicinal chemistry, three classes of KOR-related agonists, i.e., peripherally restricted KOR agonists, mixed KOR/mu opioid receptor partial agonists, and G protein-biased KOR agonists, warrant additional NHP studies to improve our understanding of their functional efficacy, selectivity, and tolerability. Pharmacological studies in NHP which carry high translational significance will facilitate future development of KOR-based medications.

Keywords

Analgesics · Antipruritics · Drug abuse · Itch · Kappa opioid receptor · Macaque · Mu opioid receptor · Neuroendocrine function · Opioids · Pain · Spinal cord

1 The Dynorphin-Kappa Opioid Receptor System

In 1975, Avram Goldstein and his colleagues isolated and purified an endogenous opioid peptide named dynorphin A, a 17 amino acid polypeptide (Cox et al. 1975; Goldstein et al. 1981; Teschemacher et al. 1975). This peptide was described as "extraordinarily potent" ("dyn" from the Greek, dynamis (power) and "orphin" for endogenous morphine peptide) (Goldstein et al. 1981). Almost two decades later, the cognate receptor for this peptide family, i.e., kappa opioid receptor (*KOR*) (Cox et al. 2015), was cloned by different groups of investigators from rodents and humans (Meng et al. 1993; Yasuda et al. 1993; Zhu et al. 1995). Similar to the mu opioid receptor (*MOR*), the KOR is coupled to pertussis toxin-sensitive Gi/o proteins which inhibit adenylate cyclase and modulate the conductance of voltage-gated calcium channels and inward rectifying potassium channels (Bruchas and Chavkin 2010; Meng et al. 1993; Yasuda et al. 1993).

In the past few years, Positron Emission Tomography (*PET*) radiotracers for the KOR have been developed (Kim et al. 2013; Li et al. 2019). Although these newly developed KOR agonist and antagonist tracers do not have ideal selectivity at KOR over MOR and displayed binding discrepancies (Placzek et al. 2019), radiotracers for PET imaging of the KOR are valuable tools to investigate the functional roles of KOR and endogenous dynorphins in humans under different disease states, such as mood disorders and substance abuse disorders (de Laat et al. 2020). The dynorphin-KOR system has been extensively studied in the past four decades. Several articles have provided a comprehensive overview about the biological actions, medicinal chemistry, pharmacology, and therapeutic applications of this ligand-receptor system (Butelman and Kreek 2015; Chavkin and Koob 2016; Cunningham et al. 2011;

Tejeda and Bonci 2019). Given a similar distribution of the KOR between human and NHP central nervous system (Peckys and Landwehrmeyer 1999; Sim-Selley et al. 1999; Simonin et al. 1995), this review highlights the functional profiles of KOR-related ligands in non-human primates (*NHP*). In particular, we discuss the therapeutic potential of KOR-related ligands based on findings from NHP studies which may facilitate the development of KOR-targeted ligands for different therapeutic applications.

2 Effects of Dynorphins in Non-human Primates

Dynorphins in different chain lengths have been administered through different delivery routes to characterize their functional roles in NHP. Following intravenous administration, dynorphin A-(1-17) decreased food-maintained operant behavior which was not mediated by KOR. Unlike the prototypical KOR agonist U69,593 producing antinociception, systemic dynorphin A-(1-17) produced mild antinociceptive effects (Butelman et al. 1999c). Nonetheless, both dynorphin A-(1-17) and U69,593 increased serum levels of prolactin and such neuroendocrine effects were antagonized by an opioid antagonist quadazocine (Butelman et al. 1999c). These findings suggest that systemic dynorphin A-(1-17) produced both opioid and non-opioid effects in NHP. On the other hand, dynorphin A-(1-17) coadministered with capsaicin into the tail of the monkey produced peripheral antiallodynic effects, which could be blocked by a KOR antagonist (Ko et al. 2000). This early study provides the first functional evidence that activation of peripheral KORs in primates could be a viable therapeutic target for alleviating peripherally elicited pain. Indeed, KORs are present in rodent and human dorsal root ganglion (*DRG*) neurons and dynorphin A-(1-17) suppressed evoked Ca^{2+} transient in human DRG neurons (Ji et al. 1995; Moy et al. 2020; Snyder et al. 2018).

Unlike β-endorphin, intrathecal administration of dynorphin A-(1-17) did not produce antihyperalgesic effects in NHP. Nevertheless, dynorphin A-(1-17) dose-dependently attenuated robust itch scratching responses elicited by intrathecal β-endorphin and gastrin-releasing peptide (Lee and Ko 2015). The inhibitory effect of dynorphin A-(1-17) on centrally elicited scratching supports the notion that patients suffering from chronic itch may have a decreased activity of the endogenous dynorphin-KOR system (Inan and Cowan 2005; Kardon et al. 2014). Importantly, a recent study demonstrates that plasma dynorphin A-(1-17) level inversely correlated with the pruritus severity in patients with chronic liver disease (Moniaga et al. 2019). These findings document a pivotal role of the dynorphin-KOR system in regulating itch sensation.

As noted, the effects of dynorphin A-(1-13) were also studied, but not as extensively as dynorphin A-(1-17). Intravenous administration of dynorphin A-(1-13) produced antinociception which was due to its partial KOR agonist activities (Butelman et al. 1995). Subcutaneous administration of dynorphin A-(1-13) with local capsaicin injection in the NHP tail also produced antiallodynic effects (Ko et al. 1999a). In addition, a synthetic dynorphin A-(1-8) analog, E-2078,

was found to be stable without biotransformation products in human and NHP blood samples (Yu et al. 1997). Subcutaneous or intramuscular administration of E-2078 did not produce antinociception in NHP. Following intravenous administration, E-2078 produced non-KOR-mediated antinociceptive effects (Butelman et al. 1999d). Interestingly, E-2078 produced other KOR-mediated effects, including diuresis, sedation, KOR agonist-like discriminative effects, and increased serum prolactin levels (Butelman et al. 2004, 1999b). Overall, dynorphin A-(1-17) and E-2078 did not produce equivalent antinociceptive effects to non-peptidic KOR agonists, except with local administration peripherally. Thus, although dynorphin A-(1-17) and E-2078 produced other KOR-mediated effects, their functional profiles are not identical to prototypical, synthetic KOR agonists, U50,488 and U69,593, across different outcome measures in NHP. Future studies are warranted to determine whether metabolism (e.g., dynorphin A-(2-17)), site of action, degree of CNS exposure, and other mechanisms (e.g., *N*-methyl-D-aspartate receptor) contribute to non-opioid effects of dynorphin A-(1-17) and E-2078.

Mounting evidence indicates that the dynorphin-KOR system is involved in negative affect derived from pain, drug abuse, and neuropsychiatric disorders (Koob and Volkow 2016; Liu et al. 2019; Massaly et al. 2019; Tejeda and Bonci 2019). It is important to investigate how elevated dynorphin level in the supraspinal regions modulates behavior and mood in humans and NHP. With the advance of surgical techniques, an intrathecal catheter can be implanted and placed in the cisterna magna of NHP for supraspinal drug delivery (Ding et al. 2015). The intracisternal administration of neuropeptides mimics the "volume transmission" of endogenous peptides transported to multiple sites in the brain (Veening et al. 2012). Future NHP studies with intracisternal administration of dynorphin A-(1-17) may improve our understanding of the supraspinal dynorphin-KOR system and the functional efficacy of KOR antagonists for modulating dynorphin-mediated effects in primates under different states.

To our knowledge, synthetic ligands targeting the KOR have been extensively studied in NHP as potential treatments in three therapeutic areas, i.e., for (1) itch (pruritus), (2) pain, and (3) substance use disorders. As the KOR is widely expressed in the peripheral and central nervous systems of humans and NHP (Peckys and Landwehrmeyer 1999; Sim-Selley et al. 1999; Simonin et al. 1995), we also highlight the pleiotropic effects of KOR-related ligands in NHP.

3 Kappa Opioid Receptor Agonists as Antipruritics

3.1 Systemic Effects

The antipruritic effect of KOR agonists was first reported by Alan Cowan in the mid-1980s (Cowan and Gmerek 1986). The KOR seems a prominent therapeutic target for inhibiting itch because a series of rodent studies demonstrate that systemic administration of KOR agonists attenuated pruritogen-elicited scratching behavior (Cowan et al. 2015; Cowan and Ko 2020; Gmerek and Cowan 1984). That this

response was consistent across chemical series confirmed that it was a property of KOR agonists and not compound specific.

One key relevant finding was that scratching behavior was a prominent withdrawal sign in NHP treated chronically with and withdrawn from a selective KOR agonist, U50,488 (Gmerek et al. 1987). Many withdrawal symptoms from opioids appear to be opposite to the acute effects of agonist administration (Ding et al. 2016; Ko et al. 2006; Martin and Eades 1964). Excessive scratching activity observed in NHP during withdrawal from the KOR agonist treatment suggests that acute administration of KOR agonists might have antipruritic effects. The first NHP study seemed to support this notion, as systemic administration of U50,488 dose-dependently prevented or attenuated morphine-induced scratching without inducing sedation (Ko et al. 2003b). Other NHP studies further demonstrated that non-antinociceptive doses of KOR agonists, such as nalfurafine, bremazocine, and GR89,696, attenuated intrathecal morphine-induced scratching without interfering with antinociception, supporting the potential clinical use of KOR agonists as antipruritics in the context of spinal opioid analgesia (Ko and Husbands 2009; Wakasa et al. 2004). Interestingly, systemic butorphanol, an opioid partial agonist, effectively blocked morphine-induced itch while maintaining morphine analgesia, through both MOR and KOR partial agonist actions (Lee et al. 2007). These findings encourage the development of opioid partial agonists with dual actions at both MOR and KOR as analgesics with fewer side effects.

More importantly, these animal studies led to a successful clinical trial of nalfurafine in hemodialysis patients suffering from uremic pruritus (Wikstrom et al. 2005). In 2009, nalfurafine was approved for clinical use as an antipruritic in Japan (Kumagai et al. 2012, 2010). However, antipruritic efficacy of a KOR agonist may be compromised by its narrow therapeutic window following systemic administration, i.e., its therapeutic effect could be associated with supraspinal KOR-mediated adverse effects, such as dysphoria and sedation (Butelman et al. 2001; Ko et al. 1999b; Pfeiffer et al. 1986). Recently, a G protein-biased KOR agonist, triazole 1.1, has been demonstrated to suppress scratching behavior without causing sedation and dysphoria in mice (Brust et al. 2016). Systemic triazole 1.1 at a single dose partially attenuated oxycodone-induced scratching without producing sedation and motor-impairing effects in NHP (Huskinson et al. 2020). It is important to have a side-by-side comparison with nalfurafine over a wide dose range to determine to what degree the therapeutic window of triazole 1.1 is wider than nalfurafine across different KOR-mediated effects in NHP.

Another viable strategy is to develop peripherally acting KOR agonists (Cowan et al. 2015). Although such agonists have not been studied in NHP models of itch, a recently completed phase 3 trial report showed that treatment with intravenous CR845 (difelikefalin), a peripherally restricted KOR agonist, for 12 weeks resulted in a marked and rapid reduction in itch intensity and improved itch-related quality of life in hemodialysis patients with chronic kidney disease-associated pruritus, compared with placebo treatment (Fishbane et al. 2020). These treatment outcomes are very encouraging as there were no adverse events of dysphoria and hallucination reported in the difelikefalin group. It is crucial for NHP experiments to investigate

and compare the functional efficacy and side-effect profiles of nalfurafine, triazole 1.1, and difelikefalin against peripherally versus centrally elicited itch in a broader context as general antipruritics.

3.2 Intrathecal Effects

Given that the spinal delivery of drugs could minimize the degree of supraspinal KOR-mediated adverse effects, intrathecal administration of KOR-related agonists may change the therapeutic window. Recent studies demonstrate that a subpopulation of spinal interneurons expressing dynorphin A tonically inhibits itch in mice (Kardon et al. 2014). However, intrathecal administration of dynorphin A only partially attenuated scratching activities elicited by intrathecal β-endorphin (Lee and Ko 2015), indicating that there are other ligand-receptor systems in the spinal cord regulates MOR-mediated itch. In general, both NHP and human studies support that mixed MOR/KOR partial agonists are effective in ameliorating spinal opioid-induced itch while maintaining spinal opioid-induced analgesia (Ko 2015).

Despite these exciting findings, the functional efficacy, selectivity, and tolerability of KOR-related agonists as spinal antipruritics in NHP remain unknown. MOR antagonists and gastrin-releasing peptide receptor antagonists are selective antipruritics because both classes of drugs are effective in alleviating central MOR- and gastrin-releasing peptide receptor-mediated itch, respectively (Ding et al. 2015; Lee and Ko 2015). If KOR agonists are found to be effective against peripherally and centrally elicited itch in NHP, such pharmacological evidence will facilitate the development of KOR-related ligands as *spinal antipruritics* and may benefit a large population of patients affected by different types of itch.

4 Kappa Opioid Receptor Agonists as Analgesics

4.1 Centrally Acting Kappa Opioid Receptor Agonists

Ample evidence indicates that KOR-related agonists exert antinociceptive and antihypersensitive effects in rodents against a variety of pain modalities (Vanderah 2010; Zöllner and Stein 2007). The first NHP study documents that KOR agonists such as U50,488 produced antinociceptive effects manifested by increased tail-withdrawal latencies to acute noxious stimulus, 50 °C water (Dykstra et al. 1987). However, these antinociceptive doses of KOR agonists also produced stupor and discriminative stimulus effects, indicating that observed antinociception is accompanied by KOR-mediated interoceptive effects such as dysphoria and psychotomimesis (Chavkin and Koob 2016; Clark and Abi-Dargham 2019; Pfeiffer et al. 1986). Subsequently, numerous NHP studies have reported similar findings, i.e., doses of KOR agonists alleviating acute pain also produced sedation, which were higher than doses that produced discriminative stimulus effects (Butelman et al. 2001, 1993; Ko et al. 1998; Negus et al. 2008). Although KOR agonists are

relatively more potent in attenuating capsaicin-induced allodynia and carrageenan-evoked inflammatory pain than acute noxious stimulus (Butelman et al. 2003; Ko et al. 1999a; Sukhtankar et al. 2014), the antiallodynic potency of KOR agonists is similar to their potency in producing discriminative stimulus effects (Butelman et al. 2002; Dykstra et al. 1987). Future studies are warranted to investigate if G protein-biased KOR agonists could display a window between antiallodynic doses and doses eliciting negative interoceptive effects in NHP.

4.2 Peripherally Acting Kappa Opioid Receptor Agonists

The functional efficacy and side-effect profile of peripherally acting KOR agonists have been demonstrated in animal models and in clinical trials (Albert-Vartanian et al. 2016; Little 2013). The most promising peptidic KOR agonist difelikefalin, which is highly hydrophilic, limiting its ability to cross the blood-brain barrier, has shown its analgesic efficacy in hysterectomy and bunionectomy patients without sedation and hallucination. As noted, like any other KOR agonists, difelikefalin increases urine output and prolactin release in humans (Albert-Vartanian et al. 2016). To our knowledge, there is no published NHP study on difelikefalin.

ICI204,448 is the only peripherally acting KOR agonist that has been studied in NHP models. Similar to spiradoline, a centrally acting KOR agonist from the same structural family, ICI204,448 dose-dependently prolonged the food pellet retrieval latency, caused sedation, and increased prolactin levels. These findings suggest that not all the in vivo effects of systemic ICI204,448 are necessarily mediated peripherally in NHP (Butelman et al. 1999b). Nonetheless, it is pivotal to conduct NHP studies to characterize the functional efficacy of peripherally restricted KOR agonists like difelikefalin against different pain modalities and the potential therapeutic window by using well-documented central and peripheral KOR-mediated outcome measures (Butelman et al. 1999b, 2001; Ko et al. 1999b).

5 Kappa Opioid Receptor-Related Ligands for the Treatment of Substance Use Disorders

5.1 Effects of Kappa Opioid Receptor-Related Agonists

Given that KORs are expressed at numerous sites in the reward neurocircuitry and activation of KORs inhibited dopamine release in the nucleus accumbens (Darcq and Kieffer 2018; Spanagel et al. 1992) and medial prefrontal cortex (Tejeda et al. 2013), KOR agonists are expected to attenuate the rewarding and reinforcing effects of abused drugs. Indeed, across different operant schedules of reinforcement, KOR agonists reduced self-administration of drug and non-drug reinforcers in NHP (Cosgrove and Carroll 2002; Negus et al. 1997). However, food-maintained responding was usually decreased at doses that decreased drug self-administration,

indicating lack of selectivity between drugs of abuse and natural rewards (Cosgrove and Carroll 2002; Mello and Negus 1998).

Both MOR and KOR are dynamically involved in drug abuse, dependence, and relapse (Darcq and Kieffer 2018; Karkhanis et al. 2017). Given that MOR and KOR agonists produce opposing effects on dopaminergic neurons (e.g., euphoria versus dysphoria) (Darcq and Kieffer 2018; Freeman et al. 2014; Negus et al. 2008), a viable strategy is to develop compounds with mixed KOR/MOR agonist activities as a treatment option for substance use disorders (Bidlack and Knapp 2013; Greedy et al. 2013). A cyclazocine analog, 8-carboxamidocyclazocine, with mixed KOR/MOR agonist actions, produced only mild sedation, but it did not show improved selectivity for inhibiting cocaine- versus food-maintained responding in NHP (Stevenson et al. 2004). However, another study reported that other mixed KOR/MOR agonists such as MCL-101 produced selective and sustained decreases in cocaine self-administration (Bowen et al. 2003). The efficacies of these mixed KOR/MOR agonists at the MOR relative to buprenorphine are not clear. Nonetheless, it is known that the mild-to-moderate reinforcing effects of buprenorphine, a low-efficacy partial MOR agonist, can be decreased by activating additional receptors such as nociceptin/orphanin FQ peptide receptors (NOR) (Ding et al. 2016). Compounds with mixed NOR/MOR low-efficacy partial agonist activities display improved side-effect profiles and effectively block drug abuse-related effects in NHP (Ding et al. 2018; Flynn et al. 2019; Kiguchi et al. 2019). In a similar approach, future studies using compounds with mixed KOR/MOR *low-efficacy* partial agonist activities in different ratios of efficacy at KOR versus MOR will advance our understanding of the functional role of KOR in modulating reinforcing effects of abused drugs.

Another viable strategy is to develop G protein signaling-biased KOR agonists. Effects of triazole 1.1 (Brust et al. 2016) alone and against the reinforcing effects of abused drugs have not been studied in NHP yet. As both MOR and KOR oppositely modulate dopaminergic neurons (Darcq and Kieffer 2018; Spanagel et al. 1992), it is important to know if G protein-biased MOR and KOR agonists have decreased euphoric and dysphoric effects, respectively. Two reported G protein-biased MOR agonists such as TRV130 and PZM21 produced oxycodone-like reinforcing effects in rodents and NHP (Ding et al. 2020; Zamarripa et al. 2018), indicating that biasing an agonist towards G protein signaling pathways does not change MOR-dopamine receptor-mediated interoceptive effects. As noted, the in vitro assay amplification and the degree of biased agonism may confound how investigators determine which ligands are biased enough (Gillis et al. 2020). Whether such G protein-biased signaling would change KOR-dopamine receptor-mediated interoceptive effects remains to be determined. Intriguingly, low intrinsic efficacy for G protein activation may also contribute to an improved side-effect profile of G protein-biased MOR agonists (Azevedo-Neto et al. 2020; Gillis et al. 2020). It is pivotal to further investigate the similarities and differences between triazole 1.1 and KOR agonists with partial versus full efficacy in NHP.

5.2 Effects of Kappa Opioid Receptor Antagonists

Mounting evidence shows that enhanced KOR signaling during drug dependency and withdrawal may contribute to the anhedonic component of the addiction process, indicating that KOR antagonists may show greater therapeutic effects than agonist-based treatments (Chavkin and Koob 2016; Karkhanis et al. 2017). To our knowledge, there are only two NHP studies conducted to examine the effectiveness of the KOR antagonist, nor-binaltorphimine (norBNI), against drug abuse-related effects. Acute injection of norBNI at a single dose of 3 mg/kg (30 min before the session) reduced ethanol-reinforced responding and ethanol intake (Williams and Woods 1998). However, on the next day, ethanol intake returned to levels similar to those at baseline or after saline pretreatment (Williams and Woods 1998). Given the long duration of KOR antagonism by norBNI extending to several weeks in NHP (Butelman et al. 1998; Ko et al. 1999b), its acute attenuation of ethanol intake may not be mediated via KOR blockade. The other NHP study shows that norBNI did not alter cocaine choice or extended-access cocaine intake (Hutsell et al. 2016). In the past decade, several short-acting selective KOR antagonists have been synthesized and characterized (Carroll and Carlezon Jr. 2013; Guerrero et al. 2019). Currently, there are numerous human studies initiated to investigate the therapeutic potential of KOR antagonists. A recent human study shows that an orally active KOR antagonist, CERC-501 (also called LY-2456302, JNJ-67953964, and aticaprant), did not affect cigarette craving, nicotine withdrawal, and subjective effects of smoking, indicating ineffectiveness of CERC-501 in the treatment of nicotine use disorder (Jones et al. 2020). To date, there are no positive findings from NHP or human studies regarding the functional efficacy of KOR antagonists in the context of substance use disorder-related endpoints. Nonetheless, future studies may explore the functional efficacy of newly developed KOR antagonists in NHP under different states (e.g., withdrawal and relapse) (Gerak et al. 2016; Kiguchi et al. 2020; Ko et al. 2006).

6 Pleiotropic Effects of Kappa Opioid Receptor-Related Ligands

As the KOR is widely expressed in the central and peripheral nervous systems (Ko et al. 2003a; Peckys and Landwehrmeyer 1999; Sim-Selley et al. 1999), it is not surprising that KOR agonists and antagonists produce pleiotropic effects in NHP and humans. Other than the abovementioned antipruritic and analgesic effects and as a potential treatment for substance use disorders, a few KOR-mediated effects in NHP are briefly discussed herein.

I. Discriminative Stimulus Effects Early drug discrimination studies have provided convincing evidence that KOR and MOR possess distinct interoceptive effects (i.e., dysphoric/hallucinogenic versus euphoric subjective effects) in NHP (Dykstra et al. 1987; Herling and Woods 1981; Pfeiffer et al. 1986). Interestingly, in

the drug discrimination assay, NHP trained to discriminate salvinorin A generalized to centrally acting KOR agonists and such effects were mediated by KORs, not serotonergic 5HT2 receptors (Butelman et al. 2010). Salvinorin A is unique pharmacologically and chemically as it represents the first non-nitrogenous, naturally occurring KOR-selective agonist and the only known non-alkaloidal hallucinogen (Roth et al. 2002). Salvinorin A-containing products have been widely used for non-medical purposes and its related analogs in a new scaffold may lead to future development for KOR-based pharmacotherapy (Butelman and Kreek 2015; Roach and Shenvi 2018).

II. Sedation Early NHP studies also find that sedation is a common adverse effect associated with KOR agonists (Dykstra et al. 1987). KOR agonist-induced sedation was mediated by supraspinal KORs, as intracisternal pretreatment with a long-acting KOR antagonist norBNI fully blocked such an effect for more than 4 weeks (Ko et al. 1999b). Centrally acting KOR agonists generally produce more robust sedation than peripherally acting KOR agonists, mixed KOR/MOR partial agonists, and agonists selective for other opioid receptor subtypes (Butelman et al. 1999b; Lee et al. 2007; Podlesnik et al. 2011; Sukhtankar et al. 2014). It will be important to determine and compare the electroencephalographic profiles at analgesic doses derived from different classes of opioid analgesics in NHP and humans (Malver et al. 2014).

III. Neuroendocrine Effects Prolactin release from the anterior pituitary is under tonic inhibition by hypothalamic dopaminergic systems and KOR agonists increase prolactin levels by suppressing these dopaminergic neurons (Durham et al. 1996; Ur et al. 1997). Importantly, NHP studies find that increased serum prolactin level is a sensitive and quantitative neuroendocrine endpoint for the apparent efficacy of KOR agonists (Butelman et al. 1999a). As its site of action may be outside of the blood-brain barrier, prolactin release could be sensitive to the action of peripherally restricted KOR agonists (Butelman et al. 1999b). Indeed, all KOR-targeted agonists (i.e., peptides, centrally penetrating, and peripherally restricted agonists) increased the serum prolactin levels in NHP and humans (Albert-Vartanian et al. 2016; Butelman et al. 2001, 2002, 2004).

KOR agonists are also known to increase adrenocorticotropic hormone (ACTH) and cortisol levels in humans (Ur et al. 1997). A selective KOR agonist, U50,488, dose-dependently stimulates ACTH and cortisol release in both male and female NHP (Pascoe et al. 2008). This study demonstrates only KOR agonists, not MOR or delta opioid receptor agonists, can stimulate the hypothalamic-pituitary-adrenal axis activity. Unexpectedly, a KOR antagonist, norBNI, caused mild-to-moderate increases in ACTH and cortisol with unknown receptor mechanisms in NHP (Williams et al. 2003). As the stimulation of hypothalamic-pituitary-adrenal axis is highly associated with stress-related disorders (Ehlert et al. 2001; Stephens and Wand 2012), future NHP and human studies are warranted to investigate the potential adverse consequences from repeated use of KOR-related ligands including both agonists and antagonists.

IV. Diuresis Human studies have documented the diuretic effects of KOR agonists (Albert-Vartanian et al. 2016; Peters et al. 1987; Reece et al. 1994). Similarly, KOR agonists potently increased the urine output in NHP (Butelman et al. 2001, 1999d; Dykstra et al. 1987). Pretreatment with intracisternal norBNI significantly blocked KOR agonist-induced diuresis in NHP for 20 weeks, indicating central KOR-mediated diuresis (Ko et al. 2003c). Further evidence suggests the sites of KOR-mediated diuresis could be both inside and outside of the blood-brain barrier, as more peripherally restricted KOR agonists also produce diuretic effects (Albert-Vartanian et al. 2016; Butelman et al. 1999d).

V. Other Effects KOR agonists have other therapeutic applications such as cardio-protection, anti-inflammation, neuroprotection, and potential treatment for multiple sclerosis (Beck et al. 2019). In addition, KOR antagonists have been proposed and developed as potential therapeutics for neuropsychiatric disorders such as depression and schizophrenia (Clark and Abi-Dargham 2019; Jacobson et al. 2020; Zhang et al. 2007). Although NHP researchers did not study these listed effects, such effects illustrate a vast diversity of potential therapeutics from KOR-related ligands.

7 Conclusion

Taken together, pharmacological profiles of KOR-related agonists in NHP have shown therapeutic potentials for treating itch, pain, and drug abuse. Figure 1 illustrates the functional profiles of four different classes of KOR-related ligands based on NHP and human studies. NHP models offer the most phylogenetically appropriate evaluation of opioid and non-opioid receptor functions and drug effects (Chen et al. 2013; Lin and Ko 2013; Phillips et al. 2014). We have often seen that exciting findings from rodents cannot be translated to primates. For example, a newly discovered G protein signaling-biased MOR agonist, PZM21, did not exert rewarding effects in mice (Manglik et al. 2016), while others found it to induce

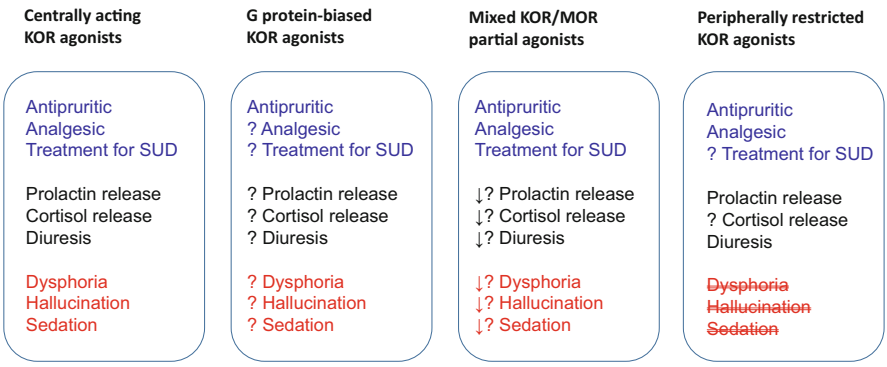

Fig. 1 Simplified scheme to compare functional profiles of kappa opioid receptor-related ligands based on NHP and human studies. Noted, ? to be determined, ↓ decreased effect

respiratory depression and develop tolerance to its analgesic effects (Hill et al. 2018). However, PZM21 produced oxycodone-like reinforcing effects and strength, i.e., the same degree of abuse liability, in NHP (Ding et al. 2020). With recent strategic advances in medicinal chemistry, three classes of KOR-related ligands, i.e., G protein-biased KOR agonists, mixed KOR/MOR partial agonists, and peripherally restricted KOR agonists, warrant additional NHP studies to improve our understanding of their functional efficacy, selectivity, and tolerability. Pharmacological studies in NHP will continue to provide a translational bridge and facilitate future drug development of KOR-based medications.

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Conflict of Interest M.C.K. and S.M.H. declare that there is no conflict of interest.

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Part V

Clinical Studies and Concepts

Clinical Profiles of Nalfurafine Hydrochloride for the Treatment of Pruritus Patients

Yohei Miyamoto, Takanori Oh, Eiji Aihara, and Akihiro Ando

Contents

Abstract

Nalfurafine hydrochloride is a selective kappa-opioid agonist that has antipruritic effects. Here we describe the clinical trials for treatment of uremic pruritus in dialysis patients and on hepatic pruritus in patients with chronic liver disease. Among cytochrome P-450 (CYP) isoforms in humans, CYP3A4 is the major isoform involved in metabolic decyclopropylmethylation of nalfurafine hydrochloride. Nalfurafine hydrochloride was found to be a substrate for P-glycoprotein (P-gp), but had no inhibitory effects on P-gp-mediated transport. The efficacy of oral nalfurafine hydrochloride at 2.5 and 5 μg for refractory pruritus in hemodialysis patients was observed within the first 7 days of treatment, and the effects persisted for the 52-week treatment period. Nalfurafine hydrochloride is also effective in the treatment of conventional refractory pruritus in peritoneal dialysis patients. Moreover, nalfurafine hydrochloride at 2.5 and

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5 μg is effective for the treatment of refractory pruritus in chronic liver disease patients within the first 7 days of drug administration. In all the clinical trials, most adverse drug reactions (ADRs) were mild and resolved quickly and there was no clinical safety problem. Following 52 weeks of treatment, hemodialysis patients did not develop physical or psychological dependence, indicating no addiction risks. In summary, nalfurafine hydrochloride administered orally at doses of 2.5 and 5 μg was safe and effective for treatment of refractory pruritus in patients undergoing hemodialysis or peritoneal dialysis and in chronic liver disease patients.

Keywords

Adverse drug reaction · Antipruritic effect · Chronic liver disease · Confirmatory study · Cytochrome P450 enzyme · Double-blind placebo-controlled design · Hemodialysis · Hepatic pruritus · Kappa opioid receptor · Long-term study · Metabolism · Nalfurafine hydrochloride · Open-label study · Peritoneal dialysis · P-glycoprotein · Uremic pruritus · Visual analogue scale

Abbreviations

ABC	ATP-binding cassette
ABCB1	ATP-binding cassette sub-family B member
ADR	Adverse drug reactions
BBB	Blood–brain barrier
CI	Confidence interval
CYP	Cytochrome P450
de-CPM	17-Decyclopropylmethylated nalfurafine
KOR	κ-Opioid receptor
MDR1	Multiple drug resistance 1
NFA-G	3-Glucuronide of nalfurafine
P-gp	P-glycoprotein
PK	Pharmacokinetics
QOL	Quality of life
SD	Standard deviation
Toray	Toray Industries, Inc.
TSH	Thyroid stimulating hormone
VAS	Visual analogue scale

1 Introduction

This chapter mainly describes the results of clinical trials of nalfurafine hydrochloride (TRK-820), which has antipruritic effects, for treatment of uremic pruritus in dialysis patients (Kumagai et al. 2010, 2012; Nakamoto et al. 2017) and hepatic pruritus in patients with chronic liver disease (Kumada et al. 2017). It acts as a

selective κ-opioid receptor (KOR) agonist (Nagase et al. 1998; Seki et al. 1999; Togashi et al. 2002; Umeuchi et al. 2003), and is the first and currently the only selective KOR agonist approved for clinical use (Nakao et al. 2010). Nalfurafine hydrochloride (Remitch®) is marketed in Japan for the treatment of the above diseases.

2 Cytochrome P450 Enzymes Involved in Metabolism

Kawamura et al. (2004) reported that nalfurafine hydrochloride was metabolized to 17-decyclopropylmethylated nalfurafine (de-CPM) and the 3-glucuronide of nalfurafine (NFA-G) by human hepatocytes (Fig. 1) and these metabolites were detected in human plasma. It is well known that cytochrome P450 (CYP) enzymes are responsible for the oxidative, peroxidative, and reductive metabolism of a variety of compounds, both endogenous and exogenous. The CYP family is associated with more than 90% of oxidative metabolic reactions of xenobiotics (Rendic and Di Carlo 1997). Nalfurafine hydrochloride was metabolized to two major metabolites, de-CPM and NFA-G in human liver microsomes or membrane fractions of *E. coli* expressing human CYP isoforms (Fig. 1, Kawamura et al. 2004). The predominant

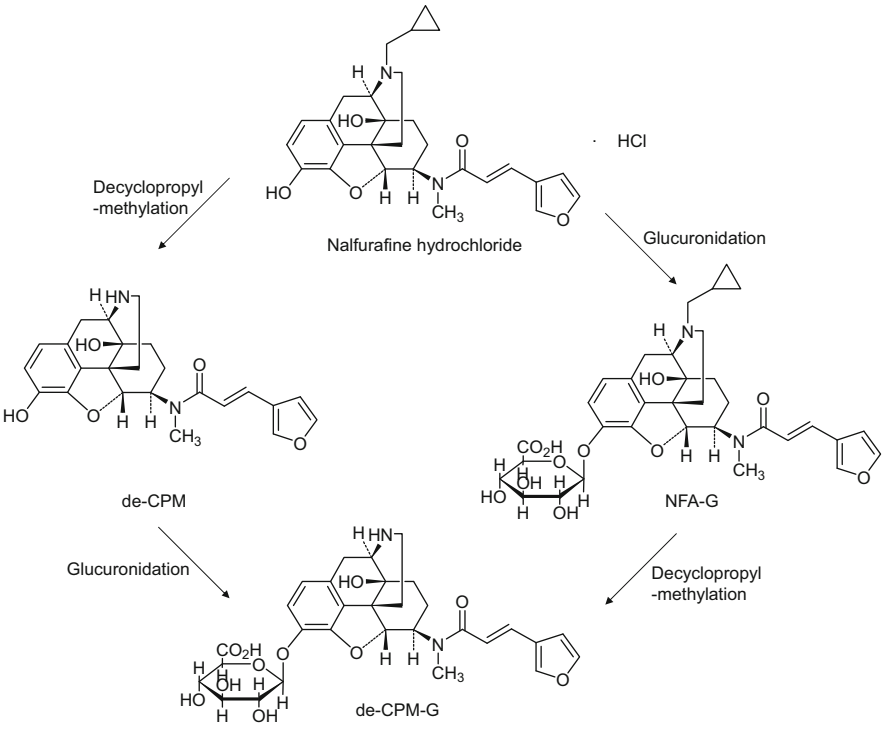

Fig. 1 Possible metabolic pathways for nalfurafine hydrochloride (Ando et al. 2012)

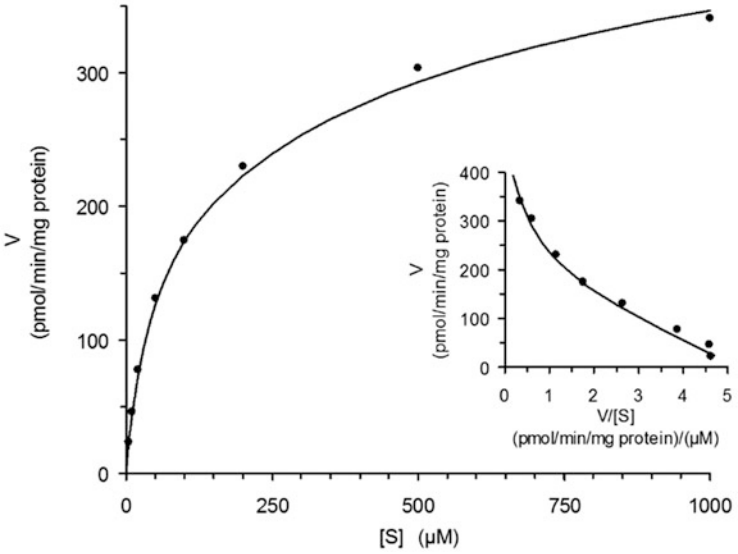

Fig. 2 Kinetics of decyclopropylmethylation of nalfurafine hydrochloride by human liver microsomes (Ando et al. 2012). The plot of velocity versus substrate concentrations is presented. Concentrations of substrates were from 5 to 1,000 μM. Lines represent rates predicted using Michaelis–Menten kinetic parameters derived from nonlinear regression analysis of the decyclopropylmethylation data. The insets show Eadie–Hofstee plots for the same data. Each point represents the mean of triplicate incubation mixtures. *V*, velocity; [S], substrate concentration

Table 1 Kinetic parameters of nalfurafine hydrochloride metabolism to de-CPM by human liver microsomes (Ando et al. 2012)

Kinetic parameter	
K_{m1} (μM)	41.3 ± 3.5
V_{max1} (pmol/min/mg protein)	202 ± 18
K_{m2} (μM)	760 ± 627
V_{max2} (pmol/min/mg protein)	270 ± 124

Data represent the mean ± SD ($n = 3$). K_{m1} and V_{max1}, Michaelis constant and maximum velocity, for the high affinity component; K_{m2} and V_{max2}, Michaelis constant and maximum velocity, for the low affinity component

human hepatic CYP isoforms are involved in the metabolic conversion of nalfurafine hydrochloride to the decyclopropylmethylated form, de-CPM (Ando et al. 2012).

Kinetics of decyclopropylmethylation of nalfurafine hydrochloride were examined using human liver microsomes (Fig. 2, Table 1). Relationships between the velocity of de-CPM formation and substrate concentration showed hyperbolic saturation kinetics. The Eadie–Hofstee plots were biphasic, indicating the involvement of multiple enzymes.

To identify CYP isoforms involved in metabolic decyclopropylmethylation of nalfurafine, ^{3}H-nalfurafine hydrochloride metabolism by membrane fractions of

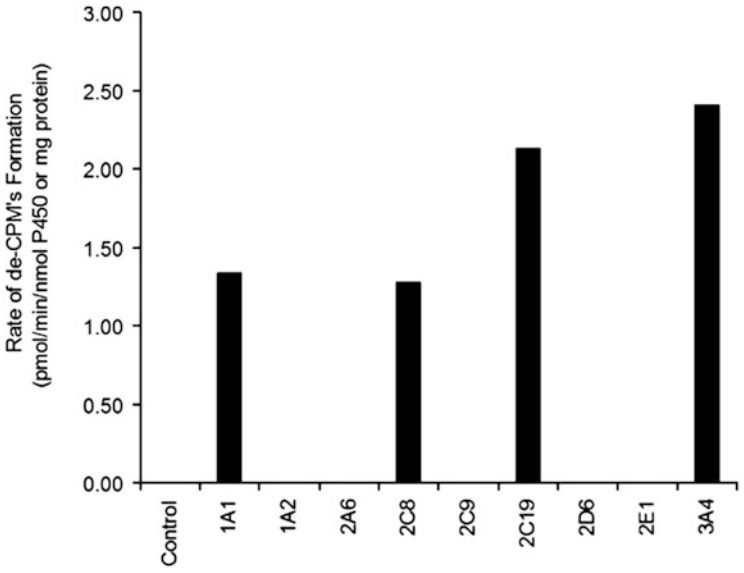

Fig. 3 Rates of de-CPM formation from ^{3}H-nalfurafine hydrochloride after a 30 min reaction with *E. coli* membrane fractions expressing human CYP isoforms (Ando et al. 2012)

E. coli expressing human CYP isoforms was examined (Fig. 3). The results showed that de-CPM was formed with membrane fractions of *E. coli* expressing human CYP1A1, 2C8, 2C19, and 3A4, suggesting involvement of these CYP isoforms in the formation of de-CPM. On the other hand, de-CPM was not formed with any membrane fractions of *E coli* expressing human CYP1A2, 2A6, 2C9, 2D6, and 2E1, or with the control.

Recombinant CYP isoforms expressed in *E. coli* are not present in the natural environment. Human liver microsomes and specific antibodies against CYP isoforms were thus used for assessing the relative importance of each isoform in the overall formation of de-CPM. de-CPM of nalfurafine by human liver microsomes was profoundly inhibited by antibodies against human 3A4 (by ~70%) and moderately by CYP2C8 (by ~25%) and 2C19 (by ~15%) (Fig. 4), but was not inhibited by anti-CYP1A1/1A2, 2A6, 2B6, 2D6, and 2E1 antibodies.

CYP1A1 is expressed only in a limited amount in the human liver (McManus et al. 1990; Shi et al. 2008) and an anti-human CYP1A1/1A2 antibody did not inhibit de-CPM metabolite formation from nalfurafine in the experiments examining the inhibition of nalfurafine metabolism by human liver microsomes. In contrast, anti-human CYP3A4 antibody profoundly, and anti-human CYP2C8 and 2C19 antibodies moderately, inhibited de-CPM formation in the inhibition experiments, respectively.

These results and those described below indicate that CYP3A4 is the major isoform in the formation of de-CPM, whereas CYP2C8 and 2C19 most likely play minor roles.

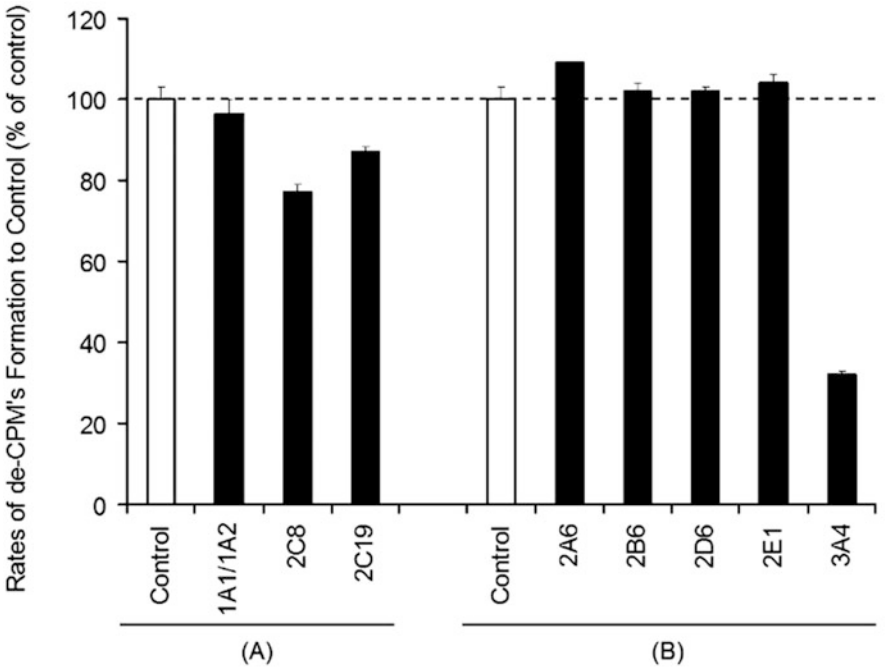

Fig. 4 Rates of de-CPM formation from nalfurafine hydrochloride after a 60-min reaction by human liver microsomes in the presence of anti-human CYP isoform antibodies relative to the control (Ando et al. 2012). Normal rabbit serum (**a**) and 25 mM Tris–HCl buffer (**b**) served as controls, respectively, and were set at 100%. Each bar represents the mean for three incubations and standard deviation of the mean. (**a**) Antibodies against CYP1A1/1A2, 2C8, and 2C19; (**b**) antibodies against CYP2A6, 2B6, 2D6, 2E1, and 3A4

The Eadie–Hofstee plot of nalfurafine decyclopropylmethylation by human liver microsomes was biphasic, suggesting the involvement of multiple enzymes (Fig. 2). This is consistent with the findings that major (CYP3A4) and minor (CYP2C8 and/or 2C19) isoforms are involved in the metabolic decyclopropylmethylation of nalfurafine.

In the metabolism of xenobiotics, CYP3A4 is the most important isoform and is considered to function in the metabolism of approximately 50% of drugs (Wrighton et al. 2000; Guengerich 2006). Therefore, competition between drugs for CYP3A4 can significantly change the plasma concentration and clearance of drugs, increasing the risk of adverse effects. Furthermore, a number of allelic variations in the CYP3A4 gene have been identified (Lamba et al. 2002). Variant CYP3A4 alleles in the population may lead to inter-individual variability in CYP3A4 activity. In the case of drugs that are metabolized by multiple enzymes, however, plasma concentrations will not be changed significantly in poor metabolizers or by the co-administration of other drugs that are catalyzed only by a particular enzyme. As described above, nalfurafine hydrochloride was metabolized by not only CYP3A4,

but also CYP2C8 and 2C19. In addition, nalfurafine is also conjugated with glucuronic acid (Fig. 1). Therefore, the risk of increased side effects in poor metabolizers and of drug–drug or drug–food interactions is unlikely to be high after the administration of nalfurafine hydrochloride either alone or with inhibitors of CYP3A4 and other CYP enzymes.

3 Drug–Drug Interactions Involving P-Glycoprotein

P-glycoprotein (P-gp) is a member of a human ATP-binding cassette (ABC) protein superfamily, a large group of proteins composed of membrane transporters, ion channels, and receptors. P-gp is encoded in humans by the multidrug resistance gene MDR1 (ABC sub-family B membrane 1: ABCB1) (Chan et al. 2004; Eyal et al. 2009). P-gp is ubiquitously expressed in normal tissues, including small intestines, blood–brain barrier (BBB), liver, and kidneys (Chan et al. 2004; Eyal et al. 2009; Tsuji 2002) and has broad substrate specificity, with a tendency towards lipophilic, cationic compounds (Schinkel and Jonker 2003; Smit et al. 1998). P-gp-mediated drug–drug interactions due to alterations in the disposition of drugs have been widely recognized and affect their bioavailability and effectiveness (Eyal et al. 2009; Han 2011). The potential transport and inhibition via P-gp in cell permeation of nalfurafine hydrochloride were investigated using human P-gp-expressing LLC-PK1 cells (Ando et al. 2016).

The cellular transport (cleared volume) of nalfurafine across the monolayers of control and P-gp-expressing cells was evaluated (Fig. 5). ^{3}H-digoxin was used as the positive control of P-gp-mediated transport, and ^{14}C-mannitol was used as the positive control of intercellular flux. Sufficient effluxes of the intercellular transport marker ^{14}C-mannitol were observed in control and P-gp-expressing cells. The cellular transport activity ratio in cells (cleared volume ratio, i.e., the ratio of the cleared volume from basal to apical to that from the apical to basal) is known as a characteristic index of cellular transport. As P-gp is expressed on the apical surface of P-gp-expressing cells, the cleared volume from basal to apical was higher than that from apical to basal, thus the cleared volume ratio of P-gp substrate in P-gp-expressing cells was significantly higher than that in control cells (Kawahara et al. 2000). The cleared volume ratios of ^{3}H-digoxin and nalfurafine hydrochloride in the P-gp-expressing cells were greater than 10.8 and 5.6, respectively, whereas those in the control cells were below 1.8 and 1.0, respectively. Therefore, specific P-gp-mediated transport of ^{3}H-digoxin was observed and a distinct involvement of P-gp in the membrane permeation of nalfurafine hydrochloride suggests that it is a substrate for P-gp.

To evaluate possible inhibition of P-gp-mediated transport by nalfurafine hydrochloride, transport of ^{3}H-digoxin with or without nalfurafine hydrochloride was examined using monolayers of control and P-gp expressing cells with verapamil as a reference inhibitor (Fig. 6). ^{14}C-mannitol was used as an intercellular transport marker. The basal to apical cleared volume of digoxin in P-gp-expressing cells is equal to the apical to basal cleared volume when digoxin transport mediated by P-gp

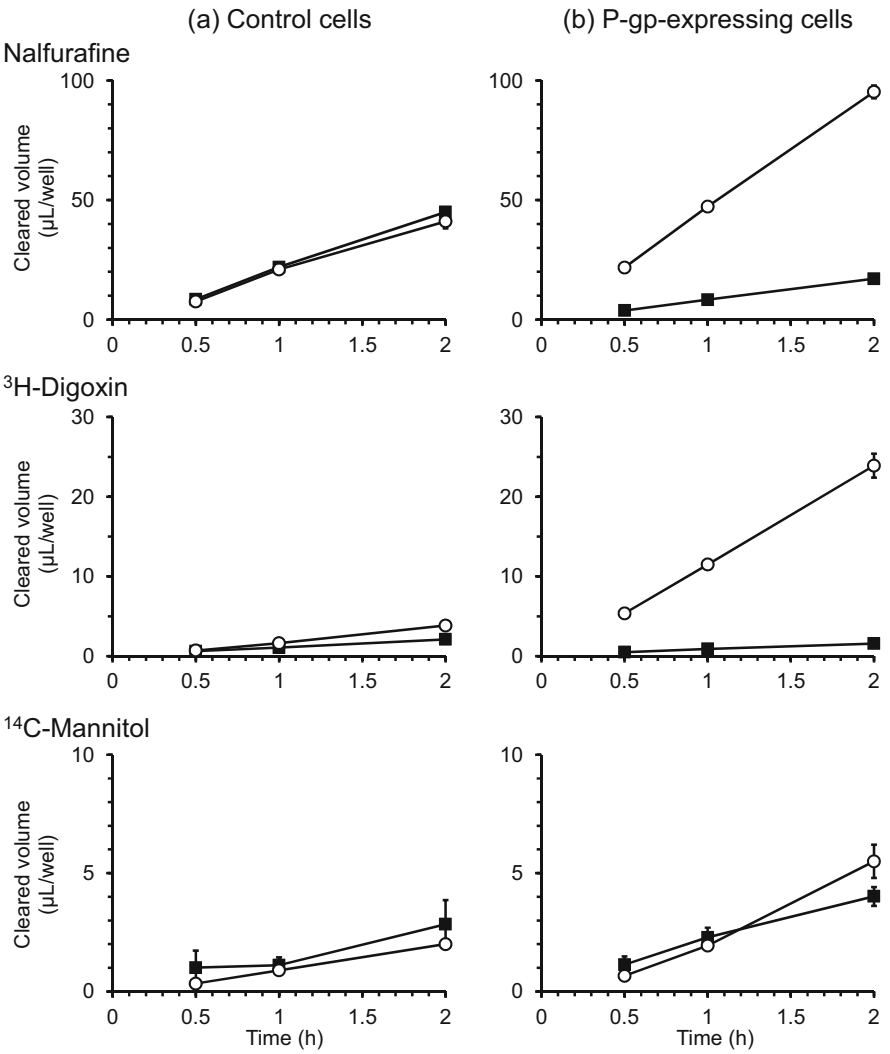

Fig. 5 Cellular transport of nalfurafine hydrochloride, ^{3}H-digoxin, and ^{14}C-mannitol across the monolayers of control (**a**) and P-gp-expressing (**b**) cells (Ando et al. 2016). Each point represents the mean ± SD of three experiments. Data are the cleared volume from apical to basal (filled squares), and the cleared volume from basal to apical (open circles). The permeability of nalfurafine hydrochloride, ^{3}H-digoxin, and ^{14}C-mannitol (cleared volume, lL/well) from apical to basal and basal to apical was calculated by dividing the amount permeated by the initial concentration in the donor compartment

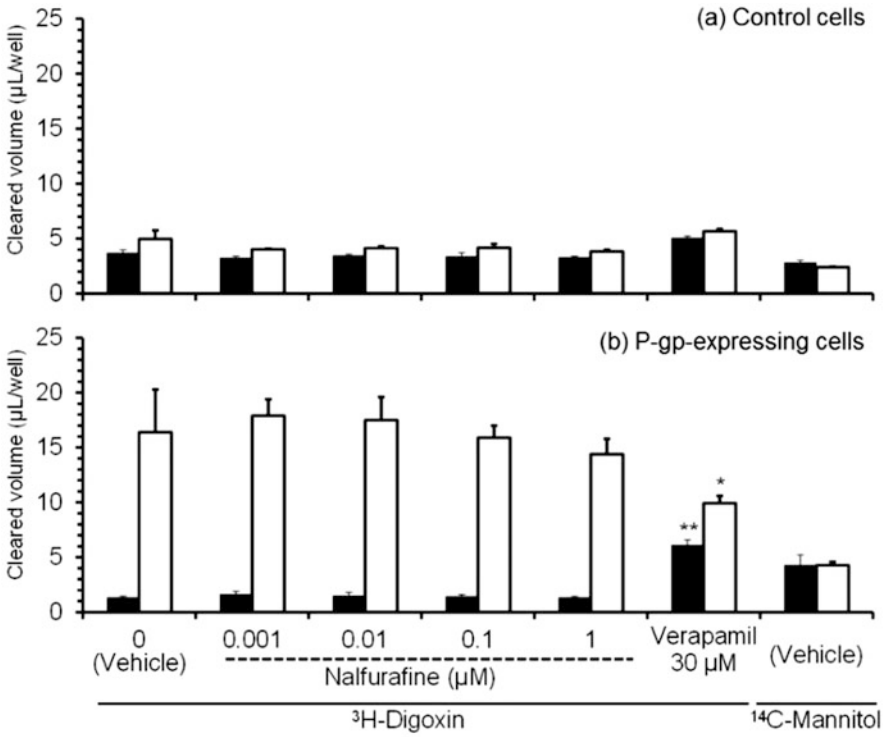

Fig. 6 Effects of nalfurafine on ^{3}H-digoxin transport across the monolayers of control and P-gp-expressing cells (Ando et al. 2016). Each column and bar represents the mean ± SD of three experiments. Closed columns are the cleared volumes from apical to basal, and open columns are the cleared volumes from basal to apical. Control and P-gp-expressing cells were pre-incubated in the presence of vehicle, nalfurafine hydrochloride (0.001, 0.01, 0.1, and 1 μM), or verapamil as a positive control (30 μM), or without either for 1 h. $*P < 0.05$; $**P < 0.01$

is inhibited (Kawahara et al. 2000). No differences between each group were detected in the control cells, but in the P-gp-expressing cells, verapamil (30 μM) inhibited significantly the cleared volumes of ^{3}H-digoxin from the basal to apical side ($P < 0.05$), compared to vehicle. In the presence of verapamil, the cleared volume ratio in the control and P-gp-expressing cells was 1.1 and 1.6, respectively. On the other hand, nalfurafine hydrochloride (0.001–1 μM) did not inhibit the cleared volumes of ^{3}H-digoxin from either the apical to basal side or the basal to apical side compared to vehicle, which indicates that nalfurafine hydrochloride (up to 1 μM) had no inhibitory effects on the P-gp-mediated transport of digoxin.

Based on these results, nalfurafine hydrochloride was found to be a substrate of P-gp, but it had no inhibitory effects on P-gp-mediated transport.

4 Efficacy and Safety in Dialysis Patients with Uremic Pruritus

4.1 Uremic Pruritus in Hemodialysis Patients

4.1.1 Efficacy

The efficacy of nalfurafine hydrochloride (the add-on effects on itching in addition to pre-existing treatment) was prospectively investigated in Japan using a double-blind placebo-controlled design in hemodialysis patients with conventional treatment-resistant pruritus (Kumagai et al. 2010). Patients were randomly treated using nalfurafine hydrochloride (2.5 μg or 5 μg) or placebo once daily for 14 days. The primary endpoint was the change in the visual analogue scale (VAS) of itch during the later 7 days of the treatment period. A total of 337 patients received the investigational drug including placebo capsules in this study: 114 patients (111 patients completed treatment) in the 5 μg group, 112 patients (109 patients completed treatment) in the 2.5 μg group, and 111 patients (109 patients completed treatment) in the placebo group.

Changes in VAS in the second week of treatment were 22 mm in the 5 μg group and 13 mm in the placebo group, respectively, with a significant difference of 9 mm at $P < 0.025$ (Fig. 7). In the 2.5 μg and placebo groups, changes in VAS were 23 mm and 13 mm, respectively, with a significant difference of 10 mm at $P < 0.025$ (Fig. 7). Thus, changes in VAS were significantly greater in the 5 and 2.5 μg groups than in the placebo group. Decreases in VAS during the first 7 days of the treatment period in the both groups were also significantly larger than that in the placebo group (Fig. 7).

These results demonstrate the effectiveness of nalfurafine hydrochloride at 5 and 2.5 μg for treatment-resistant pruritus in hemodialysis patients and show that the antipruritic effects of the drug appear within the first 7 days of treatment.

Regarding the long-term efficacy of nalfurafine hydrochloride, an open-label study (long-term study) was conducted in Japan to examine the effects of 52-week administration at 5 μg/day in 211 hemodialysis patients with treatment-resistant pruritus (Kumagai et al. 2012).

The mean VAS value was 75.2 mm ($n = 211$) [95% confidence interval (CI): 73.5–76.9] during the later 7 days of the pre-observation period, which was significantly decreased to 50.9 mm ($n = 208$) [95% CI: 47.6–54.3] at week 2, decreased to 33.6 mm ($n = 163$) [95% CI: 29.3–37.9] at week 24, and remained there through 52 weeks (Fig. 8).

Shiratori's severity scores (nighttime pruritus scores) were used to assess the impact on the quality of life (QOL), such as sleep disturbance due to pruritus (Shiratori et al. 1983). The changes in Shiratori's severity scores were consistent with those of VAS values. The means of decreases were 0.97 [95% CI: 0.86–1.07], 1.55 [95% CI: 1.40–1.70], and 1.57 [95% CI: 1.41–1.73] at weeks 2, 24, and 52, respectively, demonstrating that nalfurafine hydrochloride helps to improve the QOL of patients.

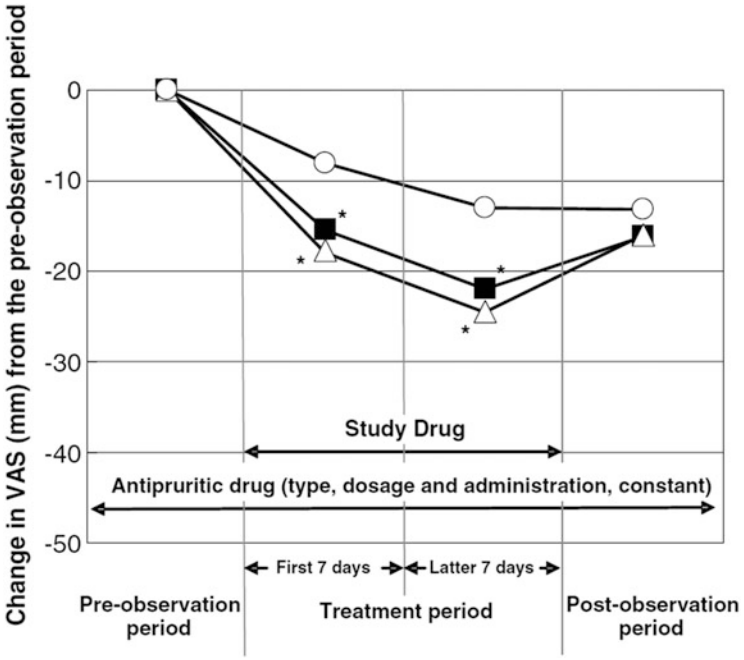

Fig. 7 Changes in VAS values from the pre-observation period. Open circle, placebo group; open triangle, nalfurafine hydrochloride 2.5-μg group; filled square, nalfurafine hydrochloride 5-μg group. All symbols show the mean value of VAS changes. *$P < 0.025$ vs. the placebo group, one-sided analysis of covariance (Kumagai et al. 2010)

These results indicate that nalfurafine hydrochloride remains effective for 52 weeks.

PK parameters in hemodialysis patients after single and repeated oral administration of nalfurafine hydrochloride are summarized in Table 2 (Nakao et al. 2010). After single oral administration of nalfurafine hydrochloride, the T_{max}, C_{max}, $t_{1/2,}$ and $AUC_{0\text{-}\infty}$ values at a dose level of 2.5 μg were 4.25 h, 3.15 pg/mL, 14.21 h, and 66.26 pg·h/mL, respectively and those at a dose level of 5 μg were 3.00 h, 6.51 pg/mL, 14.03 h, and 120.59 pg h/mL, respectively. After repeated oral administration of nalfurafine hydrochloride for 12 days, the T_{max}, C_{max}, $t_{1/2,}$ and $AUC_{0\text{-}\infty}$ values at a dose level of 2.5 μg were 4.14 h, 5.70 pg/mL, 25.33 h, and 210.25 pg h/mL, respectively, and those at a dose level of 5 μg were 3.86 h, 10.25 pg/mL, 28.34 h, and 358.86 pg h/mL, respectively.

4.1.2 Safety

Adverse drug reactions (ADRs) that developed with an incidence rate of ≥3% were insomnia (14.0%), somnolence (3.5%), and constipation (7.0%) in the 5 μg group, and insomnia (7.1%) and somnolence (4.5%) in the 2.5 μg group. On the other hand, no ADR with an incidence of ≥3% was observed in the placebo group.

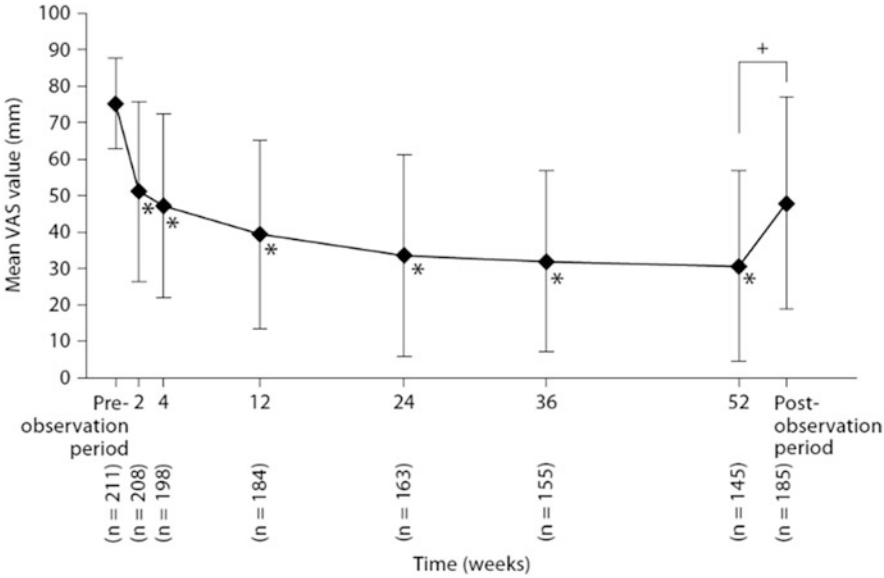

Fig. 8 Effect of long-term use of nalfurafine hydrochloride on average VAS values as indicators of itch severity. The results represent means ± SD. *$P < 0.01$ vs. pre-observation period by paired *t*-test. VAS values in all treatment periods significantly decreased compared to that in the pre-observation period. The post-treatment values were significantly larger than that at week 52 (+$P < 0.01$ by paired *t*-test) (Kumagai et al. 2012)

Table 2 PK parameters of nalfurafine hydrochloride after single and repeated (12 daily) oral administration in hemodialysis patients

	Dose (μg)	C_{max} (pg/mL)	T_{max} (h)	$AUC_{0-\infty}$ (pg h/mL)	$t_{1/2}$ (h)
Single	2.5	3.15 ± 0.82	4.25 ± 1.58	66.26 ± 15.54	14.21 ± 4.93
	5	6.51 ± 2.76	3.00 ± 0.93	120.59 ± 71.09	14.03 ± 7.44
Repeated	2.5	5.70 ± 3.85	4.14 ± 1.35	210.25 ± 144.28	25.33 ± 10.52
	5	10.25 ± 1.74	3.86 ± 1.21	358.86 ± 179.24	28.34 ± 8.55

Data are the mean ± SD. C_{max}, maximum plasma concentration; T_{max}, time to reach the maximum plasma concentration; $AUC_{0-\infty}$, area under the plasma concentration-time curve from zero to infinity; $t_{1/2}$, terminal elimination half-life

In the long-term (52-week) study, ADRs such as insomnia (15.2%) and constipation (3.3%) frequently developed in first 2 weeks. This was similar to the results of the 14-day study. There were no clinically delayed-onset ADRs which required prolonged (24 weeks or longer) treatment.

Insomnia is one of the most frequent ADRs and the attention should be paid when using nalfurafine hydrochloride. However, most cases of insomnia are likely to be mild and readily resolved without problem.

Several abnormalities on clinical examination were reported including transient increased blood prolactin, increased blood thyroid stimulating hormone (TSH), and

decreased blood testosterone, which were mostly mild and resolved without problem. Therefore, they were not considered as clinical safety problems.

Addiction liability was assessed in the long-term study using a questionnaire. The questionnaire data were analyzed by the addiction evaluation committee composed of six medical doctors, who were experts in drug addiction. There was no evidence of psychological or physical dependence, therefore, nalfurafine hydrochloride has no risk of abuse.

In summary, most ADRs of nalfurafine were mild and resolved quickly and no addiction risk was associated with long-term use. Therefore, nalfurafine hydrochloride administered orally at doses of 2.5 and 5 μg was safe in hemodialysis patients with pruritus resistant to other available treatments and there was no clinical safety problem.

4.2 Peritoneal Dialysis Patients with Uremic Pruritus

4.2.1 Efficacy

An open-label study was conducted in Japan to investigate the efficacy of nalfurafine hydrochloride (the add-on effects on itching in addition to the existing treatment) in 37 peritoneal dialysis patients with conventional treatment-resistant pruritus (Nakamoto et al. 2017). Nalfurafine hydrochloride was administered once daily for 4 weeks at 2.5 μg during treatment weeks 1 and 2 and at 5 μg during treatment weeks 3 and 4. Because of the small number of peritoneal dialysis patients in Japan, no placebo control group was included. Instead, the efficacy of nalfurafine hydrochloride for pruritus in these patients was assessed by comparing the VAS changes with those in the preceding 14-day study in which nalfurafine hydrochloride was administered at a dose of 2.5 μg for 2 weeks to hemodialysis patients with pruritus. A threshold was set at 15.24 mm, based on a point estimate of the average VAS change in the placebo group during treatment week 2. Nalfurafine hydrochloride was deemed effective when the lower limit of the 90% CI of the average VAS change in a peritoneal dialysis patient was larger than the threshold.

The mean VAS change in treatment week 2 was 24.93 mm [90% CI: 18.67–31.19] and the lower limit of the CI (18.67 mm) was larger than the threshold (15.24 mm), demonstrating nalfurafine hydrochloride to be effective.

Individual VAS changes in the evaluation period were 16.71 mm [90% CI: 10.11–23.31], 24.02 mm [90% CI: 16.52–31.53], 28.94 mm [90% CI: 21.38–36.50], 32.13 mm [90% CI: 24.08–40.18], and 19.48 mm [90% CI: 14.10–24.85] in treatment weeks 1, 2, 3, and 4 and in follow-up week 1, respectively (Fig. 9). Thus, the efficacy of nalfurafine hydrochloride was maintained during the treatment period.

In addition to VAS changes, Shiratori's severity scores were obtained to assess the impact on the QOL. Shiratori's severity scores revealed that nalfurafine hydrochloride helps improve the QOL of patients.

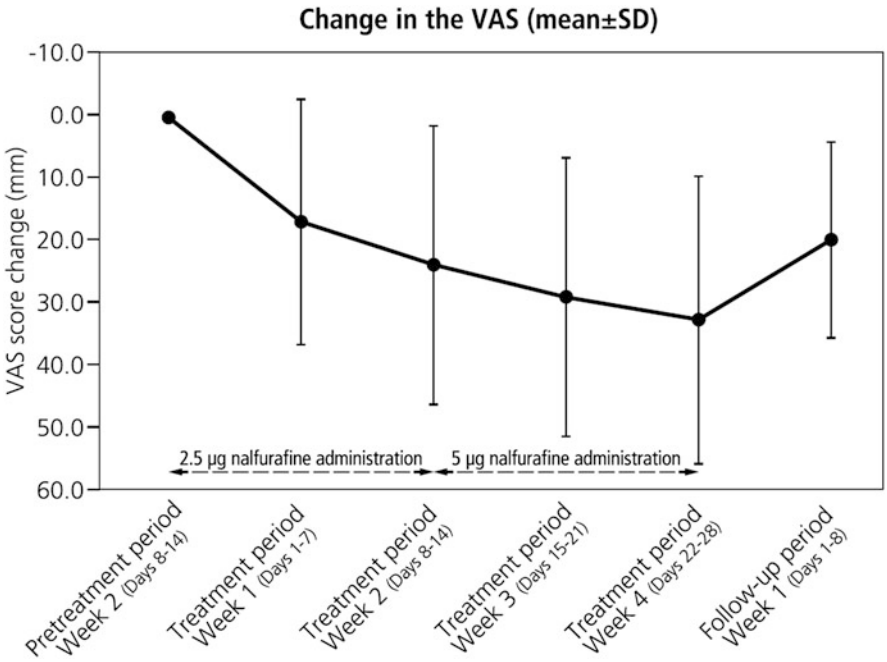

Fig. 9 Changes in VAS values from the pre-observation period. The results represent means ± SD (Nakamoto et al. 2017)

4.2.2 Safety

ADRs in this study were insomnia and increased blood prolactin in 5/37 patients (13.5%), somnolence and decreased blood free testosterone in 3/37 patients (8.1%), and vomiting in 2/37 patients (5.4%). These were mild and resolved without treatment, and were previously reported in the study in hemodialysis patients. Therefore, no special cautionary criteria for peritoneal dialysis patients need to be added to those currently use for hemodialysis patients.

Based on these results, nalfurafine hydrochloride administered at doses 2.5 or 5 μg was safe in peritoneal dialysis patients with treatment-resistant pruritus and there was no clinical safety problem.

5 Efficacy and Safety in Chronic Liver Disease Patients with Hepatic Pruritus

5.1 Hepatic Pruritus in Chronic Liver Disease Patients

5.1.1 Efficacy

The efficacy of nalfurafine hydrochloride at a dose of 2.5 μg or 5 μg was prospectively investigated in Japan using a double-blind placebo-controlled design in chronic liver disease patients with refractory pruritus (Kumada et al. 2017). Patients

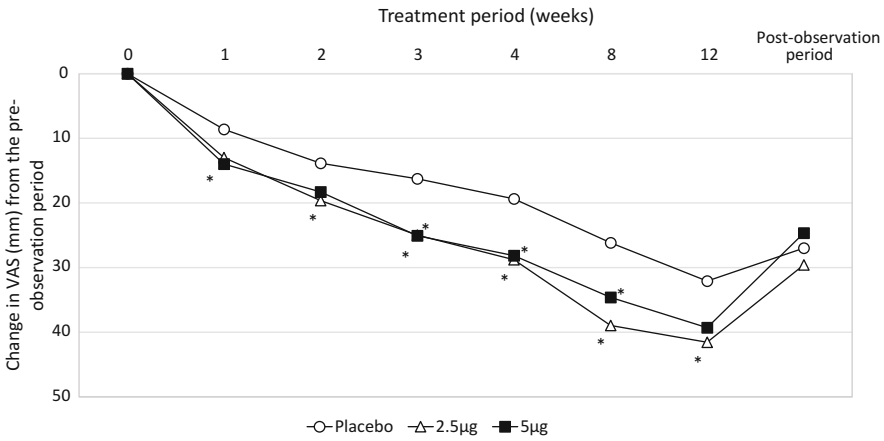

Fig. 10 Changes in VAS values from the pre-observation period. Open circle, placebo; open triangle, nalfurafine 2.5 μg; filled square, nalfurafine 5 μg. All symbols show the mean value of VAS changes. *$P < 0.025$ vs. the placebo group, one-sided analysis of covariance

were randomly assigned to receive 2.5 or 5 μg of nalfurafine hydrochloride or placebo orally once daily after the evening meal for 12 weeks. The primary endpoint was the change in VAS during treatment week 4. Kawashima's criteria of pruritus severity (Kawashima et al. 2003) were also used as a secondary endpoint. A total of 317 patients were enrolled in this study: 109 in the 5 μg group, 105 in the 2.5 μg group, and 103 in the placebo group.

During the treatment week 4, changes in VAS as the primary endpoint were 27.46 mm, 28.56 mm, and 19.25 mm in the 5 μg, 2.5 μg and placebo groups, respectively, with a significant difference from the placebo group of 8.22 mm at $P < 0.025$ for the 5 μg group and 9.31 mm at $P < 0.025$ for the 2.5 μg group, respectively (Fig. 10). Changes in VAS were significantly greater in the 5 and 2.5 μg groups than in the placebo group. Similar changes in the secondary endpoint, Kawashima's criteria of pruritus severity, during treatment week 4 were noted. The average changes in VAS increased during treatment week 12 and was consistently greater in nalfurafine hydrochloride groups than in the placebo group (Fig. 10).

These results indicate that nalfurafine hydrochloride at 2.5 and 5 μg is effective for the treatment of refractory pruritus in chronic liver disease patients and reveal that the antipruritic effects of the drug appear within the first 7 days of treatment.

5.1.2 Safety

The incidences of ADRs were 51.5%, 60.0%, and 54.1% in the placebo, 2.5 μg, and 5 μg groups, respectively.

Pollakiuria including nocturia, somnolence, insomnia, and constipation were ADRs observed in ≥5% patients in the nalfurafine hydrochloride groups, and they developed more frequently in the nalfurafine hydrochloride groups than in the

placebo group. Most of these ADRs were mild. Regarding the incidence rates of these ADRs, pollakiuria developed in 1.0%, 5.7%, 7.3%, somnolence developed in 1.0%, 5.7%, and 7.3%, insomnia developed in 2.9%, 5.7%, and 4.6%, and constipation developed in 1.9%, 3.8%, and 7.3% in the placebo, 2.5 μg, and 5 μg groups, respectively.

The most frequent abnormality on clinical examination was increased blood prolactin with an incidence rate of 8.7%, 13.3%, and 7.3% in the placebo, 2.5 μg, and 5 μg groups, respectively. However, increases in all groups were mild.

Most ADRs were mild and resolved quickly. Thus, there is no clinical safety problem when nalfurafine hydrochloride was administered orally at doses of 2.5 or 5 μg to chronic liver disease patients with refractory pruritus.

6 Perspectives

In Korea, an orphan drug application for the treatment of uremic pruritus in hemodialysis patients was approved in June 2013, and the drug is currently marketed.

In China, a phase I study to investigate the pharmacokinetics and safety of an oral formulation in healthy volunteers is currently planned.

In Europe, a marketing authorization application of an injectable formulation was submitted to the European Medicines Agency in June 2012. However, the Committee for Medicinal Products for Human Use decided that it was not sufficiently effective as compared with placebo and had a negative risk-benefit balance, and the application was withdrawn. Currently an oral formulation is under development, and a phase I study to investigate PK, safety, and anti-pruritus effects in hemodialysis patients with or without uremic pruritus was completed in 2017.

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Kappa Opioid Receptor Antagonists as Potential Therapeutics for Mood and Substance Use Disorders

Brian Reed, Eduardo R. Butelman, and Mary Jeanne Kreek

Contents

Abstract

The kappa opioid receptor (KOR) and its primary cognate ligands, the dynorphin peptides, are involved in diverse physiological processes. Disruptions to the KOR/dynorphin system have been found to likely play a role in multiple neuropsychological disorders, and hence KOR has emerged as a potential therapeutic target. Targeting KOR is complicated by close homology to the mu and delta opioid receptors (MOR and DOR), and many KOR ligands have at least moderate affinity to MOR and/or DOR. Animal models utilizing primarily very long-lasting selective KOR antagonists (>3 weeks following a single dose) have

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demonstrated that KOR antagonism attenuates certain anxiety-like and depression-like behaviors and blocks stress- and cue-induced reinstatement to drug seeking. Recently, relatively selective KOR antagonists with medication-like pharmacokinetic and pharmacodynamic properties and durations of action have been developed. One of these, JNJ-67953964 (also referred to as CERC-501, LY2456302, OpraKappa or Aticaprant) has been studied in humans, and shown to be safe, relatively KOR selective, and able to substantially attenuate binding of a KOR PET tracer to CNS localized KOR for greater than 24 h. While animal studies have indicated that compounds of this structural class are capable of normalizing withdrawal signs in animal models of cocaine and alcohol dependence and reducing cocaine and alcohol intake/seeking, additional studies are needed to determine the value of these second generation KOR antagonists in treating mood disorders and substance use disorders in humans.

Keywords

Cocaine · Depression · Opioids · Pharmacodynamics · Pharmacokinetics

Abbreviations

5′AMN	5′(2-aminomethyl) naltrindole
5′GNTI	5′-guanidinonaltrindole
5′MABN	N-((Naltrindol-5-yl)methyl)pentanimidamide
ANTI	5′acetamidinoethylnaltrindole
AZ-MTAB	3-((1R,3r,5S)-8-((5-MethylThiophen-2-yl)Methyl)-8- Azabicyclo [3.2.1]octan-3-yloxy)Benzamide
DOR	Delta opioid receptor
i.p.	Intraperitoneal
KOR	Kappa opioid receptor
MOR	Mu opioid receptor
norBNI	Norbinaltorphimine
ROS	Reactive oxygen species
TENA	$(\text{Triethylenedioxynaltrexamine})_2$
THC	Δ-9-tetrahydrocannabinol

1 Introduction

As extensively documented in the current volume, the kappa opioid receptor (KOR), a component of the endogenous opioid system, is involved in modulation of diverse neurophysiological pathways. We hypothesized the KOR system to be a putative target for the development of medications to treat psychostimulant addictions as early as 2002 (Kreek et al. 2002; Butelman et al. 2012). More recently KOR has been hypothesized as a potential therapeutic target for pain (Martínez and Abalo 2020), pruritus (Erickson et al. 2020), mood disorders (Jacobson et al. 2020), and diverse

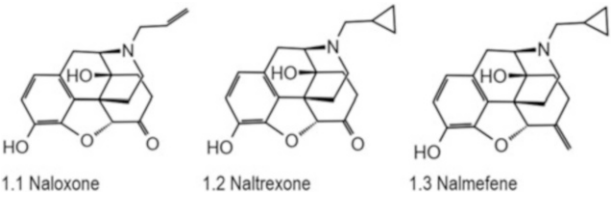


Fig. 1 Morphinan-based MOR antagonists, also with binding to KOR

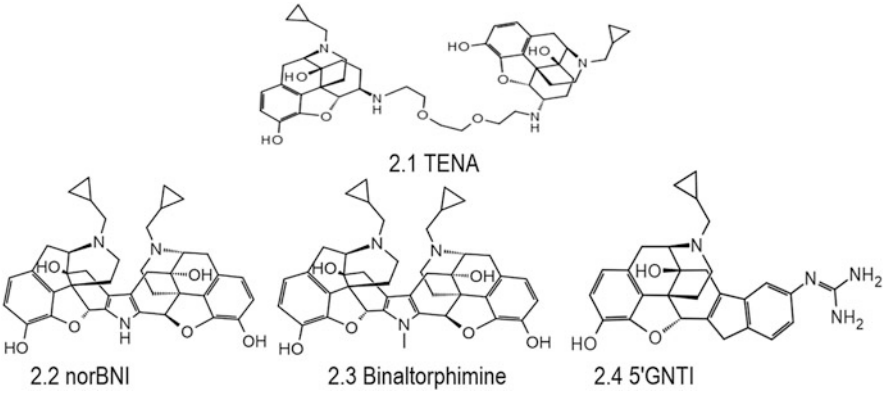


Fig. 2 Morphinan-based selective KOR antagonists

other conditions (Ved and Doshi 2020; Ellingson and Vanderah 2020). Specifically, we review here the development of selective KOR antagonists over the last few decades, and the current prospects of KOR antagonists to be developed into therapeutic agents.

The earliest antagonists of the endogenous opioid receptors were synthesized and developed prior to the full pharmacological and later molecular biological determination of multiple classes of opioid receptors. These anatagonists, such as naloxone or naltrexone were found to be relatively nonselective amongst the opioid receptors (Fig. 1, 1.1) and naltrexone (Fig. 1, 1.2) (Martin 1983). In fact, naltrexone and nalmefene (Fig. 1, 1.3) were later shown to have partial KOR agonist activity (Butelman et al. 2020; Bart et al. 2005; Dunn et al. 2019; Stahl et al. 2015). Earlier developments in the quest for a selective KOR antagonist have been previously reviewed (Metcalf and Coop 2005; Urbano et al. 2014; Carroll and Carlezon 2013; Carlezon and Krystal 2016). Exploration of naltrexone derivatives led to the discovery of the first modestly selective KOR antagonist, a dimer of the triethylenedioxy derivative of naltrexamine (TENA, Fig. 2, 2.1) (Portoghese and Takemori 1985).

2 Long-Duration KOR Antagonists

2.1 Nor-Binaltorphimine

The next reported selective antagonist of KOR versus MOR and DOR was a combination of two naltrexone moieties connected via a pyrroline ring, nor-binaltorphimine (norBNI, Fig. 2, 2.2) and binaltorphimine (Fig. 2, 2.3) by the laboratory of Portoghese (Portoghese et al. 1987). Binaltorphimine actually exhibits greater potency and selectivity for KOR in comparison with norBNI (Portoghese et al. 1988a). Interestingly, early studies of the norBNI pharmacophore has shown that only one naltrexone pharmacophore was responsible for the activity and the compound with one naltrexone pharmacophore had greater potency as an antagonist, with diminished KOR selectivity (Portoghese et al. 1988b; Larson et al. 2000). Also of interest, norBNI was found to exhibit greater KOR selectivity in vivo than in vitro (Takemori et al. 1988). In contrast to MOR antagonists as well as KOR agonists, central administration of norBNI did not result in conditioned place aversion behavior (Bals-Kubik et al. 1989). From a structural standpoint, the spacing between the two naltrexone-like moieties in norBNI seems responsible at least in part for the KOR selectivity (Portoghese et al. 1991).

NorBNI emerged as the prototypic KOR antagonist for in vivo studies of KOR function and activity, despite the somewhat enhanced potency and selectivity for KOR of binaltorphimine (Portoghese et al. 1987). Animal studies clearly demonstrated that norBNI is capable of selectively inhibiting the analgesic effect of exogenous KOR agonists in animal pain behavior assays (Takemori et al. 1988). Pharmacological selectivity studies in vivo in rodents indicated that norBNI actually has a slow onset of KOR antagonism, with full antagonism of KOR-mediated analgesia not apparent for 1–8 h following norBNI administration (Endoh et al. 1992; Horan et al. 1992). Further, at earlier time-points norBNI results in antagonism of MOR-mediated analgesia (Endoh et al. 1992; Horan et al. 1992). Thus, typically for effective selective KOR antagonism in vivo, norBNI is administered 18–24 h prior to beginning KOR-mediated behavioral or physiological measurements.

NorBNI was also shown to produce antidepressant-like effects in the forced swim test in rodents (Mague et al. 2003; Reed et al. 2012). Similarly, norBNI prevented non-contingent shock-induced learning of adaptive behaviors, in a model interpreted as reflecting learned helplessness (Joynes and Grau 2004). Sex differences in the effect of norBNI on forced swim test behavior were noted in mice, with norBNI reducing immobility in male, but not female, C57BL/6 and California mice (Laman-Maharg et al. 2018). Studies in rodents with norBNI also indicated that the KOR antagonism may result in decreased feeding behaviors (Levine et al. 1990, 1994; Cole et al. 1997; Bodnar et al. 1995; Leventhal et al. 1995; Khaimova et al. 2004; Kotz et al. 1993; Calcagnetti et al. 1990).

In investigations of the role of KOR in the models of drug use disorders, norBNI augmented sensitization to morphine locomotor activity and increases in extracellular striatal dopamine in rodent models (Spanagel and Shippenberg 1993). NorBNI was also shown to reverse the attenuating effect of inflammatory pain on morphine

conditioned placed preference (Narita et al. 2005). NorBNI dose-dependently attenuated Δ-9-tetrahydrocannabinol (THC)-induced conditioned place aversion in mice (Cheng et al. 2004), consistent with the kappa-dynorphin modulation of the aversive effects of THC in rodents (Zimmer et al. 2001; Ghozland et al. 2002). NorBNI was also shown to reduce the social avoidance and excessive grooming effects of withdrawal following induction of heroin dependence in male C57BL/6 J mice (Lalanne et al. 2017).

Remarkably, the ability of norBNI to block the antinociceptive activity of KOR agonists such as U69,593 was observed to be extremely long-lasting, up to 8–20 weeks following a single dose in rodents or non-human primates (Horan et al. 1992; Butelman et al. 1993; Ko et al. 2003; Picker et al. 1996; Jewett and Woods 1995). This was later found to also be the case for some other KOR selective antagonists such as JDTic (see below) (Munro et al. 2012).

The mechanisms underlying this extremely persistent antagonism of most KOR activity by norBNI may involve both pharmacokinetic and signaling properties. Utilizing high sensitivity mass spectrometric techniques, it was shown in separate studies that norBNI can be detected in the brain at least 21 days following acute injection in male mice (Patkar et al. 2013; Kishioka et al. 2013). Other studies suggest that an intracellular signaling event triggered by binding of antagonists to KOR, resulting in transient phosphorylation of c-Jun N-terminal kinases (JNK), correlates with the persistence of the antagonism (Melief et al. 2011; Bruchas et al. 2007). Downstream of phosphorylated active JNK, peroxiredoxin 6-mediated reactive oxygen species (ROS) and de-palmitoylation of $G_{\alpha i}$ is thought to reduce KOR signaling long-term and re-accumulation via fresh KOR protein synthesis is required to recover the receptor activity (Schattauer et al. 2017, 2019). Questions regarding the relevance of the JNK-ROS intracellular signaling pathway to in vivo duration as well as specificity of $G_{\alpha i}$ de-palmitoylation resulting in KOR versus other GPCR inactivation remain to be addressed.

2.2 5′-Guanidinonaltrindole

Subsequently another KOR antagonist was also developed by the group of Portoghese, with guanidine substitution of the indole moiety of the DOR antagonist naltrindole. This yields a novel KOR antagonist, 5′-guanidinonaltrindole (5′-GNTI, Fig. 2, 2.4), with enhanced potency and KOR selectivity in in vitro assays (Stevens et al. 2000). The peculiar slow onset and extended duration (several weeks) of activity of norBNI were also observed with 5′-GNTI (Munro et al. 2012).

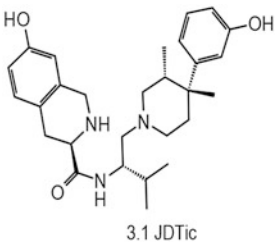
Whereas 5′-GNTI has limited BBB penetrability (Munro et al. 2012), another KOR antagonist from this series, 5′acetamidinoethylnaltrindole (ANTI), was shown to exhibit centrally mediated effects following systemic administration (Todtenkopf et al. 2004). Subsequent efforts by the group of Husbands also used the naltrindole scaffold to develop novel KOR antagonists. The developed compounds, 5′ (2-aminomethyl) naltrindole (5′AMN), and N-((Naltrindol-5-yl)methyl)

pentanimidamide (5′MABN), had similar extended duration of activity to that of norBNI and 5′-GNTI in vivo (Casal-Dominguez et al. 2012).

2.3 JDTic

Up until the early 2000s, all KOR antagonists had included the morphinan scaffold structure. In 2001, a non-morphinan specific KOR antagonist, the trans-(3R,4R)-dimethyl-(3-hydroxyphenyl)piperidine derivative JDTic (Fig. 3, 3.1), with enhanced potency in binding KOR compared to norBNI, was developed by the group of Carroll (Thomas et al. 2001). The duration of action of JDTic was found to be similar to those of norBNI and 5′-GNTI, with multi-week blockade of KOR activity following systemic administration of a single dose (Munro et al. 2012). An extensive array of JDTic analogs have since been generated, some of which may have preferable pharmacokinetic/pharmacodynamics/signaling properties which may result in these compounds having more drug-like qualities (Ondachi et al. 2018; Kormos et al. 2017). In an extinction-reinstatement model of relapse following acquisition of cocaine self-administration, JDTic blocked stress-induced reinstatement of responding (Beardsley et al. 2005). This is consistent with an interpretation of elevated dynorphin tone subsequent to extended cocaine exposure, the stress-induced release of which leads directly to cocaine-responsive behaviors.

A clinical trial of JDTic was initiated for determination of its safety profile in healthy volunteers (Buda et al. 2015). The long-acting nature of JDTic in inhibiting endogenous KOR activity for up to several weeks is an unusual property for a medication candidate. Initial investigation of such an extended duration compound requires special attention to safety. In the case of JDTic, the trial was terminated after modest cardiac abnormalities developed in a small subset of patients in response to JDTic (Buda et al. 2015).



3.1 JDTic

Fig. 3 Long-acting selective non-morphinan containing KOR antagonist, JDTic

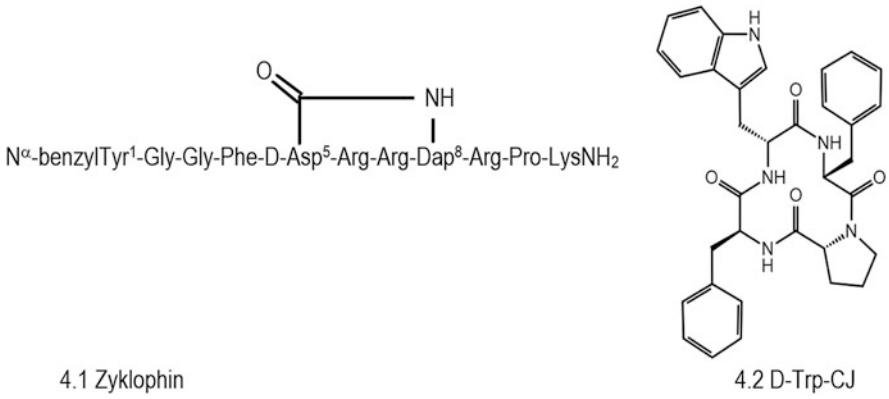


Fig. 4 Short-duration peptidic selective KOR antagonists

3 Short Duration KOR Antagonists

3.1 Peptidic Antagonists

A peptidic selective KOR antagonist, zyklophin (Fig. 4, 4.1), a protected cyclic dynorphin A(1–11) analog, was developed by the group of Aldrich (Patkar et al. 2005). In contrast to prior selective KOR antagonists, zyklophin exhibited rapid onset of antagonism in vivo, and duration of action of 12–24 h (Aldrich et al. 2009).

A separate cyclic peptide, isolated as a natural product from the fermentation broth of a fungus, *Ctenomyces serratus* ATCC15502, CJ-15,208, was found to have relatively KOR selective antagonism in rabbit vas deferens bioassay (Saito et al. 2002). Subsequent investigation of isolated stereoisomers of CJ-15,208 showed D-Trp-CJ-15,208 (Fig. 4, 4.2), to be a relatively short-acting kappa antagonist (Ross et al. 2012). Activity of D-Trp-CJ-15,208 is limited to less than 18 h following administration (Eans et al. 2013). This compound was also found to be orally active, and to prevent both stress-primed and cocaine-primed reinstatement of conditioned place preference in C57BL/6 mice (Aldrich et al. 2013).

3.2 PF-4455242

A different non-morphinan selective KOR antagonist, PF-4455242 (Fig. 5, 5.1), containing a pyrrolidinylsulfonylbiphenyl backbone based on initial identification via a high-throughput screen, was developed by Pfizer (Verhoest et al. 2011). This compound was moderately selective for KOR in vitro (21-fold KOR/MOR in binding assays) and had a narrow window for KOR versus MOR selectivity in vivo. Thus PF-4455242 inhibited U50488-induced analgesia at a 3.2 mg/kg pretreatment dose with no significant inhibition of morphine analgesia, but with significant inhibition of morphine analgesia at 10 mg/kg systemic dose (Grimwood

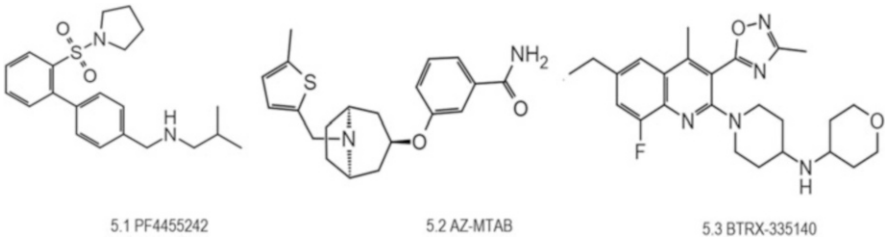


Fig. 5 Diverse short-duration small molecule selective KOR antagonists

et al. 2011). Of interest, this compound was also shown to have a limited duration of action, with KOR inhibition by 10 mg/kg sharply declined within 24 h, in sharp contrast to prior KOR antagonists norBNI and JDTic (Melief et al. 2011). A study in humans demonstrated that 18 or 30 mg PF-4455242 was capable of inhibiting the kappa agonist spiradoline from inducing prolactin release, a biomarker of kappa agonist-induced effects (Chang et al. 2011). In PET imaging of KOR with [^{11}C] GR103545 as a radiotracer, 30 mg PF-4455242 delivered orally in humans led to less than 50% occupancy of brain KOR at 1.5 h, substantially less than observed with oral naltrexone (Naganawa et al. 2014). Clinical trials of this compound were terminated soon thereafter and this compound is not currently under evaluation as a candidate medication.

3.3 AZ-MTAB

An effort was also made by AstraZeneca to develop a KOR antagonist. Initial discovery of KOR selective bis-amide alkoxypiperidines via high-throughput screening led to the identification of 3-((1R,3R,5S)-8-((5-MethylThiophen-2-yl) Methyl)-8- Azabicyclo [3.2.1]octan-3-yloxy)Benzamide (AZ-MTAB, Fig. 5, 5.2) as a potential KOR selective compound with typical medication-like properties (Brugel et al. 2010). In vivo, AZ-MTAB showed substantially shorter duration of action compared to norBNI, but in a head-to-head comparison with LY2456302 (see below), it was substantially less potent (~25 fold) (Peters et al. 2011).

3.4 LY2456302: Animal Studies

Around the same time as the development of PF4455242 and AZ-MTAB, another relatively short-acting non-morphinan KOR antagonist was developed by Eli Lilly, LY2456302 (Fig. 6, 6.1) (Rorick-Kehn et al. 2014b). LY2456302 has also been referred to as Opra Kappa (Reed et al. 2018), CERC-501 (Jones et al. 2020), and currently JNJ-67953964, the licensing having since been transferred to Janssen Pharmaceuticals (Krystal et al. 2020). The initial studies in rats indicated that LY2456302 had a half-life of 2–4 h, greater than 25-fold selectivity for KOR versus MOR or DOR, and close to 100% occupancy of in vivo KOR receptors in rats and

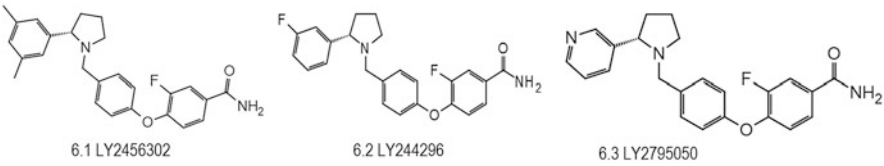


Fig. 6 Small molecule short-duration selective KOR antagonists originally developed by Eli Lilly, with a common pyrrolidine-methylene-phenoxy-benzamide backbone

mice for up to 8 h following administration of 10 mg/kg. Studies in rhesus monkeys showed that LY2456302 dose-dependently blocked binding of the PET radiotracer [^{11}C]LY2795050, a selective KOR antagonist, in multiple brain regions (Zheng et al. 2013).

Similar to longer-acting KOR antagonists, LY2456302 was shown to reduce immobility in the rodent forced swim stress test, and low sub-threshold doses of this compound were synergistic with the tricyclic antidepressant imipramine in reducing immobility (Rorick-Kehn et al. 2014b).

LY2456302 was effective in reducing the intake of alcohol in an ethanol-preferring rat strain, with no significant tolerance to this effect over the course of 4 days (Rorick-Kehn et al. 2014b). This is in contrast to the tolerance which quickly developed to the ethanol-intake reducing effects of naltrexone, a potent MOR antagonist with G-protein biased KOR partial agonist activity (Rorick-Kehn et al. 2014b; Dunn et al. 2019).

The effect of LY2456302 on alcohol intake and withdrawal in rodent models was further explored in a subsequent study in Wistar rats (Domi et al. 2018). This study demonstrated that the KOR antagonist blocked alcohol withdrawal-induced anxiety-like behavior in elevated plus maze models as well as alcohol withdrawal-induced prolactin increase, consistent with elevated dynorphin/KOR tone during alcohol withdrawal. LY2456302 blocked stress-induced, but not cue-induced, reinstatement of alcohol seeking. Finally, this compound reduced alcohol drinking in the subgroup of animals that escalated their intake of alcohol over the several-week study, normalizing the intake levels to that of the subgroup which did *not* escalate their alcohol intake (Domi et al. 2018).

In later rodent studies, LY2456302 was directly compared with JDTic and two novel KOR antagonists CYM-52220 and CYM-52288, which have higher apparent in vitro selectivity (Guerrero et al. 2019). While in vivo selectivity was not specifically addressed, all compounds were capable of inhibiting U50,488-induced analgesia. Plasma half-lives of the two novel compounds were shorter than that of LY2456302 [1.9 and 1.6 h $t_{1/2}$ for CYM-52220 and CYM-52288 (Page et al. 2019) versus 3.8 h for LY2456302 (Rorick-Kehn et al. 2014b)]. Brain accumulation was comparable to LY2456302, with brain to plasma ratios exceeding 4:1(Page et al. 2019). The duration of activity in suppressing U50,488-induced analgesia was longer (duration of action LY2456302 < CYM-52288 < CYM52220 <<JDTic), which differs from the rank order of plasma half-life for unclear reasons.

LY2456302 was also able to block a measure of U50,488-induced anhedonia, namely decreased intracranial self-stimulation (ICSS) responding (Page et al. 2019).

3.5 LY2456302: Human Studies

In initial clinical safety studies, LY2456302 was shown to be safe when administered acutely from 2 to 60 mg orally, with no substantial increase in adverse events, in two separate studies of 21 and 37 persons (Lowe et al. 2014). The half-life of LY2456302 in humans was 30–40 h, and steady state plasma levels were reached within 6–8 days of once daily oral dosing. This compound did not affect circulating levels of serum prolactin, luteinizing hormone, and cortisol. There was no interaction of LY2456302 and ethanol on either pharmacokinetic parameters of LY2456302 or cognitive measures.

In a parallel study to determine the appropriate dose of LY2456302 to avoid interaction with MOR while still allowing for KOR binding, pupillometry was used to measure MOR agonist-induced pupil constriction ("miosis") in humans (Rorick-Kehn et al. 2014a). Comparison of LY2456302 with naltrexone in blockade of fentanyl-induced miosis established that 4–10 mg of LY2456302 did not significantly attenuate fentanyl-induced miosis, yielding "minimal-to-no blockade." Larger doses of LY2456302 did produce MOR antagonist effects, with partial blockade of fentanyl-induced miosis (Rorick-Kehn et al. 2014a).

A dosing evaluation of receptor occupancy by LY2456302 in humans was performed using a KOR antagonist PET ligand, [^{11}C]LY2795050 (Naganawa et al. 2016). In this acute study, a dose of 10 mg LY2456302 was found to result in 94% occupancy of brain KOR 2.5 h after oral administration, and 72% occupancy was observed following 24 h. Lower doses (0.5, 2, and 4 mg) resulted in reduced occupancy of brain KOR, with up to 79% occupancy 2.5 h after dosing and 48% occupancy 24 h after the 4 mg dose. Although only 1 participant was evaluated at a higher dose, 25 mg, occupancy at 2.5 h (93%) and 24 h (82%) were not substantially different than for the 10 mg dose. These collective findings (Naganawa et al. 2016) are supportive of the notion of using 10 mg LY2456302 as a KOR antagonist dose.

We further investigated the activity of LY2456302 in a total of 70 humans, consisting of healthy volunteers ($n = 40$) and participants with prior ($n = 7$) or current ($n = 23$) cocaine dependence diagnosis, in the stress-minimized facilities of the Rockefeller University Hospital (Reed et al. 2018). This inpatient study involved monitoring on an initial baseline day followed directly by four consecutive days of 10 mg/day oral LY2456302, with measurements of prolactin (Bart et al. 2005), as well as stress hormones adrenocorticotropic hormone (ACTH) and cortisol, which have been found to increase in response to MOR antagonists (Schluger et al. 1998). LY2456302 was found to be safe and tolerable, with the most common adverse event being pruritus, generally in distal body parts (hands and feet). Pruritus was interestingly not included in the list of adverse events in the earlier human study (Lowe et al. 2014), but was also noted as elevated in a subsequent clinical trial (Krystal et al. 2020). This was a study in a stress-minimized setting, and was not

designed to examine the effects of LY2456302 in decreasing craving or other therapeutic-like endpoints in persons with cocaine dependence. Thus, cocaine craving levels of participants with cocaine dependence diagnosis were generally low, and were not altered by treatment with LY2456302, as expected (Reed et al. 2018). While LY2456302 did not alter prolactin levels, consistent with its activity as a KOR antagonist, modest but statistically significant elevations in circulating ACTH and cortisol were observed, suggesting that 10 mg doses resulted in levels sufficient for at least transient MOR antagonism. The greater number of subjects included as well as the stress-minimized nature of the study setting in the Rockefeller University Hospital may have impacted in part on the discrepancy in this study (Reed et al. 2018) compared to the prior studies reporting hormone levels and interaction with MOR (Lowe et al. 2014; Rorick-Kehn et al. 2014a).

Subsequently, a randomized, double-blind, placebo-controlled study on the effectiveness of LY2456302/CERC-501 on human smoking behavior was conducted by Columbia University and University of Kentucky researchers (Jones et al. 2020). Each participant underwent two 8-day 15 mg/day oral administration of LY2456302 (or placebo), with the last 2 days being inpatient nicotine abstinent periods. LY2456302 was found to be safe, but was not efficacious in altering cigarette craving, latency to smoke, number of cigarettes smoked, or associated mood alterations of withdrawal or smoking (Jones et al. 2020).

An 8-week double-blind placebo-controlled randomized "Fast-Fail" study investigated fMRI activity in the ventral striatum during reward tasks, a marker of hedonia, as a primary outcome of treatment (Krystal et al. 2020). The results of the study, which involved longer sustained duration than the prior studies, support the prior conclusions that the compound is safe with chronic daily administration in humans. Further, LY2456302/JNJ-67953964 administration results in gradual reduction in anhedonic symptoms over 8 weeks, as well as corollary reductions in ventral-striatal activity as assessed by fMRI during reward-based tasks after 8 weeks of treatment (Krystal et al. 2020). Subsequent analysis of the data from this study suggests that chronic KOR antagonism can attenuate anhedonic behavior in participants with mood and anxiety spectrum disorders (Pizzagalli et al. 2020).

3.6 LY2444296

LY2444296, a compound similar to LY2456302, was synthesized by Lilly (Fig. 6, 6.2) [referred to as FP3FBZ in (Melief et al. 2011)], and made available for use in animal studies. This compound also has relatively short-duration KOR antagonism, and 3 mg/kg was shown to antagonize KOR completely at 1 h in male Sprague Dawley rats, with complete return to baseline within 24 h (Valenza et al. 2017). This compound normalized cocaine withdrawal-induced anxiety and depression-like behaviors in rats (Valenza et al. 2017). We also showed LY2444296 attenuates escalation of cocaine use in a rodent model of extended access, escalating dose cocaine self-administration (Valenza et al. 2020). LY2795050 (Fig. 6, 6.3) is a more recently developed analog as a potential PET radiotracer and also exhibits selective

KOR antagonism (Zheng et al. 2013). LY2795050 has been studied in parallel with LY2444296, and both block KOR agonist-induced grooming deficits in rodents (Butelman et al. 2019). Relatively large doses of LY2444296 can also block oxycodone-induced antinociception, showing a moderate degree of kappa versus mu-selectivity of this antagonist (Butelman et al. 2019).

In the mouse forced swim stress test, LY2444296 acted synergistically with the selective delta agonist ADL5859 to reduce immobility (Huang et al. 2016a). For the anti-anxiety effects in rodent models, in a head-to-head comparison of LY2444296 with the short-duration peptidic antagonist zyklophin and the long-duration antagonist norBNI, all three compounds had anxiolytic activity in the novelty-induced hypophagia assay. In contrast, only norBNI had anxiolytic activity in the elevated plus maze assay (Huang et al. 2016b).

3.7 BTRX-335140

A novel chemical scaffold, containing oxadiazol, quinolone, pyranyl, and piperidine moieties, with KOR antagonist activity and selectivity was recently developed at the Scripps Research Institute. The most promising candidate, based on potency and selectivity, was licensed for development by Blackthorn, as BTRX-335140 (Fig. 5, 5.3, also referred to as CYM-53093) (Guerrero et al. 2019). BTRX-35140 has in vitro KOR selectivity of 138 versus MOR and 8,125 versus DOR. This compound and similar compounds were orally active in C57BL/6 mice (Guerrero et al. 2019). BTRX-35140 was shown to block U69,593 (0.3 mg/kg)-induced prolactin release at doses as low as 0.01 mg/kg and to block U50,488 (10 mg/kg)-induced analgesia in the tail flick assay when administered i.p. at 1 mg/kg (Guerrero et al. 2019). BTRX-335140 is currently being investigated in a phase 2 clinical trial at multiple diverse sites in participants with major depressive disorder. (https://www.clinicaltrials.gov/ct2/show/NCT04221230).

4 Conclusions

Animal models have suggested that KOR antagonists might prove effective in treating major depressive disorder (Li et al. 2016), stress-induced anxiety (Varlinskaya et al. 2020), and psychostimulant withdrawal-associated depression and anxiety (Valenza et al. 2017). In animal models of drug addiction, KOR antagonists have been shown to cause decreases in alcohol self-administration (Rorick-Kehn et al. 2014b), and attenuation of ethanol-induced depressive-like behaviors (Jarman et al. 2018). Further, KOR antagonism blocks stress-induced reinstatement (but not drug-induced reinstatement) of cocaine self-administration as a model of relapse (Beardsley et al. 2005). As mentioned, there are ongoing clinical trials of two short-duration selective KOR antagonists, LY2456302/JNJ-67953964 and BTRX-335140, for potential activity in mood disorders/major depressive disorder. Although LY2456302 was not efficacious in attenuating nicotine/

smoking related behaviors in a laboratory study, the potential therapeutic effectiveness in mood disorders and also both alcohol and psychostimulant use disorders strongly argues for further clinical research in these realms. Particularly, given the repeated indications of the safety of LY2456302/Opra Kappa/CERC-501/JNJ-6793964 both in in-patient and out-patient settings (Jones et al. 2020; Krystal et al. 2020; Lowe et al. 2014; Reed et al. 2018), the prospect of studying the effects of multi-week chronic administration in persons with alcohol or psychostimulant dependence on drug intake and associated symptoms could result in major advancements in our understanding of the potential therapeutic utility of KOR antagonists in treating substance use disorders.

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Kappa Opioid Receptors in the Pathology and Treatment of Major Depressive Disorder

Caroline A. Browne, Hildegard Wulf, and Irwin Lucki

Contents

Abstract

The kappa opioid receptor (KOR) is thought to regulate neural systems associated with anhedonia and aversion and mediate negative affective states that are associated with a number of psychiatric disorders, but especially major depressive disorder (MDD). Largely because KOR antagonists mitigate the effects of stress in preclinical studies, KOR antagonists have been recommended as novel drugs

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for treating MDD. The purpose of this review is to examine the role of KORs and its endogenous ligand dynorphins (DYNs) in the pathology and treatment of MDD derived from different types of clinical studies. Evidence pertaining to the role of KOR and MDD will be reviewed from (1) post mortem mRNA expression patterns in MDD, (2) the utility of KOR neuroimaging agents and serum biomarkers in MDD, and (3) evidence from the recent Fast Fail clinical trial that established KOR antagonism as a potential therapeutic strategy for the alleviation of anhedonia, a core feature of MDD. These findings are compared with a focused evaluation of stress-induced alterations in *OPRK* and *PDYN* mRNA expression. Finally, the current status of the effects of KOR antagonists on behavioral phenotypes of stress in preclinical studies related to MDD is summarized.

Keywords

Anhedonia · DYN – dynorphin · KOR – kappa opioid receptor · MDD – major depression disorder · PDYN – prodynorphin

1 Introduction

Meta-analysis of over 522 clinical trials for the treatment of major depressive disorder (MDD) indicates that the widely used classes of antidepressants, selective serotonin norepinephrine reuptake inhibitors, selective serotonin reuptake inhibitors, and tricyclic antidepressant compounds, are overall helpful, although their efficacy is relatively low (Cipriani et al. 2018). The odds ratio (OR) for 21 antidepressants ranged from the least efficacious reboxetine [1.37 (95% Crl 1.16–1.63)] to the most efficacious amitriptyline [OR 2.3 (95% Crl 1.89–2.41)] (Cipriani et al. 2018). An odds ratio over 1.0 indicates that the drug treatment is more likely to be effective than placebo. Moreover, the acceptability of many of these compounds was poor, with a greater dropout rate favoring placebo. In addition to their relatively modest efficacy, there is a common lag of 6–12 weeks between the initiation of treatment and the appearance of clinical improvement (Nierenberg et al. 2010). In contrast to the older antidepressants, the introduction of ketamine for the treatment of depression has dramatically raised expectations for the introduction of new clinical antidepressants. Ketamine has been shown to produce more consistent antidepressant effects, including diminishing suicidal ideation, after the application of only a single treatment. Relative to conventional antidepressants reviewed by Cipriani (above), ketamine produced greater efficacy at 24 h (OR = 10.09; 95% CI: 4.96–20.52), 72 h (OR = 7.42; 95% CI: 3.97–13.88) and 7 days (OR = 5.66; 95% CI: 2.92–10.97) following a single infusion (Han et al. 2016). Even more striking is the fact that these data were obtained in patients characterized as having treatment-resistant depression (TRD), defined as not exhibiting a therapeutic response to two or more antidepressant compounds (Nierenberg et al. 2010). Although ketamine has altered the accepted symptom reduction trajectory for MDD, it is not a silver bullet. Approved by the Food and Drug Administration in March 2019, intranasal esketamine

(Spravato) remains a scheduled drug with an unenviable acute side effect profile and currently used only by those severely ill MDD patients with a risk evaluation and mitigation strategy (Bahr et al. 2019). Moreover, the effects of ketamine fade within days of administration unless additional treatments are administered. Nevertheless the appearance of ketamine has reinforced the urgency of the need to develop novel therapeutics that are fast acting and have greater efficacy than currently used antidepressant compounds.

Mounting preclinical evidence suggests that kappa opioid receptor (KOR) antagonism may provide a mechanism leading to an alternative strategy to treat MDD (Browne and Lucki 2019). This hypothesis has been strengthened by recent clinical studies highlighting the beneficial effects of a selective KOR antagonist aticaprant (formerly JNJ-67953964, CERC-501 and LY2456302) in modulating anhedonia (a key feature of MDD) in a transdiagnostic patient population (Krystal et al. 2020; Pizzagalli et al. 2020). These exciting findings support the need for more extensive clinical evaluation of KOR in the context of MDD. To this end, this review assesses the available clinical information regarding alterations in *OPRK* and prodynorphin (PDYN) mRNA expression, KOR and dynorphin concentrations, and biomarkers of KOR engagement in subjects with MDD in order to evaluate the role of KOR in the pathology of MDD (Table 1). Preclinical studies that recapitulate these molecular endpoints after stress will also be evaluated (Table 2). Finally, preclinical evidence supporting the use of KOR antagonists will be summarized (Table 3) and the utility of clinically targeting KOR to alleviate specific endophenotypes or constructs of MDD will be addressed.

2 Literature Search

A systematic review of relevant literature was conducted using the Cochrane Central Register of Controlled Trials, CINAHL, Embase, MEDLINE, MEDLINE In-Process, PsycINFO, and NCBI PubMed including only articles available in English, from the date of their inception to July 21, 2020. The search terms were "kappa opioid receptor*" AND "major depressive disorder*" or "dynorphin*" AND "major depressive disorder*". Supplemental manual searches for published, unpublished, and ongoing trials in ClinicalTrials.gov were conducted using the search term "kappa opioid receptor*" AND "major depressive disorder*" or "dynorphin*" AND "major depressive disorder*". For the preclinical literature NCBI PubMed was searched from the date of inception to July 21, 2020 for articles available in English with the following search terms "kappa opioid receptor" AND "stress" or "dynorphin" AND "stress" or "kappa opioid receptor antagonist" AND "stress".

Table 1 Clinical studies supporting the role of KOR in MDD

Reference	Subjects	Demographics	Treatment History	Brain region	Endpoint	Outcome
(Hurd et al. 1997)	Controls ($n = 6$)	Female 2, male 4; 1 white, 4 black, 1 Asian	Toxicology negative for antidepressants or illicit drugs	Neostriatum – CPu and NAc	*PDYN* mRNA expression	Elevated *PDYN* in CPu patch
	Suicide completers MDD diagnosis ($n = 6$)	Female 1, male 5; 4 white, 1 black, 1 Asian	One MDD and two control subjects had a history of SUD			
(Peckys and Hurd 2001)	Controls ($n = 15$)	Female 6, male 9; 14 Caucasian, 1 African American	Three MDD subjects had a current SUD	dlPFC and ACC	*PDYN* & *OPRK1* mRNA expression	No significant alterations in *PDYN* or *OPRk1*
	MDD ($n = 15$)	Female 6, male 9; Caucasian	No treatment history reported			
(Hurd 2002)	Controls ($n = 15$)	Female 6, male 9; 14 white, 1 black	TCA $n = 3$; SSRIs $n = 4$; SNRI $n = 4$; lithium $n = 2$ and anxiolytics $n = 5$	Periamygdaloid cortex nucleus, AHA and AB	*PDYN* mRNA expression	Decreased *PDYN* in AHA, ABpc and ABmc
	Suicide completers MDD diagnosis ($n = 15$)	Male 9, female 6; Caucasian				
(Anderson et al. 2013)	Controls ($n = 10$)	Female 3, male 7; European Caucasian	N/A	Periamygdaloid cortex nucleus, AHA and AB	*PDYN* mRNA expression	Decreased *PDYN* in AHA, ABpc and ABmc
	Suicide completers MDD diagnosis ($n = 14$)	Female 6, male 8; European Caucasian				
(Lutz et al. 2018b)	Controls (C; $n = 34$ AI, 26 ACC and 27 MDT)	Not reported	N/A	ACC, MDT and AI	*DYN* and *OPRK1* mRNA expression	AI only – decreased *Oprk1* in CA subjects
	Suicide completers history of child abuse			AI	KOR isoform splicing	Selective KOR splicing in CA subjects

	(CA; $n = 30$ AI, 26 ACC and 26 MDT					
	Suicide completers no child abuse (SNA; $n = 30$ AI)			AI	DNA methylation	Hypomethylation of *OPRK1* -i2
(Lutz et al. 2018a)	Controls ($n = 30$)	Female 6, male 24	SUD in 18 depressed suicide subjects and 4 controls	ACC, MDT and AI	INDEL rs35566036 in KOR promoter region	INDEL was associated with MDD
				AI		No effect of INDEl on KOR expression in ACC, MDT & AI
	Suicide completers MDD diagnosis ($n = 52$)	Female 10, male 42	No treatment history reported	AI		INDEL resulted in less DNA methylation of 8 CpG sites within 500 bp of the *OPRK1* start codon
(Miller et al. 2018)	Controls ($n = 13$)	Female 6, male 7; 1 Asian, 3 African American, 7 Caucasian, 1 Hispanic, 1 > 1 race	Treatment resistant	Amygdala, ventral striatum, Hippocampus and raphe nuclei	[11C] GR103545 binding at KORs	No difference between controls and MDD in KOR occupancy
	MDD ($n = 10$), HDRS score ≥ 16	Female 5, male 5; 1 Asian, 2 African American, 2 Caucasian, 1 Hispanic, 1 > 1 race	No antidepressant therapy during image acquisition		Life stress association with KOR binding	KOR binding not associated with childhood trauma or current (6 months) life stress
					TSST evoked cortisol association with KOR binding	Inverse correlation between TSST cortisol secretion and binding

(continued)

Table 1 (continued)

Reference	Subjects	Demographics	Treatment History	Brain region	Endpoint	Outcome
(Al-Hakeim et al. 2020)	Controls ($n = 30$)	Male	First MDD episode, no treatment	N/A	Serum KOR & DYN levels	Biomarker Z scores for elevated serum KOR & DYN separates MDD from control subjects
	MDD ($n = 60$) HRDS of >21					
(Krystal et al. 2020)	Anhedonia SHAPS ≥30		Placebo or Aticaprant (10 mg/day) for 8 weeks	Ventral striatum	Ventral striatal activation during monetary incentive task	Greater mean and maximum fMRI ventral striatal in Aticaprant group
	Placebo ($n = 44$; SHAPS 33.4 (SD = 5.9)	Female 29, male 16; 65.1% Caucasian, 18.6% African American, 18.6% Asian, 9.3% 0.1 race				Lower baseline adjusted mean SHAPS score in Aticaprant group
						Higher post treatment for PRT response bias in aticaprant group, but no difference between blocks
	Aticaprant ($n = 44$; SHAPS 36.4 (SD = 8.5)	Female 21, male 23; 70.5% Caucasian, 22.7% African American, 4.5% 0.1 race				Baseline striatal activity predicted PRT responders
						Ventral striatal activity correlated with SHAPS score

(Pizzagalli et al. 2020)	Anhedonia SHAPS ≥20		Placebo or Aticaprant (10 mg/day) for 8 weeks	N/A	Reward learning	Greater response bias post Aticaprant treatment
	Placebo ($n = 31$; SHAPS 33.03 (SD = 5.91)	Female 19, male 12; 19 Caucasian, 5 African American, 2 Asian, 4 > 1 race, 1 unknown				Higher posttreatment rich hits for Aticaprant relative to the placebo group
	Aticaprant ($n = 24$; SHAPS 37.29 (SD = 8.89)	Female 14, male 10; 18 Caucasian, 3 African American, 2 > 1 race, 1 unknown)				

This table details postmortem studies of *OPRK* and *PDYN* mRNA expression, KOR binding, serum concentrations, and the outcomes of the Fast Fail clinical trial for the KOR antagonist Aticaprant. *OPRK* human opioid receptor kappa gene, *OPRK1-i2* OPRK splice variant 1, intron 2, *PDYN* human prodynorphin gene, *KOR* kappa opioid receptor, *DYN* dynorphin, *MDD* major depressive disorder, *SUD* substance use disorder, *TCA* tricyclic antidepressant (TCA), *SSRI* selective serotonin reuptake inhibitor, *SNRI* serotonin norepinephrine reuptake inhibitor, *CPu* Caudate Putamen, *NAc* Nucleus Accumbens, *dlPFC* dorsolateral prefrontal cortex, *ACC* anterior cingulate cortex, *AHA* amygdalohippocampal area, *AB* accessory bundle of the amygdala, *ABpc* parvicellular accessory bundle of the amygdala, *ABmc* magnocellular accessory bundle of the amygdala, *MDT* mediodorsal thalamus, *AI* anterior insular cortex, *INDEL* insertion deletion, *PET* positron emission tomography, *fMRI* functional magnetic reason imaging, *HDRS* Hamilton Depression Rating Scale, *TSST* Trier Social stress Test, *SHAPS* Snaith-Hamilton Pleasure Scale, *MID* monetary incentive delay task, *PRT* probabilistic reward learning task

3 Clinical Studies

Evidence in support of altered KOR/DYN signaling in the psychopathology of MDD has been derived primarily from a group of studies that investigate *OPRK* and *PDYN* mRNA expression in postmortem tissues from depressed subjects. Studies have also examined the concomitant roles of childhood abuse and substance use disorder in altered KOR signaling (Fig. 1).

3.1 Postmortem Studies

The dysphoric and aversive qualities of KOR agonists in humans were first documented in the 1980s (Pfeiffer et al. 1986) from dose-dependent increases in subjective reports of dysphoria and psychotomimesis produced in male subjects administered a benzomorphan KOR agonist MR 2033. Subsequently, Hurd and

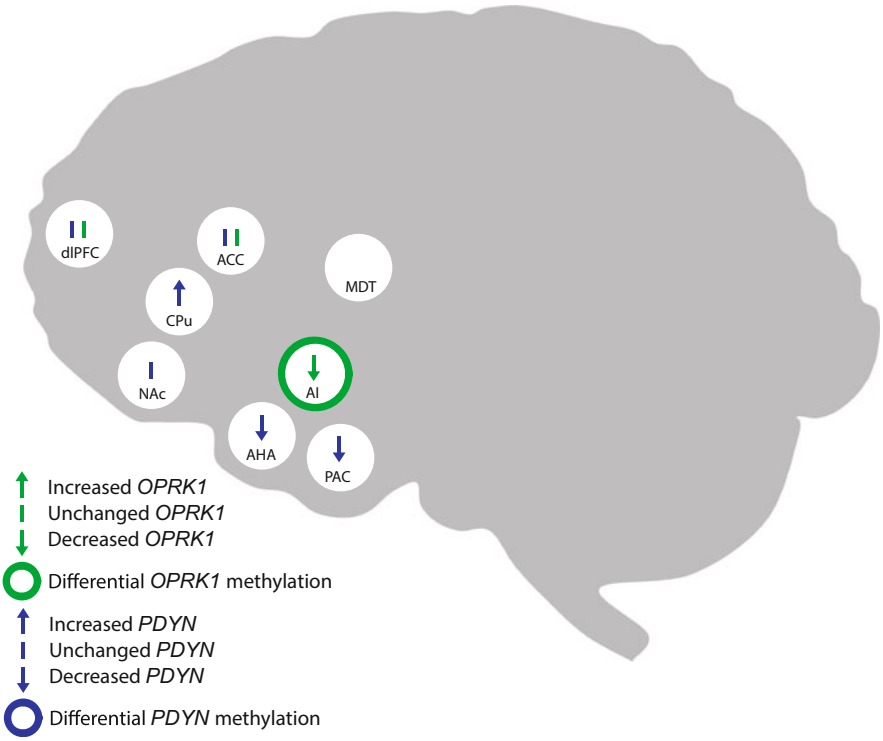

Fig. 1 Clinical study findings. This figure summarizes the postmortem *OPRK* and *PDYN* findings detailed in Table 1. *OPRK* opioid receptor kappa gene, *PDYN* prodynorphin gene, *dlPFC* dorsolateral prefrontal cortex, *NAc* Nucleus Accumbens, *CPu* Caudate Putamen, *ACC* anterior cingulate cortex, *AHA* amygdalohippocampal area, *AI* anterior insular cortex, *MDT* mediodorsal thalamus, *PAC* periamygdaloid cortex nucleus

colleagues reported elevations of *PDYN* mRNA transcript in the neostriatum of a small ($n = 6$) cohort of suicide completers with a history of MDD, relative to schizophrenic patients and controls (Hurd et al. 1997). The pattern of increased expression of *PDYN* was most evident in patch striosomes from the caudate putamen (CPu). None of the subjects had positive toxicology for antidepressant treatment or other illicit drug use, indicating that these robust effects were most likely attributable to suicide and potential MDD diagnosis. In contrast to marked differences found in the CPu and periamygdaloid cortex (PAC), no significant alterations in *PDYN* or *OPRK* mRNA expression in two other key regions, the dorsolateral prefrontal cortex (dlPFC) and anterior cingulate cortex (ACC), were associated with any affective disorder diagnosis (MDD, bipolar disorder, or schizophrenia) (Peckys and Hurd 2001). Following this study, Hurd (2002) reported decreased mRNA expression of *PDYN* within discrete nuclei of the PAC in 15 suicide completers with a diagnosis of MDD relative to healthy controls. The average reduction in expression for MDD diagnosis was 41.9%, 68.9%, and 55% in the parvicellular (ABpc) and magnocellular division (ABmc) of the accessory bundle and the amygdalohippocampal area (AHA), respectively. Antidepressant medication did not correlate with the alterations in *PDYN* transcript in this study (Hurd 2002). This finding was replicated in a more recent cohort of MDD subjects who died of other causes (Anderson et al. 2013), suggesting that decreased *PDYN* expression in the amygdala is a replicable maker of MDD.

As indicated in Table 1, these postmortem studies of *PDYN* and *OPRK* expression were conducted in relatively small cohorts of MDD subjects and contain some inconsistencies. For example, in the 1997 study one sample had very low PDYN levels throughout the neostriatum, in direct opposition to the elevations detected in the other five subjects (Hurd et al. 1997). Likewise, in the Peckys and Hurd (2001) study most subjects exhibited low to moderate expression of *PDYN* in the lateral nucleus and almost no expression of *PDYN* in the basal nucleus of the amygdala. However, one MDD subject expressed clusters of high expression in both regions. The subjects interrogated in this study included individuals with a prior or current substance use disorder, which were accordingly used as covariates. As such, a significant association of marijuana and stimulant use with *OPRK* mRNA expression was evident (Peckys and Hurd 2001). These data emphasize the diversity of PDYN and OPRK transcript levels that can be evoked by environmental experiences. Consequently, it is important for postmortem clinical studies to carefully construct inclusion criteria for future evaluations of *PDYN* and *OPRK* expression in specific patient populations.

More recent clinical studies have emphasized the potential contribution of a history of childhood abuse (CA) on KOR. KOR downregulation in the anterior insular (AI) cortex was reported in a postmortem sample of suicide completers with a history of severe CA relative to controls and suicide completers without a history of CA (Lutz et al. 2018b). No significant changes in the expression of *PDYN*, *PENK*, *POMC*, *OPRD1,* or *OPRM1* genes were evident in this study. Specifically, decreased *OPRK1 variant 1* mRNA was associated with CA, but not suicide. In contrast, *OPRK1 variant 2 mRNA* expression was not associated with either suicide

or CA (Lutz et al. 2018b). *OPRK1* variant 1 mRNA encodes the full-length hKOR protein. *OPRK1* variant 2 mRNA lacks the sequence for the first two transmembrane domains that are necessary for trafficking of the receptor protein to the plasma membrane and appears not to be translated into a protein. Altered DNA methylation was most prominent at clusters of CG in Intron 2 of variant 1 (OPRK1-i2) and the variant 2-exon incorporated into *OPRK*1 (Lutz et al. 2018b). Although decreased DNA methylation of the *OPRK1* variant 2-exon was not associated with group differences, *OPRK1-i2* hypomethylation was associated with the CA group relative to the suicide completers without a history of CA (Lutz et al. 2018b). Low levels of DNA hydroxymethylation facilitate glucocorticoid binding and subsequent regulation of *OPRK1* expression (Lutz et al. 2018b). These data indicate that CA can evoke long-lasting epigenetic regulation of *OPRK1* transcript in the AI, a region that is critical for regulating emotional salience and introspection.

Lutz and colleagues also assessed the consequences of a specific insertion deletion (INDEL) sequence, rs35566036, in the *OPRK1* promoter region of 30 healthy controls and 52 suicide completers with a history of MDD (Lutz et al. 2018a). They hypothesized that the INDEL would decrease transcriptional activity at the promoter, ultimately downregulating KOR in the regions of interest. INDEL rs35566036 did not alter the *OPRK1* expression of MDD subjects relative to controls in the AI, ACC, or mediodorsal thalamus. However, the INDEL did decrease average DNA methylation in eight of the *OPRK1* CpG clusters interrogated within the AI samples, located within 500 base pairs of the promoter. Notably, INDEL rs35566036 occurred more frequently in suicide completers relative to controls ($p = 0.014$; OR = 3.55; 95%CI 1.30–9.70), suggesting that at the very least this KOR INDEL may confer a vulnerability for MDD.

Together these postmortem data underscore a complex region-specific pattern of *PDYN* and *OPRK* expression that is associated with MDD diagnosis. Although SUD diagnosis was not associated with any alterations in *OPRK* expression in the ACC in the Lutz (2018b) study, it agrees with that of Peckys and Hurd (2001) in which *OPRK* expression was not associated with suicide or MDD diagnosis (Lutz et al. 2018b). However, it should be noted that the variability in the *PDYN and OPRK* mRNA transcript in the dlPFC and ACC was driven by an association with a SUD diagnosis in the Peckys study (Peckys and Hurd 2001). Thus, future exploration of these regions is warranted. Otherwise, it may be the discrete allocortex nuclei, as suggested by Hurd, that prove to be of most relevance to *OPRK* and *PDYN* associated with MDD, as borne out by positive associations with *PDYN* in the PAC (Hurd 2002; Anderson et al. 2013) and KOR downregulation in AI (Lutz et al. 2018b).

3.2 KOR and DYN as Potential Biomarkers of MDD

Positron emission tomography (PET) imaging has the potential to address the understudied contribution of the KOR to psychiatric illness by categorizing differences between patient populations and to follow the emergence and remission

of MDD over time in the same patient. So far, only one published study has quantified KOR occupancy with PET ligands (Table 1). KOR binding was evaluated in 13 healthy controls and 10 medication-free TRD subjects with a current MDD episode using a highly selective KOR agonist radiotracer [^{11}C]GR103545 at a dose of 0.02 μg/kg (Miller et al. 2018). Total volume of distribution (V_T) was comparable between controls and MDD subjects in the four regions of interest examined: amygdala, hippocampus, raphe nucleus, and ventral striatum (Miller et al. 2018). KOR V_T was not associated with depression severity, measured with either the Beck Depression Inventory (BDI) or the Hamilton Depression Rating Scale (HDRS), self-reported childhood trauma questionnaire scores, or with the domains determined using the Interview for Recent Life Events scale which documented life stress during the 6 months prior to imaging. Interestingly, in a subset of individuals who completed the Trier Social Stress Test, cortisol secretion during the test was inversely correlated with [^{11}C] GR103545 V_T, although this correlation did not reach the level of statistical significance (Miller et al. 2018). Despite these negative findings, further evaluation of KOR occupancy with other improved radiotracers is recommended. Although [^{11}C]GR103545 has a 6×10^2 fold greater selectivity for KOR over MOR (Talbot et al. 2005), the low specific binding of this radiotracer in the hippocampus and raphe nuclei is a concern. As the kinetics of the compound are slow (Tomasi et al. 2013; Naganawa et al. 2014), it may be most suited to detecting high levels of occupancy. Critically, there was poor test-retest reproducibility in the amygdala reported for this radiotracer (Naganawa et al. 2014). This is a significant limitation because the positive associations between *PDYN* and MDD diagnosis determined for this region in the previously reviewed postmortem studies would make the amygdala an important area of interest (Hurd 2002; Anderson et al. 2013). Other radiotracers that overcome these limitations are available. One such radiotracer is a KOR antagonist ligand [^{11}C]LY2795050, which has been employed to determine KOR availability in alcohol use disorder (Vijay et al. 2018), sex differences in basal KOR occupation (Vijay et al. 2016), and receptor occupancy of the KOR antagonist aticaprant (Naganawa et al. 2016).

Recently published biomarker research focused on establishing a neuroimmune or opioid-associated fingerprint for discrimination of individuals with affective disorders has yielded some exciting data regarding serum KOR levels (Al-Hakeim et al. 2020). This study initially screened 60 male, drug-naïve patients in their first depressive episode and 30 healthy controls. Patients were included with a HDRS of $>$21. Overall levels of dynorphin and KOR measured in serum were higher in MDD patients, although it should be noted that these subjects were nicotine users and the possibility that the levels of dynorphin and KOR may be dependent on nicotine was not evaluated. In addition, elevated mu opioid receptor, β-endorphin, and the cytokines interleukin-10 and interleukin 6 were detected in the MDD group (Al-Hakeim et al. 2020). This biomarker dataset was then mined to build prediction models that were used to identify and authenticate soft independent modeling of class analogies (SIMCA) for classification of unknown patients. The data demonstrate a clear separation of controls and MDD patients with a remarkable 100% accuracy and a specificity of 93.3% (Al-Hakeim et al. 2020). Many questions are

inspired by this article. Methodological concerns regarding the specificity of the antibodies for the KOR and MOR call for validation of the data set with additional antibodies to enhance confidence in the results. Other pertinent questions include identification of the origin of cells expressing KOR (peripheral immune cells or centrally derived) and whether the stratification of subjects would be altered by recurrent episodes of illness, biological sex, treatment or disease remission, and aging. Validation of this opioid-associated fingerprint in a larger, more diverse population of MDD patients would be highly informative. If subsequent studies can obtain consistent findings, these data could be very important in showing that serum KOR could be used as a biomarker for MDD.

3.3 Aticaprant: NIH Fast Fail Initiative

Since the 1980s, non-selective KOR antagonists, such as naltrexone and buprenorphine, have been employed in humans for treatment of substance use disorder. Buprenorphine also carried FDA approval as a therapeutic for chronic pain. More recently, positive outcomes on measures of depression have been reported with adjunctive use of these non-selective KOR antagonists (Ehrich et al. 2015a; Fava et al. 2016; Guerdjikova et al. 2017; Mischoulon et al. 2017; Fava et al. 2020). Although some selective KOR antagonists were in development that would have been tested in MDD populations, their progression was discontinued because of potential toxicities.

Recently, the selective KOR antagonist aticaprant (JNJ-67953964) has shown some promise in alleviating anhedonia in a transdiagnostic sample (Clinical trial NCT02218736), see Table 1. As part of the National Institute on Mental Health "New Experimental Medicine Studies: Fast-Fail Trials Program," aticaprant was screened in a randomized multi-center trial with a diverse group of subjects diagnosed with mood and anxiety disorders (MAS). For inclusion in this Fast–MAS trial, subjects were required to exhibit severe anhedonia, measured as a Snaith-Hamilton Pleasure Scale (SHAPS) score of greater than 30. It was the first demonstration of the Fast-Fail Trials utility in streamlining novel therapeutic development (Krystal et al. 2018). The primary outcome was ventral striatal activation detected with functional magnetic resonance imaging (fMRI) provoked during a monetary incentive delay (MID) task in anticipation of a gain (win) relative to activation on a no-incentive trial (neutral). During MID, wins were associated with greater mean and maximum ventral striatal activity in aticaprant treated subjects following 8 weeks of treatment relative to placebo (Krystal et al. 2020). However, this finding was driven in part by a decrease in post treatment responding exhibited by the placebo group (baseline 0.64 (SD = 0.8) to 0.33 (SD = 0.68), an effect apparently of repeated testing (Green et al. 2019)). Irrespective of the drift in the placebo score, those treated with aticaprant maintained engagement in the MID task on repeated testing and did exhibit a noticeable though insignificant increased score at 0.72 (SD = 0.67) relative to the pretest 0.63 (SD = 0.9) (Krystal et al. 2020). Further exploration of the fMRI data also revealed greater activation in the ventral

striatum of subjects administered aticaprant in the anticipation of loss relative to the neutral state (Krystal et al. 2020), suggesting an overall change in response bias and greater engagement with the task in general. Moreover, baseline ventral striatal activity predicted responders with a 76% accuracy (Krystal et al. 2020). These imaging biomarker-based outcomes demonstrate that aticaprant does engage with, and can reverse hypoactivation within the ventral striatum, an important target region in mitigating the abnormal reward processing observed in MDD subjects (Borsini et al. 2020).

The secondary outcomes evaluated in the Fast-MAS trial included (1) changes in mean SHAPS and (2) a change in response bias in the probabilistic reward task (PRT). Using a baseline adjusted mean (the groups differed significantly on this variable at pretest), there was a modest but significant improvement in the SHAPS scores of individuals randomized to aticaprant treatment (Krystal et al. 2020). In the additional exploratory analysis, ventral striatal activity in the primary outcome measure correlated with SHAPS scores. Furthermore, an increase in the Temporal Experience of Pleasure Scale (TEPS) consummatory subscale was evident in the aticaprant group post treatment 29.3 (SD = 4.2) relative to baseline (26.3 (SD = 4.4) (Krystal et al. 2020). These positive findings on measures of anhedonia are encouraging. However, these relatively small changes in SHAPS and TEPS subscale scores do not have a clinically significant meaning. At the end of the treatment period, all subjects continued to exhibit significant rates of anhedonia. It is possible that higher doses of aticaprant may produce a more robust effect on these self-report questionnaires. The dose of aticaprant used in this trial (10 mg) was based on prior PET imaging studies showing approximately 95% occupancy (Naganawa et al. 2016) with peak plasma concentrations detected 2–3 h post administration (Lowe et al. 2014) and acceptable toxicity. This dose also exhibits good occupancy (72%) of receptors 24 h post treatment (Naganawa et al. 2016). However, doses as high as 60 mg at a single time point and 35 mg daily for 14 days appear to have been well tolerated by humans in other studies with no serious adverse events reported (Lowe et al. 2014). Therefore, future evaluation of aticaprant for anhedonia outcomes should concentrate on specific diagnostic populations and evaluate higher doses that may produce more pronounced changes on this endophenotype of MDD.

The PRT is a challenging discrimination task that requires subjects to discriminate whether a cartoon face, presented for 100 ms, had a long or short mouth. Of the two stimuli presented (long and short faces), one was deemed the "rich" stimulus for which subjects were rewarded with a monetary incentive three times more frequently than the "lean" stimulus. Although the proposed outcome (change from block 1 to 2) was not achieved, secondary measures determined a significant increase in the response bias in the aticaprant group relative to placebo post treatment (Krystal et al. 2020). In the 66 subjects who completed the PRT, there was a trend for ventral striatal activity to correlate with the change from block 1 to block 2 (Krystal et al. 2020).

In a secondary analysis of the Fast-MAS Trial, a significant enhancement of response bias in block 2 compared to block 1 was established in a subset of patients scoring $\geq$20 on the SHAPS (Pizzagalli et al. 2020). Response bias in the task was

detected in 16/24 subjects treated with aticaprant pre to post, whereas only 13/31 of placebo treated subjects exhibited this response. In addition, there was a higher number of posttreatment rich hits for aticaprant relative to the placebo group. This occurred irrespective of whether the preceding trial had been rewarded or not (Pizzagalli et al. 2020). No effect on reward sensitivity was detected in this secondary analysis. The higher posttreatment responses in aticaprant treated individuals without altering reward sensitivity is indicative of aticaprant's capacity to induce a sustained preference for a rewarding stimulus and overall improvement in reward learning. The blunted reward learning between blocks is a characteristic of MDD subjects (Pechtel et al. 2013), which emphasizes the striking selective effect of aticaprant on this behavioral measure.

It should be noted that there was a 25% drop out rate during in the Fast Fail KOR antagonist trial. Although only four subjects in each group dropped out due to an increase in their symptoms, the failure to retain subjects may have limited the power to determine statistically significant alterations. Nevertheless, these studies demonstrated that the KOR antagonist aticaprant engaged with ventral striatal activity (the neurobiological biomarker of interest), enhanced reward valuation and reward learning (behavior) and had a positive effect on self-report of anhedonia (SHAPS). These encouraging findings support the further evaluation of novel KOR antagonists for the eventual treatment of MDD and related disorders.

4 Preclinical Studies

Evidence in support of altered KOR/DYN signaling in the psychopathology of MDD is supported by preclinical literature showing changes in KOR gene expression and protein concentrations after exposure to environmental stress (Table 2).

4.1 Stress-Induced Changes in *Oprk* and *Pdyn* Expression

Preclinical studies of stress effects on alterations in *Oprk* and *Pdyn* mRNA expression are important because they could provide information about the regulation of gene expression following various stressors in developing and mature rodents and in different regions of the limbic system (Table 2). Reviewed in this section are studies that examined Oprk and Pdyn expression following acute and chronic stress exposure after different durations of recovery. However, as a result of the varying parameters used in the preclinical studies reported so far, it is often difficult to compare the results between many of these reports. The results of many of these studies revealed that, just as in humans, stress exposure in rodents consistently altered *Oprk* in the striatum, with many studies differentiating between the effects in NAc and CPu. The amygdala also appears to be a region sensitive to the effects of stress.

Exposure of rodents to stress during early life could evoke patterns of gene expression that would be reminiscent of the impact of child abuse in humans

Table 2 Preclinical stress-induced alterations in gene transcript and protein levels of KOR and Dyn

	Reference	Stressor	Subjects	Region	Tissue collection	Result
Early life	(Haj-Mirzaian et al. 2019)	Social isolation	Male NMRI mice	Hippocampus and amygdala	PND 21–25	*Oprk1* decreased in Hippocampus and amygdala
	(Nakamoto et al. 2020)	Maternal separation 6 h/day during PND 15–21. Single housing post PND21	Male ddY mice	Hypothalamus, PAG, RVM, LC, amygdala, mPFC, ACC, NAc and Spinal Cord	10 weeks	*Oprk1* increased in amygdala and decreased in PAG
						No alterations in *Pdyn*
	(Chang et al. 2019)	Neonatal predator odor exposure – cat, rat and ferret odor presented on PND 1, 2 and 3, respectively	Male and female Sprague-Dawley rats	NAc	PND 3	Reduced O*prk1* in stressed females only
					PND 33	Elevated *Oprk1* in stressed females only
Adolescence	(Varlinskaya et al. 2018)	Forced swim stress (10 min) – 2 days	Male and female Sprague-Dawley rats	BLA	PND 33–35 immediately following social interaction	No alterations in *Oprk1* or *Pdyn*
Adulthood	(Shirayama et al. 2004)	Forced swim stress – 15 min	Male Sprague-Dawley rats	Hippocampus (DG and CA3)	0,2,5 and 25 h post stress	Elevated Dyn A immunoreactivity in DG and CA3 2 h post stress, but not at later time points
		Immobilization stress – 3 h		Hippocampus (DG and CA3), NAc (core and shell) and CPu	Immediately after stress	Increased Dyn A & B positive cells in all regions, except the CPu

(continued)

Table 2 (continued)

	Reference	Stressor	Subjects	Region	Tissue collection	Result
		LH – sixty inescapable footshocks (0.65 mA, 30 s duration) on day 1 & 2, 30 escapable shocks on day 3, 30 inescapable trials on day 4		Hippocampus DG & CA3, NAc (core and shell) and CPu	After retesting on day 8	Increased Dyn A & B positive cells in all regions, except the CPu
	(Carr et al. 2007)	Genetic rat model of anxiety and depression	Male WKY and Sprague-Dawley rats	Piriform cortex and NAc shell	N/A	WKY rats exhibited greater KOR and Dyn A protein in the piriform cortex relative to SD rats
	(Lucas et al. 2011)	Immobilization stress	Male Sprague-Dawley rats	NAc (core and shell), CC and dorsolateral CPu		
		Acute – 2 h			2 days post stress cessation	*Oprk1* increased in all regions
					9 days post stress cessation	No alterations in *Oprk1* or *PDYN*
		Chronic 2 h/day – 10 days			2 days post stress cessation	Increased *Oprk1* in dorsolateral and dorsomedial CPu
					9 days post stress cessation	Elevated *Pdyn* in dorsomedial CPu and shell of NAc
	(Nocjar et al. 2012)	Social isolation – 28 days + resident intruder – five 30 min defeats separated by 48–72 h intervals	Male long Evans rat	mPFC, NAc, VTA and hypothalamus	2 days after the last defeat	Dyn A concentrations reduced in hypothalamus of defeated animals
	(Reed et al. 2012)	Swim stress – 1 pretest (15 min) and 1 test (5 min)	Male Sprague-Dawley rats	CPu, NAc, hypothalamus, FC, Hippocampus, and amygdala	30 min post test	Elevated *Pdyn* in the CPu relative to swim naive controls

	(Berube et al. 2013)	Social defeat – 7 days	Male Sprague-Dawley rats; stratified into stress resilience and stress vulnerable	CPu and NAc (shell)	Resilient	Enhanced Dyn concentrations in striatum
					Vulnerable	Increased Dyn concentrations in NAc (shell)
	(Donahue et al. 2015)	Social defeat	Male C57BL/6J mice; stratified into stress resilience and defeated	NAc		No significant alterations in *Oprk1* under any condition
		Acute – 1 defeat bout (10 min)			48 h following acute defeat	Increased *Pdyn* in defeated mice
		Chronic – 10 days			48 h following chronic defeat	Decreased *Pdyn* in both defeated and resilient mice
					37 days stress cessation	Decreased *Pdyn* in both defeated mice
	(Szklarczyk et al. 2015)	CFC – 5 shocks 1 mA 2 s duration	Male C57BL/6J mice	Hippocampus and amygdala	2 h post footshock	No changes in *Pdyn*
			Male SWR/J mice			*Pdyn* elevated in amygdala of shocked mice
	(Wang et al. 2015)	Five footshocks (1.5 mA, 2 s duration)	Male Sprague-Dawley rats; stratified into high and low responders	NAc, lateral and medial CPu, BNST and Amygdala	HR – 14 days post footshock	No alterations in *Pdyn*
					LR – 14 days post footshock	No alterations in *Pdyn*

(continued)

Table 2 (continued)

	Reference	Stressor	Subjects	Region	Tissue collection	Result
	(Falcon et al. 2016)	Unpredictable chronic mild stress – 21 days	Male C57BL/6J mice	Hippocampus, amygdala, Striatum and FC	14 days post stress cessation	*Oprk1* increased in striatum and decreased in amygdala
						Decreased *Pdyn* in hippocampus
	(Browne et al. 2018)	Social defeat – 10 days	Male C57BL/6J mice; stratified into stress resilience and stress susceptible	Hippocampus, amygdala, Striatum and mPFC	Susceptible mice – 14 days post stress cessation	Decreased *Oprk1* in amygdala. Increased *Oprk1* expression in FC
					Resilient mice – 14 days post stress cessation	No change in *Oprk1*

Stress exposure during critical periods in early life and adolescence is contrasted with the impact of stress induction in adulthood on protein and mRNA expression of the genes and proteins of relevance to KOR/Dyn signaling. *Oprk* mouse opioid receptor kappa gene, *Pdyn* mouse prodynorphin gene, *KOR* kappa opioid receptor protein, *Dyn* dynorphin protein, *PND* post natal day, *NMRI* Naval Medical Research Institute, *ddY* Deutschland, Denken, and Yoken, *WKY* Wistar Kyoto, *LH* learned helplessness, *PAG* periaqueductal gray, *RVM* rostroventral medulla, *LC* locus coeruleus, *mPFC* medial prefrontal cortex, *ACC* anterior cingulate cortex, *NAc* nucleus accumbens, *DG* dentate gyrus, *CA3* cornu amos 3, *CPu* caudate putamen, *VTA* ventral tegmental area, *BNST* – bed nucleus of the stria terminals, *FC* – frontal cortex, *CC* – corpus callosum and *BLA* basolateral amygdala

reported in Table 1. For example, one study showed that early maternal separation of mice caused changes in *Oprk* in the amygdala and the PAG when measured later as adults at 10 weeks of age, although other regions were not affected (Nakamoto et al. 2020). Another study showed that social isolation of mice during early life decreased *Oprk* expression in the hippocampus and amygdala at weaning (Haj-Mirzaian et al. 2019). Exposure to predator stress during early life elevated expression of *Oprk* in adult rats, but only in females (Chang et al. 2019). Indeed, in other early life studies, marked sex differences occur that have been reported to impact the stress response (McCarthy et al. 2018). Additional work is needed to replicate these developmental patterns of response and extend them to other parameters.

The majority of studies have focused on the response of KOR/Dyn system in rodents when exposure to stress as adults. After acute or chronic immobilization stress, *Oprk* expression was increased in the NAc core and shell of adult male Sprague-Dawley rats 2 days after the cessation of stress and disappeared by 9 days post stress (Lucas et al. 2011). Dyn A and Dyn B contents were also found to be increased in the NAc shell and core of adult rats immediately following acute immobilization stress and following exposure to a learned helplessness paradigm (Shirayama et al. 2004). Increased *Pdyn* mRNA expression in the CPu, but not in other regions, was found immediately following exposure of rats to swim stress (Reed et al. 2012). Summarizing these data, stressors evoked an initial surge in *Oprk* expression in the NAc and CPu that diminished over time. In addition, genetic differences can contribute to variations as WKY rats, a genetic model of stress responsiveness, had higher levels of KOR protein and dynorphin in the NAc and piriform cortex than Sprague-Dawley rats (Carr et al. 2010).

Gene expression in the KOR/Dyn system has also been studied in mice and rats in response to chronic social defeat as a model of severe social stress. *Oprk* mRNA transcript in the NAc was unchanged in adult male mice when examined 48 h following the last defeat bout from a chronic social defeat paradigm for 10 days (Donahue et al. 2015). This time point may have been too late to detect the initial elevation described in other reports. When C57BL/6J mice were divided into either susceptible or resilient groups based on their social interaction scores, no significant changes in *Oprk* expression were found in the striatum of mice exposed to 10 consecutive days of social defeat (Browne et al. 2018). In contrast, regional differences in Sprague-Dawley rats subjected to social defeat for 7 days were reported according to their behavioral response pattern. Dyn levels were increased in the striatum of resilient rats but increased in the NAc shell of vulnerable rats (Berube et al. 2013). Finally, male Long-Evan rats given five defeat sessions showed reduced Dyn A concentrations in the hypothalamus of defeated animals.

Although the parameters of administering social defeat likely vary between laboratories, a species difference may contribute to this divergent pattern of results as elevated *Oprk* may be a positive indication in rats, but not in mice. On the other hand, the differences reported between these studies for mice may represent a specific stressor effect, as marked increases in striatal *Oprk* mRNA expression have been reported in the striatum of male C57BL/6J mice exposed to 21 days of unpredictable chronic mild stress (Falcon et al. 2016). Overall, these data suggest

that careful selection of stressor, species and evaluation time must be incorporated in future studies to determine differential *Oprk* expression in the ventral striatum.

A number of studies have focused on modulation of the KOR/Dyn system in the amygdala as this region is a critical hub in regulation of emotional salience and the behavioral responses to fearful stimuli. Early life (Haj-Mirzaian et al. 2019; Nakamoto et al. 2020), adolescent (Varlinskaya et al. 2018), and adult stress exposure (Falcon et al. 2016; Browne et al. 2018) have been reported to modulate the amygdala (Table 2). Divergent effects of early life stress paradigms on *Oprk* mRNA may be determined by the severity of the stressor employed. The stress paradigm utilized in the Nakamoto study incorporated maternal separation for 6 h per day during postnatal day 15–21 in male ddY mice followed by social isolation from PND 21 until the tissue collection at 10 weeks of age (Nakamoto et al. 2020). The stressor combination increased *Oprk* transcript in the amygdala. In contrast, the report of Haj-Mirzaian et al. (2019) identified marked decreases in *Oprk* mRNA expression in the amygdala of male NMRI mice that were socially isolated from PND 21–25. Similarly in adulthood, robust reductions in amygdala *Oprk* mRNA expression were found in male C57BL/6J mice that were susceptible to the behavioral deficits produced by 10 days of social defeat (Browne et al. 2018). These data suggest that social instability following weaning consistently decreases *Oprk* expression in the amygdala. Moreover, in adulthood, chronic stressors in general diminish *Oprk* mRNA expression in the amygdala. In contrast, forced swim stress on PND 33–35 was not sufficient to induce alterations in either *Oprk* or *Pdyn* expression in the basolateral nucleus of the amygdala (BLA) (Varlinskaya et al. 2020).

Overall, these data suggest that early life and adolescent stress, rather than social isolation alone, may alter the trajectory of *Oprk* and *Pdyn* expression in a differential manner to stressors which occur later in life. Clearly, more comprehensive analysis of stress evoked KOR mRNA and protein expression are required within defined regions of the amygdala and other regions. There remains a dearth of information pertaining to KOR expression and function during adolescence and development.

4.2 Rescue of Stress-Induced Behavioral Deficits by KOR Antagonists

Evidence endorsing the importance of KOR modulation in models of substance use disorders has been reviewed by others in recent years (Charbogne et al. 2014; Chavkin and Koob 2016; Karkhanis et al. 2017) and therefore the effects of KOR antagonists on stress-induced deficits in rodent models of substance use disorders will not be evaluated here. Just as reported in humans, KOR agonist administration induces dysphoria and aversion in rodents (Bruchas et al. 2007b, 2009; Land et al. 2008; Muschamp et al. 2011; Ehrich et al. 2015b). KOR antagonists consistently block these effects when administered prophylactically or concurrent with stress (Jacobson et al. 2020a).

There is an important difference between studies of drugs that prevent the effects of stress and drugs capable of reversing the effects of stress. From the literature review, it is apparent that in most preclinical studies KOR antagonists were administered prior to or concurrent with stress. These study designs were most likely influenced by the long-lasting activity of the KOR antagonists like norbinaltorphimine (norBNI), 6′-guanidinonaltrindole (GNTI), and JDTic (Munro et al. 2012). These antagonists reduce c-Jun N-terminal kinase (JNK) mediated signaling that prevents dynorphin or exogenous KOR agonist activation of KOR and may also last for up to 21 days following a single injection (Bruchas et al. 2007a; Melief et al. 2011). Studies that utilized prophylactic and concurrent administration of KOR antagonists have yielded important information regarding the role of KOR in mediating the response to stressful stimuli. However, to truly demonstrate the utility of KOR antagonists as potential psychotherapeutic compounds, they must be shown capable of reversing behavioral deficits induced by stress, rather than merely acting to prevent or dampen the effect of stress. A class of tests already exist where clinically effective antidepressants can reverse behavioral deficits that have been induced by chronic stress.

The sample of studies highlighted in Table 3 interrogated the effects of KOR antagonists administered following exposure to stress. These studies confirmed that KOR antagonists can reverse stress-induced behavioral deficits providing a solid rationale for developing KOR antagonists clinically as therapeutics for MDD and other long-lasting psychiatric disorders. As shown in Table 3, norBNI effectively reversed behavioral deficits produced by translationally relevant stress models that employ footshock, including learned helplessness (LH), contextual fear conditioning (CFC), and fear potentiated startle (FPS). Specifically, systemic administration of norBNI attenuated FPS (Knoll et al. 2007) and reduced the time spent freezing in the chamber (context) in which rodents were shocked during CFC training (Rogala et al. 2012; Szklarczyk et al. 2015). Microinfusion of norBNI into specific brain regions highlights the NAc, hippocampus, and amygdala as key sites of KOR antagonist engagement in rats exposed to the LH paradigm; decreased escape failures and escape latency were evident following bilateral infusion of norBNI into these regions (Newton et al. 2002). Reversal of FPS was also reported following systemic (Knoll et al. 2007) and intra-amygdala administration of JDTic (Knoll et al. 2011). In agreement with the norBNI studies, microinfusion studies with JDTic implicated the BLA and CeA as the critical sites for KOR mediated restoration of normal response in the presence of novel fearful and threatening stimuli. In one study, WKY rats were used as a genetic model of stress, compared to unstressed Sprague-Dawley rats, and were sensitive to norBNI reducing immobility in the FST when administered systemically or when infused directly into the piriform cortex, a region associated with the amygdala (Carr et al. 2010). Moreover, generalized fear in CFC exposed rats, assayed using the defensive withdrawal test, was attenuated by systemic norBNI injection (Rogala et al. 2012). Considering these data, evaluating the benefits of KOR antagonists in MDD subjects with a comorbid diagnosis of post-traumatic stress disorder is warranted.

Table 3 Reversal of stress-induced behavioral deficits by KOR antagonists in rodents

Reference	Subjects	Stressor	Antagonist	Test time	Behavior	Behavioral outcome
(Newton et al. 2002)	Male Sprague-Dawley rats	LH – 120 shocks 0.45 mA, 15 s duration	norBNI 2.5 μg, lateral ventricles (bilateral)	3 days after infusion	LH – escape failures and latency	Decreased escape failures and escape latency
			norBNI 2.5 μg infusions NAc (bilateral)			Decreased escape failures and escape latency
			norBNI 2.5 μg infusions hippocampus (bilateral)			No effect
(Shirayama et al. 2004)	Male Sprague-Dawley rats	LH – 60 shocks 0.65 mA, 30 s duration × 2 days	norBNI, 0.25 and 2.5 μg infusions DG (bilateral)	3 days after infusion	LH – escape failures and latency	2.5 μg decreased escape failures and latency
			norBNI, 0.25 and 2.5 μg infusions CA3 (bilateral)			Infusion of both doses into CA3 radiatum, but not oriens, decreased escape failures and latency
			norBNI, 0.25 and 2.5 μg infusion NAc (bilateral)			Both doses decreased escape failures
(Knoll et al. 2007)	Male Sprague-Dawley rats	FPS – 10 shocks 0.6 mA 0.5 s duration paired with 3.7 s light presentation	norBNI 3, 10, 30 mg/kg (i.p.)	24 h after injection	FPS – % startle	10 mg/kg decreased startle
			JDTic 3, 10, 30 mg/kg (i.p.)			10 mg/kg decreased startle
(Carey et al. 2009)	Male C57BL/6J mice	FSS – 1 pretest swim and 4 swim trials (6 min)	norBNI 10 mg/kg (i.p.)	1 h after injection	NOR – recognition index	Restored object recognition to control levels

(Rogala et al. 2012)	Male Sprague-Dawley rats	CFC- 5 shocks 1.5 mA 2 s duration	norBNI 15 & 30 mg/ml (s.c.)	6 days after injections	Novel square chamber – % time freezing	Non-significant decrease at 30 mg/kg
				7 and 25 days after injection	CFC – % time freezing	Decreased % freezing at 30 mg/kg in shocked animals 7 but not 25 days post injection
				22 days after injection	Large open field – % time freezing	Non-significant decrease at 30 mg/kg
				28 days after injection	Defensive withdrawal – latency to enter light compartment, time in withdrawal box	30 mg/kg decreased latency and time in withdrawal box in shock exposed rats
(Knoll et al. 2011)	Male Sprague-Dawley rats	FPS – 10 shocks 0.6 mA 0.5 s duration paired with 3.7 s light presentation	JDTic 0, 3 & 10 μg/5 μL bilateral infusions into BLA	24 h after injection	FPS – startle	Both doses decreased startle
			JDTic 0, 3 & 10 μg/5 μL bilateral infusions into CeA			10 μg does decreased startle
			JDTic 0, 3 & 10 μg/5 μL bilateral infusions into Str			No effect
(Peters et al. 2011)	Male and female Sprague-Dawley rats	CVS – third week of gestation E14–20	AZ-MTAB 30 μmol/kg, (s.c.)	2 h after injection (adults > PND 70)	EPM – time in open arm	Restored stress-suppressed open arm exploration
			Aticaprant 24 μmol/kg, (s.c.)			Restored stress-suppressed open arm exploration

(continued)

Table 3 (continued)

Reference	Subjects	Stressor	Antagonist	Test time	Behavior	Behavioral outcome
(Bruchas et al. 2011)	Male C57BL/6 mice	SD – 10 days	norBNI 10 mg/kg, (i.p.)	24 h after injection	SI – avoidance	Reversed social interaction deficits
(Bruning et al. 2015)	Male Swiss mice	SNI – 4 weeks post ligation	(m-CF3-PhSe)2 0.1–10 mg/kg (i.g.)	30 min after injection	FST – immobility	10 mg/kg reduced immobility
			(m-CF3-PhSe)2 0.1 mg/kg (i.g.) × 2 weeks	24 h after injection		Repeated 0.1 mg/kg decreased immobility scores
(Szklarczyk et al. 2015)	Male SWR/J mice	CFC – 5 shocks 1 mA 2 s duration	norBNI 10 mg/kg (i.p.)	2 days post injection	CFC – % freezing	No effect
(Browne et al. 2018)	Male C57BL/6J mice	SD – 10 days	Aticaprant 1 mg/kg (i.p.) × 1 or 7 days	24 h after final injection	SI – avoidance	No effect
(Laman-Maharg et al. 2018)	Female California mice	SD – 3 days	norBNI 10 mg/kg (i.p.)	24 h after injection	FSS – immobility across all four trials	No effect
(Williams et al. 2018)	Female California mice	SD – 3 days	AZ-MTAB 10 mg/kg (i.p.)	2 h after injection	SI – time in interaction zone, head orientation	No effect
					SPT – % sucrose	No effect
(Haj-Mirzaian et al. 2019)	Male NMRI mice; PND 21–25	SIS – 5 weeks	norBNI 5 mg/kg (i.p.)	30 min after injection	FST – immobility	Reduced immobility in the FST in socially conditioned but not socially isolated mice
					ST – reduced grooming	No effect
					HBT – reduced head dips	No effect
					OFT – time in center, rearing and distance moved	No effect

(Jacobson et al. 2020b)	Male C57BL/6J mice	UCMS – 28 days	Aticaprant 10 mg/kg (i.p.) 11 days	24 h after injection. 5 days post stress cessation	SPT – % sucrose	Normalized sucrose preference
				10 days after final injection. 15 days post stress cessation		Normalized sucrose preference
				24 h after injection. 1-day post stress cessation	SI – discrimination index	No effect
				11 days after final injection. 16 days post stress cessation		No effect
				24 h after injection. Day 22 of stress	NB – final nest score	Reversed stress-induced nesting impairments
				24 h after injection. Day of stress cessation		Reversed stress-induced nesting impairments
				13 days after final injection. 18 days post stress cessation		Reversed stress-induced nesting impairments
				24 h after injection. 2 days post stress cessation	LD – time in light	No effect

(continued)

Table 3 (continued)

Reference	Subjects	Stressor	Antagonist	Test time	Behavior	Behavioral outcome
				12 days after final injection. 17 days post stress cessation		No effect
				24 h after injection. 3 days post stress cessation	FST – immobility	Normalized immobility scores
				15 days after final injection. 20 days post stress cessation		Normalized immobility scores
				24 h after injection. 4 days post stress cessation	HP – latency to lick hind paw/jump	Reversed stress-induced hyperalgesia
				14 days after final injection. 19 days post stress cessation		Reversed stress-induced hyperalgesia

Summarized in this table are the effects of KOR antagonists **JDTic** – (3R)-7-Hydroxy-N-[(2S)-1-[(3R,4R)-4-(3-hydroxyphenyl)-3,4-dimethylpiperidin-1-yl]-3-methylbutan-2-yl]-1,2,3,4-tetrahydroisoquinoline-3-carboxamide, **norBNI** – norbinaltorphimine), **AZ-MTAB** – (3-((1R,3r,5S)-8-((5-MethylThiophen-2-yl) Methyl)-8-Azabicyclo[3.2.1]octan-3-yloxy)Benzamide, **aticaprant** and **(*m*-CF3-PhSe)$_2$ –** *m*-Trifluoromethyl-diphenyl diselenide on stress-induced behavioral deficits in mice and rats. *LH* learned helplessness, *CFC* contextual fear conditioning, *FPS* fear potentiated startle, *SIS* social isolation stress, *CUS* chronic unpredictable stress, *CVS* chronic variable stress, *SD* social defeat, *FSS* forced swim stress, *SNI* sciatic nerve ligation, *NOR* novel object recognition, *EPM* elevated plus maze, *SI* social interaction, *FST* forced swim test, *SPT* sucrose preference test, *ST* splash test, *HBT* hole board test, *OFT* open field test, *NB* nesting behavior, *LD* light dark box, *HP* hot plate, *NMRI* Naval Medical Research Institute, *NAc* nucleus accumbens, *CA3* cornu amos 3, *DG* dentate gyrus, *BLA* basolateral nucleus of the amygdala, *CeA* central nucleus of the amygdala, *Str* striatum, *mA* milliamp, *i.p.* intraperitoneal, *i.g.* intragastric, *s.c.* subcutaneous

It is clear from Table 3 that more extensive evaluation of KOR antagonists across a range of behavioral endophenotypes will be required to evaluate their value. For example, only one study explored stress-induced cognitive deficits in mice (Carey et al. 2009). In that study, norBNI effectively returned novel object recognition to the levels exhibited by stress naïve mice (Carey et al. 2009). If this pro-cognitive effect of KOR antagonists in stressed subjects is replicated, it would further strengthen the rationale for pursuing KOR antagonists as therapeutics for MDD, as very few antidepressant compounds target reversal of cognitive deficits in MDD (Jacobson et al. 2018).

Social withdrawal is another common feature of depressed subjects (Fernández-Theoduloz et al. 2019; Suffel et al. 2020) and studies have evaluated the effects of KOR antagonists on deficits of social behavior in rodents. With regard to social avoidance or social interaction deficits produced by stress, only norBNI effectively restored social interaction in mice (Bruchas et al. 2011). The shorter acting compounds AZ-MTAB (Williams et al. 2018) and aticaprant (Browne et al. 2018; Jacobson et al. 2020b) were unable to reverse social avoidance produced in mice by chronic social defeat or chronic stress. This lack of effect on social avoidance may be specific to the shorter acting KOR antagonists as repeated administration of aticaprant failed to alter social avoidance in chronically stressed mice (Browne et al. 2018; Jacobson et al. 2020b). Conversely, 1 week of aticaprant treatment was sufficient to restore sucrose preference in stressed mice, an effect that persisted for at least 10 days following cessation of treatment (Jacobson et al. 2020b). Repeated administration of AZ-MTAB was not evaluated on reward related behaviors (Williams et al. 2018), but it is possible that AZ-MTAB may also rescue deficits in sucrose preference from chronic stress upon repeated dosing. These findings suggest that going forward it may be useful to screen and differentiate KOR antagonists on constructs of social and non-social anhedonia. This differentiation may be helpful in evaluating the likelihood of whether preclinical findings can be translated successfully into clinical practice. It has already been established that aticaprant engages the ventral striatum during rewarding tasks in subjects with clinically severe anhedonia (Krystal et al. 2020; Pizzagalli et al. 2020). However, the effects on the overall SHAPS score and depression rating scales were quite minimal, although the dose of aticaprant might be increased in future trials. Based on the preclinical evidence, it may be worthwhile to review the data pertaining to social reward and social avoidance relative to non-social anhedonia parameters on the rating scales of the subjects included in the FAST MAS trial.

5 Conclusion

The data collated in this review support the potential for KOR antagonists to alleviate the symptoms of MDD. Clear regional differences in *PDYN* and *OPRK* mRNA expression are evident in clinical MDD populations that are recapitulated in preclinical stress studies. The potential to exploit serum KOR and DYN as a biomarker capable of differentiating depressed subjects relative to controls in their

first depressive episode is intriguing. This biomarker has not seen development in preclinical studies. Similarly, the utility of KOR binding as a neuroimaging biomarker of MDD may be developed to help address the lack of studies following the course of KOR function in MDD. However, these tools are in the nascent stages of development. Translationally relevant preclinical studies continue to inform clinical evaluation of KOR antagonists in terms of neurochemical correlates, sites of action, and behavior. This review illustrated only a select portion of the preclinical literature that has demonstrated the anti-stress effects of KOR antagonists. Combined with the encouraging results obtained in the NIMH sponsored Fast MAS Trial, the rationale for pursuing KOR antagonism as a therapeutic strategy for the treatment of MDD has never been stronger.

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The Role of Dynorphin and the Kappa Opioid Receptor in Schizophrenia and Major Depressive Disorder: A Translational Approach

Samuel David Clark

Contents

Abstract

The kappa opioid receptor (KOR) and its endogenous ligands dynorphins (DYN) have been implicated in the development or symptomatology of a variety of neuropsychiatric disorders. This review covers a brief history of the development

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of KOR agonists and antagonists, their effects in healthy volunteers, and the potential role of DYN/KOR dysfunction in schizophrenia and major depressive disorder from a translational perspective. The potential role of DYN/KOR dysfunction in schizophrenia is based on several lines of evidence. Selective KOR agonists induce affective states in healthy volunteers with similarities to the symptoms of schizophrenia. Studies have shown increased DYN in patients with schizophrenia, although the data have been mixed. Finally, meta-analytic data have shown that opioid antagonists are associated with reductions in the symptoms of schizophrenia. The potential role of DYN/KOR dysfunction in major depressive disorder is also based on a combination of preclinical and clinical data. Selective KOR agonists have shown pro-depressive effects in human volunteers, while selective KOR antagonists have shown robust efficacy in several preclinical models of antidepressant activity. Small studies have shown that nonselective KOR antagonists may have efficacy in treatment-resistant depression. Additionally, recent clinical data have shown that the KOR may be an effective target for treating anhedonia, a finding relevant to both schizophrenia and depression. Finally, recommendations are provided for translating preclinical models for schizophrenia and major depressive disorder into the clinic.

Keywords

Buprenorphine · Kappa opioid receptor · Major depressive disorder · Naloxone · Naltrexone · Schizophrenia

1 Introduction

The kappa opioid receptor (KOR) and its endogenous ligands dynorphins (DYN) (Chavkin and Goldstein 1981; Chavkin et al. 1982) have been implicated in the development or symptomatology of a variety of neuropsychiatric disorders including schizophrenia, depression, anxiety, and substance use disorders (cocaine, alcohol, opiate, and nicotine) (Browne and Lucki 2019; Callaghan et al. 2018; Clark and Abi-Dargham 2019; Jacobson et al. 2020; Koob and Volkow 2010; Li et al. 2016; Lutz and Kieffer 2013; Zhang et al. 2007). Indeed, there is already a large body of supporting clinical evidence for an effect of nonselective KOR antagonists on these disorders although the evidence is mixed. Despite strong preclinical evidence for a therapeutic effect of targeting KORs, there have been a number of failed attempts at translating preclinical models into clinical results and there are currently no United States Food and Drug Administration (FDA) or European Medicines Agency (EMA) approved therapeutics that selectively target the KOR.

In this review I will cover the evidence for involvement of DYN and KOR in two neuropsychiatric disorders: schizophrenia and major depressive disorder (MDD). This review will take a translational perspective and will cover the different clinical successes and failures of targeting the KOR with a focus on potential clinical strategies for translating preclinical evidence into the clinic.

2 KOR Agonists in Preclinical Models and Healthy Volunteers

In preclinical models selective KOR agonists including U50488 (Vonvoigtlander et al. 1983) and U69593 (Lahti et al. 1985) have been shown to produce depressive-like and anxiogenic effects on a number of behavioral screening tests for depression. Historically, it was during the development of new analgesics when the first selective KOR agonists were tested in humans. During this time, the selective KOR agonist spiradoline was also advanced into clinical trials for Parkinson's disease (Giuffra et al. 1993). Selective KOR agonists were shown to produce negative affective states including cognitive deficits, confusion, dysphoria, anxiety (Pfeiffer et al. 1986), and psychotomimetic side effects that included visual hallucinations, auditory disturbances (hearing real voices as distorted (Walsh et al. 2001)), full auditory hallucinations (hearing voices that were not present (Resnick et al. 1971)), and paranoid delusions of persecution (Walsh et al. 2001). In one notable example, 10 min after an acute injection of the selective KOR agonist enadoline a healthy volunteer became aggressive during a delusion that the staff were attempting to harm him by conspiring to "ruin his mind" (Walsh et al. 2001). These side effects have been reported across a number of clinical trials of synthetic selective KOR agonists including MR 2033/2034 (Pfeiffer et al. 1986), enadoline (Hunter et al. 1990; Reece et al. 1994; Walsh et al. 2001), niravoline (Gadano et al. 2000), bremazocine (Dortch-Carnes and Potter 2005), and spiradoline (Giuffra et al. 1993; Wadenberg 2003). Salvinorin A, a naturally occurring selective KOR agonist isolated from the psychoactive sage plant *Salvia divinorum* (Roth et al. 2002), produced psychotomimetic effects in both open label (Addy 2012) and double-blind placebo-controlled studies (MacLean et al. 2013; Maqueda et al. 2016). One such study also reported cognitive disruption (difficulty concentrating) (Addy 2012). Additionally, less selective KOR agonist/mu opioid receptor (MOR) antagonists cyclazocine (4.0 mg daily) and ketocyclazocine (0.6–1.2 mg daily) produce cognitive deficits, detachment, and paranoia including multiple reports of feelings of being watched, visual hallucinations which included seeing "monsters," and auditory hallucinations of voices (Hanlon et al. 1975; Kumor et al. 1986; Resnick et al. 1971).

There are some exceptions to the dysphoric and psychotomimetic effects of KOR agonists in healthy volunteers using low dosages. Fink and colleagues (Fink et al. 1970) described two studies of a range of doses of cyclazocine (0.2–3.0 mg daily) to treat depressive symptoms. In 10 patients with depressive symptoms, cyclazocine produced an antidepressant effect over 3–8 months. In a second group, 19 patients, who had treatment-resistant symptoms of depression from a variety of different primary psychiatric diagnoses, were treated with cyclazocine for 1–34 weeks. Over this time period, 10 patients showed improvements in depressive symptoms although the authors note a narrow therapeutic index. Side effects included dysphoria, visual hallucinations, and auditory hallucinations in some patients (Fink et al. 1970). In most cases the side effects occurred with dosages above 1.4 mg, however, two patients experienced side effects at the lowest dosages.

Additionally, salvinorin A has a long history of medicinal and religious use by the indigenous Mazatec people (Maqueda 2018). Low dose salvinorin A from *Salvia*

divinorum tea has been reported to have an antidepressant effect in a case report (Hanes 2001). Consistent with this, preclinical studies have shown that low dosages of salvinorin A produce anxiolytic effects (Braida et al. 2009) and conditioned place preference in mice (Braida et al. 2008).

2.1 Selective KOR Antagonists

KOR antagonists fit into two categories broadly: long-acting noncompetitive antagonists, also referred to as receptor inactivating, which cause permanent inactivation of the KOR and also activate c-Jun terminal kinase (JNK) (Bruchas et al. 2007; Schattauer et al. 2017); and short-acting competitive antagonists which cause temporary blockade of the KOR and do not activate JNK. One proposed mechanism for their long duration of effect is their very long half-life in the brain (Kishioka et al. 2013; Patkar et al. 2013). However, their effects can be blocked by pretreatment with a rapidly cleared short-acting competitive KOR antagonist indicating that the long duration of effects may not be due to their long half-lives in the brain (Bruchas et al. 2007). Béguin and Cohen (2009) provide a thorough review of the history of KOR antagonist development. Unlike KOR agonists, which exert acute effects within minutes, KOR antagonists have not shown acute effects on baseline mood when administered to healthy volunteers (Reed et al. 2018). Preclinical studies have shown that KOR antagonists do not have inherently rewarding effects as shown by a lack of effect on intracranial self-stimulation (Mague et al. 2003; Todtenkopf et al. 2004).

The first long-acting KOR antagonist, norbinaltorphimine (norBNI) was rationally designed from two naltrexone derived pharmacophores linked with a spacer (Portoghese et al. 1987). It displays unusual pharmacokinetics and KOR blockade. norBNI levels have been shown to last 21 days from a single injection (Horan et al. 1992; Jones and Holtzman 1992; Kishioka et al. 2013; Patkar et al. 2013). NorBNI was never administered to humans although it remains among the most widely used KOR antagonist for preclinical research. Interestingly, one recent study has shown that small dosages approximately 100-fold less than a typical dosage can build up to a cumulative effective dose over a 1-month period of daily administration (Chavkin et al. 2019). As the effects of a full dosage of receptor inactivating KOR antagonists cannot be rapidly reversed, this may represent a safer dosing protocol for antagonists in this class.

The second long acting KOR antagonist, 5′-guanidinonaltrindole (5'-GNTI) was developed as a more potent and selective norBNI derivative (Jones et al. 1998), although it has low bioavailability through oral administration. Its analogue 5-′-acetamidinoethylnaltrindole (ANTI) has improved lipophilicity and is orally active (Stevens et al. 2000). 5'-GNTI and ANTI were also never developed beyond use in preclinical models and have not been administered to humans.

Finally, (3R)-7-hydroxy-N-((1S)-1-[[(3R,4R)-4-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidinyl]methyl]-2-methylpropyl)-1,2,3,4-tetrahydro-3-isoquinolinecarboxamide (JDTic), a long acting receptor inactivating small molecule, was developed at the Research Triangle Institute (Thomas et al. 2001). The first

selective KOR antagonist to enter the clinic, it also showed unusual pharmacokinetics with a long half-life of ~9 days (Munro et al. 2012). Unfortunately, it was discontinued in phase 1 when two participants exhibited several beats of ventricular tachycardia, a finding that was also observed in non-human primates (Buda et al. 2015). However, questions remained as to whether JDTic was responsible for these effects (Chavkin and Martinez 2015).

JNJ-67953964/LY2456302 (Rorick-Kehn et al. 2014) is the first short-acting selective KOR antagonist to show efficacy in a clinical trial in humans (Krystal et al. 2019). The compound was formerly known as LY2456302, CERC-501, and OpraKappa. Two related compounds, LY2444296 and LY2795050, are in preclinical use, but were not advanced into the clinic (Butelman et al. 2019; Valenza et al. 2017). A second clinical stage short-acting KOR antagonist is BTRX-140 (Guerrero et al. 2019). Other small molecule antagonists include AZ-MTAB, for which development was discontinued due to hERG liability (Brugel et al. 2010), and PF-04455242 (Grimwood et al. 2011), which was discontinued in phase 1 when toxicity issues in preclinical animal models were discovered (Urbano et al. 2014). There are also a number of preclinical short-acting peptide KOR antagonists including zyklophin (Aldrich et al. 2009) and [d-Trp]CJ-15,208 (Eans et al. 2013), a derivative of CJ-15,208 which was discovered by Pfizer Japan from the fungus, *Ctenomyces serratus* (Saito et al. 2002).

2.2 Nonselective Opioid Antagonists

Naloxone, naltrexone, nalmefene, and buprenorphine are nonselective opioid antagonists that have shown efficacy in a variety of psychiatric conditions in humans. Naloxone, naltrexone, and nalmefene have historically been thought to be pan-opioid antagonists at the KOR, MOR, and delta opioid receptor (DOR), while buprenorphine has been shown to be a KOR antagonist and MOR partial agonist (Toll et al. 1998). However, some studies have shown evidence for partial KOR agonist effects of nalmefene and naltrexone (Butelman et al. 2020). Of note, neither naltrexone nor nalmefene has been shown to produce psychotomimetic effects, indicating that any partial agonism of the KOR is too weak to trigger this mechanism. Importantly, the psychotomimetic effects induced by KOR agonists can be blocked, and rapidly be reversed by treatment with naloxone (Jasinski et al. 1968; Watson et al. 1978) and naltrexone (Maqueda et al. 2016).

3 Therapeutic Targeting of the DYN/KOR System in Schizophrenia

Schizophrenia, a debilitating illness with a prevalence of 0.749% worldwide (Moreno-Kustner et al. 2018), is characterized by positive, negative, and cognitive symptoms. The positive symptoms comprise hallucinations, delusions, and disorganization in speech and behavior, and the negative symptoms comprise flattened

affect and social withdrawal. The cognitive symptoms comprise deficits in memory and cognitive function (Association 2013; Nuechterlein et al. 2004).

There are a large number of FDA- and EMA-approved antipsychotics, and a meta-analysis has revealed some variability in efficacy and tolerability (Huhn et al. 2019), however, these only treat the positive symptoms of schizophrenia and there are currently no FDA- or EMA-approved drugs for the treatment of negative or cognitive symptoms. Additionally, approximately 30% of patients do not respond to first line antipsychotic therapy (Kane et al. 1988), representing an enormous unmet medical need.

3.1 Evidence for Abnormal DYN Signaling in Schizophrenia

There is some evidence for disruption of DYN signaling in schizophrenia with at least three studies examining DYN levels, and a number of other studies looking at other opioid peptides in the cerebrospinal fluid (CSF) of patients with schizophrenia. In the first study of DYN levels in schizophrenia, Zhang et al. (1985) measured DYN (type unspecified) in the CSF of 35 first-break medication-free patients with schizophrenia and 35 neurological patients with a wide variety of neurological diseases including tumors. They found that the DYN levels in patients with schizophrenia were significantly lower than the patients with neurological diseases, however this study did not include a healthy control group. In contrast, the second study by Heikkilä et al. (1990) examined DYN A levels from the CSF of 10 unmedicated patients with schizophrenia, 10 patients with other psychiatric conditions, and 10 healthy controls. They found significantly higher DYN A levels in the unmedicated patients with schizophrenia, compared to the healthy control group and a trend effect when compared with the patients with other psychiatric illnesses. The average DYN A levels in the patients with schizophrenia showed a decrease after 1 month of treatment with antipsychotics but this did not reach statistical significance. Interestingly, there was a significant correlation between the level of DYN A and symptoms assessed via the Brief Psychiatric Rating Scale (BPRS) with higher levels of DYN A associated with worse psychopathology. Following this study, Lindtrom (1996) performed a 5-year longitudinal study of 120 patients with schizophrenia. This study measured CSF DYN A levels in medication-free patients upon admission (including 66 patients who had never been treated). After biomarker collection antipsychotic therapy was initiated and patients were followed for 5 years. Outcomes were assessed at 1, 3, and 5 years via the Strauss-Carpenter outcome scale. Lindtrom reported that higher DYN A levels at admission were significantly associated with worse outcomes at 5 years. He also found that negative symptom severity at admission, but not positive symptom severity was also associated with worse 5-year outcomes. Finally, regarding other opioid peptides that have been shown to interact with the KOR (Fricker et al. 2020), a number of studies in the late 1970s have found increased beta-endorphin and other unspecified opioid peptides referred to as “endorphins” in patients with schizophrenia compared to healthy volunteers (Domschke et al. 1979; Lindström et al. 1986; Lindtröm et al. 1978), although

the data have been mixed with at least one study finding no differences (Ross et al. 1979).

3.2 Preclinical Evidence from Selective KOR Agonists

As covered previously, in healthy volunteers KOR agonists produce acute psychotomimetic effects including paranoid delusions and auditory hallucinations that have similarities to the positive symptoms of schizophrenia. They also produce negative affective states, including dysphoria and cognitive disruption, which may have similarities to the negative and cognitive symptoms of schizophrenia.

While the positive symptoms of schizophrenia are not clearly able to be modeled in rodents, KOR agonists produce depressive-like symptoms in rodents that may have translational relevance to the negative symptoms of schizophrenia. KOR agonists are aversive to rodents as measured by conditioned place aversion (Bals-Kubik et al. 1993). Acute administration of U50488 (Dogra et al. 2016) or salvinorin A (Butelman et al. 2019) has both been shown to produce depressive-like effects on rodent measures of anhedonia. Further testing with salvinorin A shows that these measures can be reversed by treatment with short-acting KOR antagonists LY2444296 and LY2795050 (Butelman et al. 2019). U69593 has been shown to reduce rewarding effects of positive stimuli as measured by an increase in the intracranial self-stimulation (ICSS) threshold in rats, an effect that can be blocked by KOR antagonist ANTI (Todtenkopf et al. 2004). Selective KOR antagonist JNJ-67953964/LY2456302 has also shown efficacy at reducing anhedonia in patients with clinically significant anhedonia, suggesting a potential role for KOR antagonists in treating anhedonia in the context of the negative symptoms of schizophrenia (Krystal et al. 2019).

Finally, KOR agonists produce disruptions in cognition in both rodents and non-human primates that may have translational relevance to the cognitive symptoms of schizophrenia. Acute administration of U50,488 has been shown to disrupt cognition measured via prepulse inhibition (PPI) (Bortolato et al. 2005), the 5-choice serial reaction time task (5CSRTT) in rats (Shannon et al. 2007), and the differential reinforcement of low response rate task (DRL) in mice (Abraham et al. 2018). Salvinorin A has also been shown to disrupt PPI in mice (Yan et al. 2009). However, one study found that U50,488, U69,593, and salvinorin A did not have any effect on PPI in rats (Tejeda et al. 2010). Additionally, U69593 and GR89,696 (Shannon et al. 2007) and salvinorin A (Braida et al. 2011) have also been shown to disrupt performance on the 5CSRTT in rats. In non-human primates, enadoline causes disruption in cognition measured with the cognitive performance task (Davis et al. 1992). Finally, naltrexone (Shannon et al. 2007) was shown to reverse, and JDTic (Nemeth et al. 2010) and norBNI (Abraham et al. 2018) have all been shown to block the cognitive deficits induced by acute administration of KOR agonists in rodents.

3.3 Treatment with Nonselective Opioid Antagonists Is Significantly Associated with Reductions in the Symptoms of Schizophrenia

To date, there have been more clinical trials of nonselective opioid antagonists for schizophrenia than any other mood disorder, with over 50 clinical trials as well as a number of case reports spanning 4 nonselective opioid antagonists: buprenorphine, nalmefene, naloxone, and naltrexone (Clark et al. 2020). The premise for these studies was based on an initial study performed by Gunne and colleagues in 1977 who reported an acute antipsychotic effect of naloxone injection in 4 out of 6 patients with schizophrenia (Gunne et al. 1977). This high-profile finding set off a number of clinical trials attempting to replicate the results with a variety of different designs, drugs, and clinical endpoints.

The findings from these studies included acute antipsychotic effects reported in short trials and effects on negative symptoms in longer trials. Despite these interesting findings from a relatively large number of trials, many of them had very small sample sizes, and did not contain placebo controls. Overall, approximately half of these trials did not find any significant effect which makes it difficult to derive any definitive results from any individual trial (Clark and Abi-Dargham 2019). To address this, a recent meta-analysis was conducted on the double-blind placebo-controlled trials of opioid antagonists in patients with schizophrenia (Clark et al. 2020). Pooling the trials together resulted in a dataset of 434 patients from 30 trials. When combining the data from the four different opioid antagonists (buprenorphine, nalmefene, naloxone, and naltrexone) they found a significant effect of all drugs combined on the symptoms of schizophrenia combined across all clinical scales (comprising positive, negative, and general). Additionally, a significant effect of all drugs on the positive symptoms (measured by pooling results from scales that only measured positive symptoms such as hallucinations or delusions) was found. Finally, significant effects on hallucinations and delusions individually were found suggesting a potential antipsychotic effect. Unfortunately, there were not enough trials to power the analysis on negative symptoms, and while a large effect size was found using Hedge's g (Hedges and Olkin 1985) ($g = 0.66$) it did not reach significance due to underpowering.

Most of the trials assessed (22 out of 30) utilized opioid antagonists as adjunctive therapy which suggests they may have efficacy in this setting. However, a moderator analysis of trials that reported information where chlorpromazine equivalents could be calculated suggested that the effect size was reduced when combined with higher dosages of antipsychotics. Since the average dose was quite high (avg 773 mg per patient) it remains to be seen whether optimal efficacy would be achieved through adjunctive therapy or monotherapy. It is hypothesized that the potential efficacy of nonselective opioid antagonists for treating schizophrenia is due to their ability to reduce signaling through the KOR. However, effects on the MOR and DOR cannot be ruled out and one recent positron emission tomography (PET) study has reported changes in the MOR system in patients with schizophrenia (Ashok et al. 2019).

While this meta-analysis shows a significant association between opioid antagonists and reductions in the symptoms of schizophrenia, definitive conclusions

cannot be drawn until opioid antagonists have been trialed in large randomized double-blind placebo-controlled trials.

3.4 Conclusions and Recommendations for Translating Preclinical Findings to the Clinic

Based on the entirety of these data discussed here, there is strong rationale for advancing nonselective opioid antagonists into well-powered randomized double-blind placebo-controlled trials in patients with schizophrenia. However, it is proposed that selective KOR antagonists could be more effective, as off- target effects on the MOR may limit the efficacy of nonselective KOR antagonists in treating the negative symptoms. Future trials will be necessary to examine the appropriate duration of treatment and whether monotherapy or adjunctive therapy with standard of care antipsychotics would be more effective. Many of the clinical trials of nonselective opioid antagonists suggest there may be an acute antipsychotic effect, but due to the mixed data no definitive conclusions can be drawn.

As schizophrenia is a heterogeneous disorder, it is possible that DYN/KOR dysfunction affects a subpopulation of patients who might benefit most from therapeutic targeting of this system. Genetic studies have been limited with one study finding a significant interaction between a polymorphism of pDYN gene and a polymorphism of the dopamine receptor 3 gene DRD3 in 114 patients with schizophrenia compared to 138 healthy controls (Ventriglia et al. 2002). Another study in 250 patients with schizophrenia found a significant association of a pDYN polymorphism with population susceptibility to schizophrenia (Zhang et al. 2004). PET imaging with KOR selective radiotracers may help determine whether there is overactivation of the KOR system in patients with schizophrenia. Additional future studies should also re-examine whether there is elevated DYN in the CSF of patients with schizophrenia as this may provide a potential biomarker.

There are a number of preclinical studies that could provide important translational information. It has been hypothesized that DYN/KOR dysfunction may play a role in the symptoms of schizophrenia through modulating dopamine signaling and potentiating D2 receptor super-sensitivity (Clark and Abi-Dargham 2019). For review of the potential circuitry involved, please see (Shekhar 2019). It would be useful to perform preclinical studies combining KOR antagonists with different standard of care antipsychotics and measure effects on behavior as well as on cortical and striatal dopamine levels via in vivo microdialysis. Additionally, for preclinical modeling of the effects of overactive DYN/KOR, chronic administration of selective KOR agonists may provide a more useful picture of effects on dopamine than studies utilizing a single administration paradigm.

4 Therapeutic Targeting of the DYN/KOR System in Major Depressive Disorder

Major depressive disorder (MDD) is a neuropsychiatric illness with a lifetime prevalence of 20.6% in the USA (Hasin et al. 2018). Approximately 35% of patients experience a recurrent disease course (Eaton et al. 2008). Symptoms defined in the DSM-5 include depressed mood, anhedonia, appetite changes, sleep disturbances, fatigue, and cognitive dysfunction such as problems with memory, psychomotor changes, feelings of worthlessness or excessive guilt, and suicidal ideation or behavior (Association 2013).

Current first line pharmacotherapy is often selective serotonin reuptake inhibitors (SSRIs) (Marcus and Olfson 2010), however SSRIs have a slow onset of action and efficacy is often achieved over several weeks to months (Nierenberg et al. 2000). The DYN/KOR system has been shown to be an attractive target for the treatment of MDD (Browne and Lucki 2019; Callaghan et al. 2018; Jacobson et al. 2020; Li et al. 2016; Lutz and Kieffer 2013; Zhang et al. 2007). Here I will review the clinical and preclinical evidence for potential DYN/KOR dysfunction in MDD from a translational perspective.

4.1 Preclinical Evidence for KOR Antidepressant Activity

As previously mentioned, KOR agonists produce negative affective states in healthy volunteers that may have similarities to the symptoms that occur in MDD. There is also a wealth of preclinical data supporting a potential antidepressant effect of KOR antagonists. Much of the preclinical evidence comes from studies utilizing the forced swim test (FST) developed in rodent models (Porsolt et al. 1977). Both tricyclic and SSRI antidepressants reduce immobility on the FST (Detke et al. 1995).

Consistent with their depressive-like effects in humans, selective KOR agonists increase depressive-like behavior on the FST and reduce time to immobility and total immobility time as shown by U69593 in rats (Mague et al. 2003), U50488 in mice (Dogra et al. 2016), and salvinorin A in rats (Carlezon et al. 2006).

A link between stressful conditions and increased DYN/KOR signaling has been demonstrated as stress via exposure to the FST increases pDYN mRNA levels (Chartoff et al. 2009; Reed et al. 2012). pDYN knock out (KO) mice exposed to the repeated FST do not show increased immobility whereas wild type (WT) mice show increased immobility on repeated exposure (McLaughlin et al. 2003). Rats exposed to the FST, the learned helplessness (LH) model of depression, or immobilization stress showed increased dynorphin A and B in different brain regions including the hippocampus and the nucleus accumbens core (NAc) (Shirayama et al. 2004). DYN signaling may be necessary for negative associations with stressful aversive conditions as norBNI and pDYN KO both blocked conditioned aversion to a neutral odorant cue paired with stress via the FST (Land et al. 2008).

4.2 KOR Antagonists Show Efficacy in the FST Anti-depressant Screening Test

Both long- and short-acting KOR antagonists have been shown to reduce depressive-like behavior in the FST as measured by increased latency to immobility or reduced total immobility time. Studies have shown that intracerebroventricular (ICV) infusion of either norBNI (Mague et al. 2003; Pliakas et al. 2001) or 5'-GNTI (Mague et al. 2003) decreased immobility in the FST in a dose-dependent manner in rats. Additionally, systemic norBNI reduces immobility in the FST in mice (Falcon et al. 2016; Laman-Maharg et al. 2018; McLaughlin et al. 2003) and rats (Reed et al. 2012). Similarly, JDTic reduced immobility at higher dosages tested in the FST in rats; however, in this specific experiment, both norBNI and desipramine had no effect (Beardsley et al. 2005). Systemic ANTI decreases immobility in the FST in rats while systemic 5'-GNTI had no effect likely due to its poor bioavailability (Mague et al. 2003).

Short-acting KOR antagonists have shown similar effects to long-acting KOR antagonists in the FST, suggesting that this paradigm is not dependent on the receptor inactivating effects of long-acting KOR antagonists. PF-04455242 (Grimwood et al. 2011), JNJ-67953964 / LY2456302 (Rorick-Kehn et al. 2014; Wang et al. 2017), LY2444296 (Butelman et al. 2019; Huang et al. 2016a), and LY2795050 (Butelman et al. 2019) all reduce immobility in the FST. Additionally, the reduced time to immobility produced by JNJ-67953964/LY2456302 was shown to be of a similar magnitude to a therapeutic dosage of tricyclic antidepressant imipramine (Rorick-Kehn et al. 2014). Although there are some conflicting data, one study found that LY2444296 did not alter immobility time in the FST in rats (Valenza et al. 2017).

4.3 Other Preclinical Models of Depression and Anxiety

In addition to the robust effects observed in the FST, both competitive and noncompetitive KOR antagonists have shown efficacy in other preclinical models of depression and anxiety. Two tests of anxiety include the elevated plus maze (EPM) (Pellow et al. 1985) and the open field (OF) test (Hall 1934). In the EPM the evidence is mixed. Both short-acting JNJ-67953964/LY2456302 (Wang et al. 2017) and long-acting norBNI (Huang et al. 2016b; Knoll et al. 2007) and JDTic (Knoll et al. 2007) increased entries into the open arms and time spent in the open arms in mice (Wang et al. 2017), while zyklophin had no effect in mice (Huang et al. 2016b) and LY2444296 had no effect in either mice (Huang et al. 2016b) or rats (Valenza et al. 2017). In the novelty-induced hypophagia test of anxiety in mice LY2444296, zyklophin, and norBNI all decreased latency to palatable food consumption in novel, but not training, cages (Huang et al. 2016b). Pretreatment with norBNI and JDTic reduced learned fear in the fear-potentiated startle paradigm (Knoll et al. 2007) and norBNI infusion into hippocampus and NAc reduced escape failure on the LH

paradigm (Shirayama et al. 2004). Additionally, both norBNI and pDYN KO blocked conditioned aversion to foot shock (Land et al. 2008).

4.4 Nonselective KOR Antagonists as Antidepressants: Evidence from Buprenorphine and ALKS-5461

Buprenorphine has been hypothesized to exert its antidepressant activity primarily through blocking the KOR. Chronic mild stress induces changes in pDYN mRNA and KOR (Oprk1) mRNA that are normalized with 7 days of buprenorphine (Falcon et al. 2016). Studies in rodent models of WT mice and opioid receptor KO have shown that the antidepressant effects of buprenorphine in the FST and unpredictable chronic mild stress (UCMS) are mediated through the KOR in mice (Falcon et al. 2016). The ability of buprenorphine to reduce measures of anhedonia and anxiety is blocked in KOR KO mice, but not in MOR KO or DOR KO mice. Additionally, buprenorphine did not produce additional antidepressant effects beyond those already achieved by pretreatment with the selective KOR antagonist norBNI. In contrast, pretreatment with MOR antagonist clocinnamox did not affect the antidepressant effects of a dose of buprenorphine indicating that the antidepressant effects of buprenorphine are mediated through the KOR (Falcon et al. 2016).

While these studies suggest that antidepressant activity of buprenorphine may be mediated through the KOR, other preclinical studies have identified some different effects between buprenorphine and selective KOR antagonists. The KOR antagonist DIPPA reduces anxiety-like behaviors in both Wistar Kyoto and Sprague Dawley (SD) rat strains (Carr and Lucki 2010), while buprenorphine reduces immobility in the FST only in the Wistar Kyoto rats, but not SD rats (Browne et al. 2015), suggesting that selective KOR antagonists may have advantages over nonselective KOR antagonists. However, in mice exposed to chronic social defeat as a model for post-traumatic stress disorder (PTSD), 7-day treatment with either buprenorphine or the SSRI fluoxetine reversed social deficits, while JNJ-67953964/LY2456302 had no effect (Browne et al. 2018). However, this finding may be limited to this specific KOR antagonist, or other factors related to the experimental paradigm, since other studies have shown that norBNI (McLaughlin et al. 2006), JDTic (Wells et al. 2017), and PF-04455242 (Grimwood et al. 2011) all reduce stress-like behaviors induced by exposures to chronic social defeat stress.

It is important to consider the antidepressant effects of buprenorphine in humans although the dataset is small. The first trial of 0.2 mg sublingual buprenorphine (Emrich et al. 1982) utilized a double-blind crossover design with a variable time period of 1–2 weeks in 10 patients diagnosed with depression. The symptoms of patients significantly improved on buprenorphine and became worse again during the second placebo period. There were also several open label studies that showed efficacy as an antidepressant (Bodkin et al. 1995; Kosten et al. 1990), including patients with treatment-resistant depression (Karp et al. 2014; Nyhuis et al. 2008).

As buprenorphine is a MOR partial agonist, it has potential to form dependence in patients (Lewis 1985). The functional KOR antagonist ALKS 5461, a combination of buprenorphine and the MOR antagonist samidorphan was created to harness the

antidepressant potential of buprenorphine without the potential for dependence (Ehrich et al. 2015). ALKS 5461 ultimately completed five trials FORWARD1–5, which have been systematically reviewed (Peckham et al. 2018). While ALKS 5461 initially showed efficacy in phase 2, the large effect size observed in phase 2 did not translate to phase 3. Out of three phase 3 trials, one failed (ClinicalTrials.gov Identifier: NCT02158546), and the effect sizes in the largest phase 3 trial were small (ClinicalTrials.gov Identifier: NCT02218008). Ultimately the FDA rejected the application to approve the drug. However, due to the lack of selectivity for the KOR, limited conclusions can be drawn from ALKS-5461 to the potential efficacy of selective KOR antagonists in MDD.

4.5 KOR Antagonists Treatment of Anhedonia

Anhedonia, the inability to experience pleasure, is a symptom that occurs across multiple psychiatric disorders including MDD and schizophrenia. On two preclinical models of anhedonia, the sucrose preference test and the social interaction test, U50488 increased measures of anhedonia that were resistant to treatment with SSRIs (Dogra et al. 2016). U69,593 increases the ICSS threshold in rats, indicating a reduction in the ability to experience pleasurable stimulation (Todtenkopf et al. 2004). Similarly, salvinorin A has been shown to produce anhedonic effects in mice such as reduced self-grooming, an effect that is reversed by short-acting KOR antagonists LY244296 and LY2795050 (Butelman et al. 2019).

JNJ-67953964/LY2456302 was advanced into the NIMH Fast-Fail Trials in Mood and Anxiety Spectrum Disorders (FAST-MAS) program for the treatment of anhedonia. It was trialed in 89 patients with clinically significant anhedonia measured via the Snaith–Hamilton Pleasure Scale (SHAPS) score. Approximately 80% of the patients had a primary diagnosis of MDD with 20% having other mood disorders. Patients were randomized to either JNJ-67953964/LY2456302 or placebo (Krystal et al. 2019). The primary outcome measure was the mean fMRI ventral striatal activation, an imaging biomarker relevant to anhedonia, measured during a reward anticipation task. The drug produced statistically significant activation of the ventral striatum on fMRI and improved clinical measures of anhedonia measured by a significant reduction on the SHAPS (secondary endpoint). There were no significant effects on the Hamilton Depression Rating Scale (HAM-D) or the Hamilton Anxiety Rating Scale (HAM-A) (exploratory endpoints). Although this was a relatively small trial, the effects on the SHAPS score for anhedonia are consistent with preclinical models predicting efficacy in this symptom domain and support a potential role for KOR antagonists as targeted treatments for anhedonia. Finally, while there were no effects shown on HAM-D, only 80% of patients had major depressive disorder and there was no specified minimum HAM-D score for enrollment, and so no conclusions can be drawn on potential efficacy in MDD from this study.

4.6 KOR Antagonists as Monotherapy vs Adjunctive Therapy

There is evidence that SSRIs and KOR antagonists may exert their antidepressant effects through distinct pathways. In mice, deficits induced by U50488 on preclinical models of anhedonia in the sucrose preference and social interaction tests could be blocked by norBNI and the tricyclic antidepressant imipramine, but not by SSRIs fluoxetine or citalopram, suggesting that KOR agonists induce symptoms of anhedonia that are mediated through different pathways than those acted on by SSRIs (Dogra et al. 2016). Similarly, in one experiment, both norBNI and JDTic reduced anxiety-like behavior on the EPM while SSRIs had no effect, however, SSRIs were found to be effective in the OF model while both norBNI and JDTic had no effect in this paradigm (Knoll et al. 2007). This suggests that SSRIs may modulate different depressive-like and anxiety-like symptomatology than KOR antagonists and that KOR antagonists may help patients who do not fully respond to SSRIs.

Additionally, synergistic antidepressant-like effects were found in the FST in mice when a subtherapeutic dosage of JNJ-67953964/LY2456302 was administered with a subtherapeutic dosage of either SSRI citalopram or the tricyclic antidepressant imipramine (Rorick-Kehn et al. 2014). This suggests the possibility that KOR antagonists may show optimal efficacy when used as adjunctive treatments with current standard of care SSRIs.

KOR antagonists JNJ-67953964/LY2456302 and BTRX-140 are currently being investigated in two ongoing clinical trials in MDD. JNJ-67953964 / LY2456302 is being trialed as an adjunctive treatment for patients who have had an incomplete response to current serotonin-norepinephrine reuptake inhibitor (SNRI) or SSRI therapy. In this case, 10 mg JNJ-67953964/LY2456302, a KOR selective dosage (Rorick-Kehn et al. 2015) that was previously shown on PET imaging to provide 94% occupancy at the KOR at 2.5 h and 72% at 24 h from a single dosage (Naganawa et al. 2016), is added to the patient's baseline SSRI or SNRI for 6 weeks following a placebo run-in period. The primary endpoint is the change on the Montgomery–Åsberg Depression Rating Scale (MADRS) (ClinicalTrials.gov Identifier: NCT03559192). In contrast, BTRX-140 is being trialed as a monotherapy for 8 weeks with a primary endpoint as the HAM-D (ClinicalTrials.gov Identifier: NCT04221230).

4.7 Sex Differences

One potential issue that may impact the ability to translate preclinical findings to the clinic is the observed sex differences in response to KOR antagonists in rodents. Female rats have been shown to be less sensitive than male rats to the depressive-like effects of KOR agonists (Russell et al. 2014) and female guinea pigs are less sensitive than male guinea pigs to the effects on pain (Wang et al. 2011). One experiment found that the antidepressant effects of norBNI on the FST in two different strains of male mice were not found in females (Laman-Maharg et al. 2018). Interestingly, one PET imaging study of naltrexone in humans reported a

higher KOR availability in men than in women (Vijay et al. 2016). Whether any of the sex differences observed in animals will translate humans will need to be addressed in larger trials.

4.8 Conclusions and Recommendations for Translation

The preclinical data reviewed here show that both long- and short-acting KOR antagonists have shown efficacy across a variety of preclinical antidepressant screening tests as monotherapies. Furthermore, they have also been shown to have synergistic efficacy when combined with tricyclic and SSRI antidepressants, suggesting that additional efficacy may be achieved as adjunctive therapies.

While the data with nonselective KOR antagonist buprenorphine are relatively small and many of the studies were open label, significant efficacy was found in treatment-resistant populations, including those resistant to both pharmacotherapy and electroconvulsive therapy (ECT) (Nyhuis et al. 2008). This is consistent with the preclinical data showing that KOR agonists induce depressive-like phenotypes that are resistant to treatment with SSRIs, and it is conceivable that KOR antagonists may represent a potential treatment for patients with treatment-resistant depression. While ALKS 5461 showed mixed results in phase 3 ranging from low effect sizes to lack of effect, this may be due to the MOR antagonist activity of samidorphan.

Finally, KOR antagonists may have specific efficacy on anhedonia, a symptom present in multiple different neuropsychiatric disorders, including MDD and the negative symptoms of schizophrenia. KOR agonists have been shown to induce anhedonic behavioral phenotypes in rodent models that can be reversed by KOR antagonists and this effect has been successfully translated in one trial in humans. The results of ongoing phase 2 trials of KOR antagonists may help answer outstanding questions, such as whether effects on anhedonia will hold up in larger trials, whether there are specific patient populations with DYN/KOR dysfunction, whether efficacy will vary between in men and women, and whether differences will be seen between monotherapy and adjunctive therapy.

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Imaging Kappa Opioid Receptors in the Living Brain with Positron Emission Tomography

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Contents

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Abstract

Kappa opioid receptor (KOR) neuroimaging using positron emission tomography (PET) has been immensely successful in all phases of discovery and validation in relation to radiotracer development from preclinical imaging to human imaging. There are now several KOR-specific PET radiotracers that can be utilized for neuroimaging, including agonist and antagonist ligands, as well as C-11 and F-18 variants. These technologies will increase KOR PET utilization by imaging centers around the world and have provided a foundation for future studies. In this chapter, I review the advances in KOR radiotracer discovery, focusing on ligands that have been translated into human imaging, and highlight key attributes unique to each KOR PET radiotracer. The utilization of these radiotracers in KOR PET neuroimaging can be subdivided into three major investigational classes: the first, measurement of KOR density; the second, measurement of KOR drug occupancy; the third, detecting changes in endogenous dynorphin following activation or deactivation. Given the involvement of the KOR/dynorphin system in a number of brain disorders including, but not limited to, pain, itch, mood disorders and addiction, measuring KOR density in the living brain will offer insight into the chronic effects of these disorders on KOR tone in humans. Notably, KOR PET has been successful at measuring drug occupancy in the human brain to guide dose selection for maximal therapeutic efficacy while avoiding harmful side effects. Lastly, we discuss the potential of KOR PET to detect changes in endogenous dynorphin in the human brain, to elucidate neural mechanisms and offer critical insight into disease-modifying therapeutics. We conclude with comments on other translational neuroimaging modalities such as MRI that could be used to study KOR-dynorphin tone in the living human brain.

Keywords

Agonist radiotracer · Antagonist radiotracer · EKAP · FEKAP · GR103545 · Kappa opioid receptor · LY2459989 · LY2795050 · Neuroimaging · PET

1 Introduction

1.1 Neuroimaging with PET

Positron emission tomography (PET) is a translational molecular imaging modality capable of quantifying radiotracer biodistribution in vivo. For studying molecular frameworks in the human brain, PET is well-suited, with a spatial resolution of ~3 mm allowing brain subregions to be studied in detail (Catana 2019). Relevant to this chapter, the power of this imaging modality lies in its ability to (1) quantify protein density, (2) measure occupancy of exogenous ligands, and (3) measure

occupancy of endogenous ligands (e.g., dynorphins). Further, PET enables in vivo longitudinal measurements of molecular changes in healthy and diseased brains facilitating the study of molecular changes throughout disease progression.

Critical to successful utilization of these three imaging paradigms is a corresponding radiotracer that has high target affinity (K_D) and specificity (specific >> nonspecific binding), high brain uptake, ideal pharmacokinetics, metabolic stability, and low test-retest variability (Pike 2016; Hooker and Carson 2019). Radiotracer development for CNS targets, such as G protein-coupled receptors (GPCRs), represents a great challenge, requiring strict ligand physicochemical properties for optimal brain uptake while conserving target affinity and selectivity, and limiting nonspecific binding (e.g., low plasma and brain protein binding). Feasibility for developing novel radiotracers should be assessed by first understanding target density in the brain (B_{max}), typically with post-mortem brain tissue. For radiotracers to have broad utility, high dynamic range is critical, to allow measurements of small changes in B_{max}. Radiotracer K_d must be low, especially for low expressing targets (low B_{max}), and ideally the ratio of $B_{max}/K_D \geq 10$ (B_{max} = fmol/g tissue = nM; K_D = nM).

Fortunately, KOR imaging with PET is poised for success, owed in large part to a high density of KORs in the human brain and a rich history of high-affinity ligands specific for KORs. Since its inception 30 years ago, KOR neuroimaging with PET has benefited from the successful translation of several radioligands (both KOR agonist and antagonist radioligands). These successes have afforded radiotracers deemed successful for rodent, nonhuman primate, and human brain PET imaging. To those not rooted in the field of neuroimaging, it is important to contextualize the rarity of this success and highlight the immense productivity that afforded these tools. A database search within the Human Protein Atlas (http://www.proteinatlas.org/) and Allen Human Brain Atlas (https://human.brain-map.org/) shows there are over 3,700 total brain receptors identified in the human brain (Hawrylycz et al. 2012; Sjöstedt et al. 2020). Of these 3,700 targets, only 60 have been targeted by PET, through development of 171 CNS PET radiotracers (in many instances, multiple radiotracers exist for a single target) (https://www.ncbi.nlm.nih.gov/books/NBK5330/; Gunn and Rabiner 2017; Suridjan et al. 2019; McCluskey et al. 2020). The KOR represents 1 of those 60 CNS targets. In addition, among the 171 CNS PET radiotracers, 6 are both KOR-specific and translated to humans for PET neuroimaging. Even with these successes, to date, only 6 human PET studies have been reported for studying associations between KOR function and human brain function or diseases (major depression, alcohol use disorder, cocaine use disorder, social status/stress, sex differences) (Vijay et al. 2016, 2018; Miller et al. 2018; Martinez et al. 2019; Matuskey et al. 2019; de Laat et al. 2020). KOR PET imaging has been relatively under-utilized; however, we see great opportunity as both infrastructure and training continue to develop. We hope this discussion will catalyze a growth in the KOR PET community and further pursuit of KOR pharmacology in vivo with PET.

1.2 Concepts

Neuroimaging with positron emission tomography provides molecular insights on the neurobiological targets within the living brain. In its most basic sense, PET represents classic radioligand binding pharmacology, conducted in vivo. At the core of PET data analysis is a framework derived from in vitro radioligand binding and modified for in vivo quantitation. Binding potential (BP), frequently sought as the primary outcome measure of radioligand binding experiments, represents the ratio of specifically bound ligand (B) to free ligand concentration (F); or receptor density (B_{max}) to radioligand equilibrium dissociation constant (K_D) (Mintun et al. 1984):

$$\mathrm{BP} = \frac{B\,\mathrm{max}}{K\mathrm{D}}$$

When BP is not defined further with subscript, it reflects in vitro measurements of binding potential. In vivo measurements of binding potential, as is with PET, have been defined further to account for other factors such as (1) free vs bound radioligand at equilibrium (BP_F), (2) uptake uncorrected for radioligand plasma protein binding (BP_P) (Innis et al. 2007). Lastly, a tissue reference region devoid of target receptors can be used to define nondisplaceable (ND) uptake and estimate binding potential with respect to nondisplaceable uptake (BP_{ND}) in receptor-rich brain regions. In other words, BP_{ND} utilized within PET is defined as in vivo specific binding. The importance of this concept stems from performing measurements of receptor density (B_{max}) with PET. If radioligand K_D has been determined, and BP can be estimated with PET, then receptor density (B_{max}) or receptor availability (B_{avail}) can be calculated. While B_{max} can be measured with in vitro radioligand binding studies in post-mortem brain tissue, PET is the only pharmacological tool to calculate B_{max} in the living brain.

PET analysis methods that rely on a tissue reference region do not require invasive input function measurements from arterial blood sampling. If a valid reference region is not available, then PET kinetic modeling with a 1-tissue or 2-tissue compartmental model can be used to estimate volumes of distribution (V) in tissue (T) or V_T. Adapted from clinical pharmacology, V_T is defined within in vivo imaging as the ratio of radiotracer concentration in tissue (C_T) relative to that in plasma (C_P):

$$VT = \frac{CT}{CP}$$

A majority of the imaging data in this chapter and within the KOR neuroimaging literature uses V_T and BP_{ND} as an outcome measure. While it is not within the scope of this chapter to summarize all aspects of neuroimaging PET data analysis, a recent comprehensive review on PET neuroimaging has covered this topic in great depth (Hooker and Carson 2019). In addition, a more focused review on in vivo imaging nomenclature was summarized to create a unified set of descriptors for the entire field (Innis et al. 2007).

2 KOR-Targeting Radiotracers for Brain PET

Over the past two decades, research on the development of KOR-targeting PET radiotracers has been highly successful, resulting in the synthesis and translation of several agents, both KOR agonists and KOR antagonists. Figure 1 shows the KOR radiotracers that have been useful for studying KORs in the living human brain with PET. KOR-targeting radiotracers have been utilized in PET studies in mice, rats, monkeys, baboons, and humans for KOR neuroimaging. While carbon-11 KOR radiotracers are limited to imaging centers with an in-house cyclotron (carbon-11 $t_{1/2} = \sim 20$ min), we are fortunate to have KOR-specific F-18 isotopologues, such as [^{18}F]LY2459989 (fluorine-18 $t_{1/2} = \sim 110$ min), allowing off-site production and shipment to nearby imaging centers (typically within 100-mile radius). The field is fortunate to reap the benefits of these successes, including improved second-generation radiotracers such as LY2459989 and EKAP, which are superior to first generation ligands LY2795050 and GR103545. This section will describe the development, historically, of KOR-targeting PET radiotracers for neuroimaging, to help those interested in KOR imaging to choose the proper KOR radiotracer best-suited for their studies.

2.1 KOR Agonist PET Radiotracers

2.1.1 Discovery and Preclinical Evaluation of [^{11}C]GR103545

The class of arylacetamides, including U50,488 and U69,593, represent a major class of brain penetrant KOR agonists that have been used for decades as pharmacological tools to investigate KOR activation. Early reports described a chemical series of phenylacetamides as potent KOR agonists (Barlow et al. 1991; Naylor et al. 1993). Among them was GR89696, a dichlorophenylacetamide racemate (R,S),

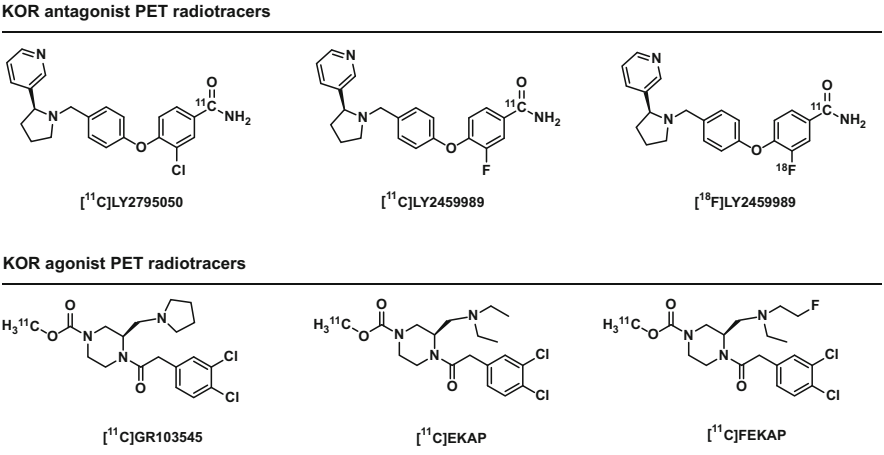


Fig. 1 KOR-targeting PET radiotracers translated to human neuroimaging

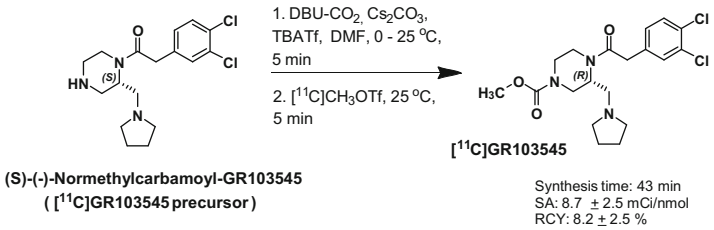


Fig. 2 High specific activity radiosynthesis of [^{11}C]GR103545 from [^{11}C]methyl triflate ([^{11}C]CH_3OTf), as described by Nabulsi et al. (2011). *SA* specific activity, *RCY* radiochemical yield (non-decay corrected)

demonstrated potent activity in a KOR-specific assay ($IC_{50} = 0.041$ nM; rabbit vas deferens, (Hayes and Kelly 1985)). Importantly, GR89696 contains an *O*-methyl carbamate functionality that allows facile C-11 labeling from the corresponding carbamic acid (Fig. 2). In mice, [^{11}C]GR89696 has high brain uptake ($C_{max} = 5\%$ injected dose/cc tissue (%id/cc) in receptor-rich regions) and a regional brain distribution that correlates KOR autoradiography studies in the rat brain (Ravert et al. 1999). Specificity for KORs was determined by measuring [^{11}C]GR89696 binding levels in animals pretreated with cold GR89696 (i.e., self-block), U69,593 (KOR), spiperone (dopamine D2 receptor), or ketanserin (5HT receptor). In both self-blocking and heterologous blocking (U69,593), [^{11}C]GR89696 binding was significantly reduced in KOR dense brain regions (olfactory tubercle, hippocampus, striatum, prefrontal cortex, thalamus). Animals treated with spiperone or ketanserin did not show any reduced binding of [^{11}C]GR89696 ruling out off-target binding to D2 or 5HT receptors.

GR89696 racemate was later purified and (R)GR89696 was determined to be the active enantiomer, which had a much higher affinity than (S)GR89696 (IC_{50} 0.018 nM vs 6.0 nM) (Naylor et al. 1993). The active enantiomer [^{11}C](R)GR89696 was renamed [^{11}C]GR103545. This isomer displayed higher brain uptake ($C_{max} = 5$ %id/cc), increased target-rich to target-void tissue ratio (olfactory tubercle vs cerebellum), and greater degree of saturable or block-able binding with homologous and heterologous blocking, compared to [^{11}C](S)GR89696 (Ravert et al. 2002).

Following the successful evaluation in mice, [^{11}C]GR103545 was studied in adult male baboon to determine translational potential for human KOR imaging (Talbot et al. 2005). Preclinical studies in nonhuman primates also allow for arterial blood sampling and subsequent kinetic modeling of PET data. Moreover, in PET neuroimaging, validating a reference region allows kinetic modeling of data to estimate binding potential or drug occupancy potentially, without the need for invasive arterial blood sampling. In these studies, adult male baboons were administered a bolus of [^{11}C]GR103545 to evaluate regional distribution (V_T) and specific binding (BP) in baboon brain. [^{11}C]GR013545 has high brain uptake (C_{max} of 0.010 %id/cc; t_{max} of 16 min), reasonable kinetics, acceptable metabolic stability (35% parent ligand remained after 30 min), and high free fraction ($f_{u,p} = 24$ %) in baboon.

Animals pretreated with naloxone (1 mg/kg, i.v.) had significantly lower V_T and BP in all brain regions examined except cerebellum, indicating specificity of [^{11}C]GR10355 for the KOR and use of the cerebellum as a nonspecific tissue region for derivation of BP. This work represented the first evaluation of KOR-specific PET radiotracer with potential for clinical translation.

While this effort represented a major milestone, the radiosynthesis utilized had resulted in low specific activity (0.150–0.495 mCi/nmol at End Of Synthesis (EOS)), hindering clinical translation. Given the potency of GR103545 (in vitro $K_i < 0.02$ nM at KORs), a follow-up PET study in baboon revealed that GR103545 in vivo K_d was ~0.048 nM at KORs in the brain (Tomasi et al. 2013). Therefore, to avoid psychomimetic effects from the associated cold-mass produced during radiosynthesis (specific activity), a strict mass limit was set for injected radiotracer dose in humans ($\leq$0.02 μg/kg). This required an improved radiosynthetic procedure that allowed higher specific activity radiolabeling. Schoultz et al. (2008) described a new radiosynthesis procedure that led to a 4–10× improvement in specific activity (1.79 ± 0.31 mCi/nmol at EOS). This was later improved upon by Nabulsi et al. (2011), whose protocol further improved specific activity by another fivefold (8.7 ± 2.2 mCi/nmol at EOS), and is the same general radiosynthesis method used today for [^{11}C]GR103545. The improvements made to increase specific activity allowed sufficient injected dose to achieve strong PET signal while avoiding pharmacological effects from the associated cold mass (^{12}C-GR103545). Even with this improved radiochemistry method for production of [^{11}C]GR103545, strict mass limits should continue to be followed, to avoid undesirable effects (e.g., dysphoria or psychotomimesis) given the potency of this ligand.

2.1.2 [^{11}C]GR103545 First-in-Human Evaluation

[^{11}C]GR103545 was the first KOR-specific PET radiotracer evaluated in humans, opening the possibility for occupancy studies with KOR-targeting therapeutics and studying changes in KOR density in relation to both health and disease (Naganawa et al. 2014a). The primary driving force behind this effort stemmed from Pfizer's KOR-selective antagonist PF-055242 which demonstrated antidepressant-like efficacy in preclinical models (Grimwood et al. 2011a). In humans, [^{11}C]GR103545 (bolus, i.v.) has high brain uptake (C_{max} of 3.5 SUV in amygdala; SUV = standardized uptake value = id/cc corrected for body weight) and a regional distribution that correlated well with previous in vitro measurements of KOR density in the primate brain (Peckys and Landwehrmeyer 1999). [^{11}C]GR103545 PET in humans demonstrated highest uptake in the amygdala, anterior cingulate cortex, and insula. There was moderate uptake in the temporal lobe, frontal lobe, globus pallidus, putamen, occipital lobe, and caudate. Low uptake in the cerebellum, posterior cingulate cortex, centrum semiovale, with lowest uptake in the thalamus (Fig. 3). When subjects were treated with naltrexone (150 mg, p.o., 75 min pretreatment), [^{11}C]GR103545 uptake was significantly reduced in highest binding regions (V_T reduced ~75%). Following kinetic modeling of the PET data, it was determined that Multilinear Analysis 1 (MA1) model (Ichise et al. 2002) provided a small standard error and is the best method for kinetic analysis in human. The

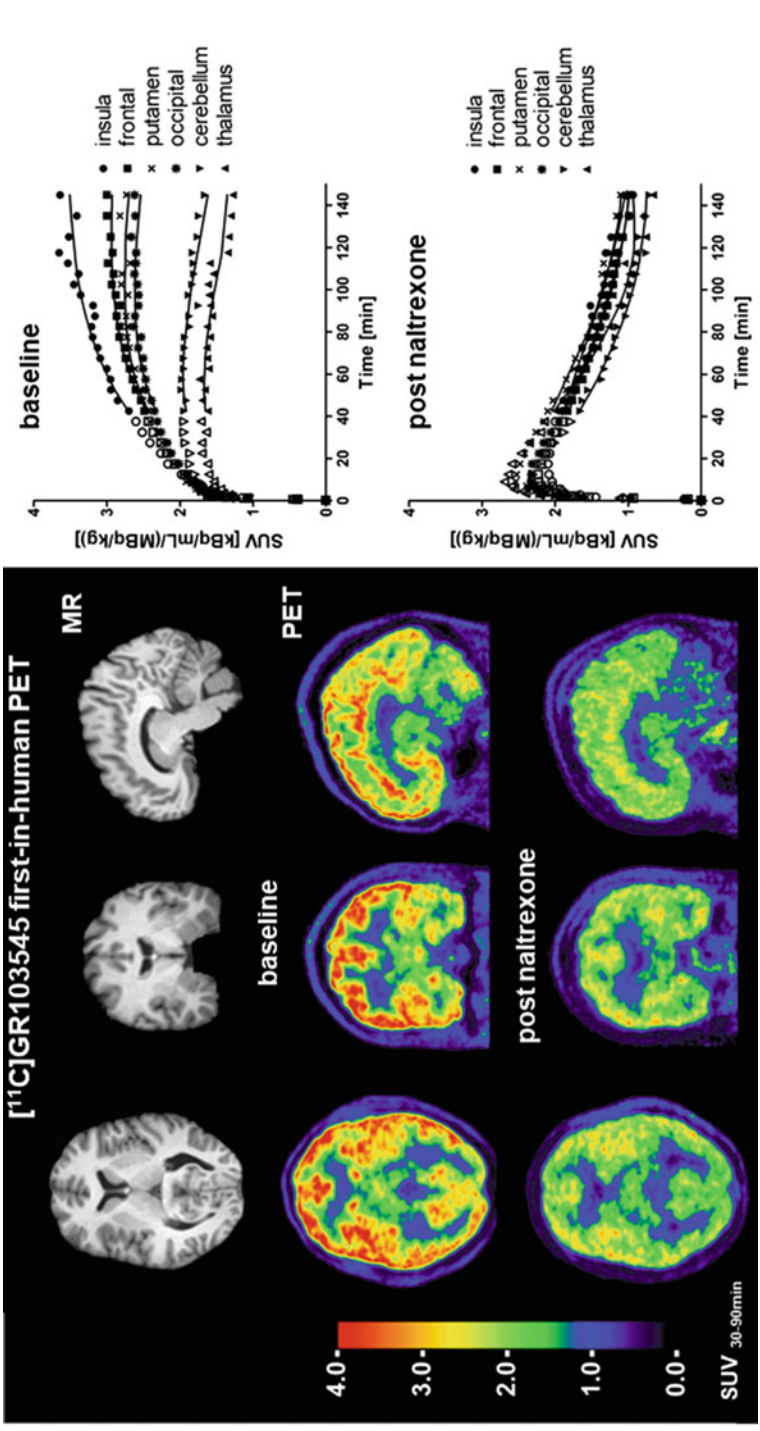


Fig. 3 [^{11}C]GR103545 first-in-human PET demonstrating saturable binding and excellent SUV dynamic range in post-naltrexone treated participants compared to baseline. Modified from Naganawa et al. (2014a)

kinetics of [^{11}C]GR103545 are less than ideal in humans (t_{max} of 120 min in high binding regions) and only adequate test-retest variability (TRV = ~15%; range 8–41%). Importantly, it was determined that there is no suitable reference region in the brain to estimate BP_{ND}, and arterial blood sampling is required for kinetic modeling of [^{11}C]GR103545 human PET data. The slow washout kinetics of this tracer result in long scan times (150 min) and strict mass requirements add challenges to injection parameters to ensure subject safety.

2.2 2nd Generation KOR Agonist Radiotracers: [^{11}C]EKAP and [^{11}C]FEKAP

2.2.1 Discovery and Preclinical Evaluation

To circumvent pitfalls encountered during human imaging with [^{11}C]GR103545, a second generation of KOR agonist radioligands were developed in hopes of improving ligand pharmacokinetics. This effort led to the identification of two lead compounds, EKAP (Li et al. 2019b) and FEKAP (Li et al. 2019a). Both molecules were designed from the GR103545 pharmacophore (dichlorophenylacetamide) but incorporated a diethylamino side chain instead of a pyrrolidine (Fig. 4). FEKAP was designed as the fluoroethyl analog of EKAP, to allow for potential F-18 labeling. In vitro potency of EKAP for KORs was comparable to GR103545 (EKAP K_i = 0.28 nM; GR103545 K_i = 0.23 nM) but FEKAP exhibited slightly lower affinity for KORs (FEKAP K_i = 0.43 nM). Both EKAP and FEKAP demonstrated good opioid KOR-selectivity compared to mu opioid receptor (MOR) (KOR:MOR = 30:1 EKAP; 17:1 FEKAP) or delta opioid receptor (DOR) (KOR:DOR = >1,000:1 EKAP; 320:1 FEKAP).

PET neuroimaging in nonhuman primates demonstrated that both molecules exhibited similar high brain uptake and improved brain pharmacokinetics (C_{max} = ~4.5 SUV; t_{max} = ~20 min). For comparison, in monkeys, [^{11}C]EKAP brain to plasma ratios reached equilibrium within 70 min, but [^{11}C]GR103545 failed to reach equilibrium within 120 min, following single bolus injection (i.v.). Both [^{11}C]EKAP and [^{11}C]FEKAP demonstrate heterogenous binding throughout the brain with highest accumulation in KOR-rich brain regions (cortical and striatal regions), but exhibited lower specific binding levels (BP_{ND}) compared to [^{11}C]GR103545, a

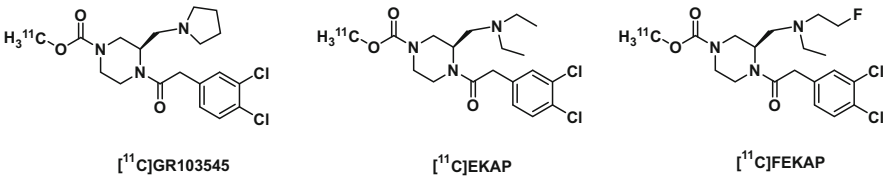


Fig. 4 Second generation KOR agonist PET radioligands [^{11}C]EKAP and [^{11}C]FEKAP. The pyrrolidine heterocycle was replaced with diethylamino side chain which led to improved binding kinetics while conserving high binding affinity at KORs (EKAP K_i = 0.28 nM; FEKAP K_i = 0.43 nM; GR103545 K_i = 0.23 nM)

(~20–40% decrease). Although [^{11}C]EKAP and [^{11}C]FEKAP BP_{ND} were lower compared to [^{11}C]GR103545, they were still relatively high for a brain PET radiotracer ($BP_{ND} > 0.5$ in KOR-dense brain regions).

2.2.2 [^{11}C]EKAP and [^{11}C]FEKAP First-in-Human Evaluation

Both [^{11}C]EKAP and [^{11}C]FEKAP demonstrated high brain uptake and faster tissue kinetics in humans compared to [^{11}C]GR103545 (Naganawa et al. 2020). [^{11}C]EKAP brain tissue clearance allows for shorter scan durations (90 min) compared to [^{11}C]FEKAP (110 min) and [^{11}C]GR103545 (140 min). Metabolic stability was acceptable for both [^{11}C]EKAP (33% parent compound remaining at 60 min) and [^{11}C]FEKAP (22% parent compound remaining at 60 min). [^{11}C]EKAP V_T (*MA1*) values across brain regions were high (V_T 5.4–21.6 mL/cm^3) compared to FEKAP (V_T 2.3–9.6 mL/cm^3) and comparable to [^{11}C]GR103545 (MA1 V_T 7.3–26.9 mL/cm^3). A higher V_T dynamic range allows the study of small changes in KOR availability. Comparing inter-subject MA1 V_T variability, [^{11}C]EKAP had lower variability across subjects (23–39%) than [^{11}C]FEKAP (14–26%). In addition, it was estimated that the minimum scan duration for [^{11}C]EKAP and [^{11}C]FEKAP should be 90 and 110 min for reliable estimations of V_T. [^{11}C]EKAP also outperformed [^{11}C]FEKAP in intrasubject test-retest variability (4–8%) vs (13–26%) across brain regions. It is recommended that [^{11}C]EKAP be the radioligand of choice for KOR agonist PET radiotracers. FEKAP has the potential for F-18 development given its fluoroethylamine moiety and would be the first F-18 KOR agonist PET radiotracer. To date, the radiosynthesis of [^{18}F]FEKAP has not been reported.

2.3 KOR Antagonist Radiotracers

2.3.1 Discovery and Preclinical Evaluation of [^{11}C]LY2795050

An investigation into the therapeutic potential of KOR antagonists led to the discovery of short-acting KOR benzyloxyarylamide antagonists (Mitch et al. 2011). One molecule in this chemical series, LY2795050, possessed physicochemical properties suitable for a brain PET radiotracer. To develop the required carbon-11 isotopologue of LY2795050, the corresponding arylbromide was synthesized for subsequent coupling with [^{11}C]cyanide followed by hydrolysis to form [^{11}C]LY2795050 (Zheng et al. 2013).

Preclinical neuroimaging in monkey revealed that [^{11}C]LY279505 bolus (i.v.) resulted in high brain uptake (C_{max} of 3.0 SUV; t_{max} 20 min), reasonable washout kinetics, good metabolic stability (40% parent ligand remained after 30 min), and heterogenous binding throughout the brain with highest signal in the basal ganglia and cingulate cortex. Both naloxone pretreatment (1 mg/kg) and LY2456302 pretreatment (96 μg/kg) reduced radiotracer binding to background levels in the brain, indicating both saturable and specific binding (Zheng et al. 2013).

These studies represented a major advancement in the field of KOR brain imaging by providing a safe alternative to KOR agonist radiotracers, avoiding potential psychomimetic effects from [^{11}C]GR103545. In addition, as we have seen from

dopamine imaging, GPCR-antagonist radiotracers have better utility at measuring receptor density (Vidal et al. 2019), while avoiding the confounding interpretation from endogenous ligand occupancy (e.g., neurotransmitters, neuropeptides).

Although [^{11}C]LY2795050 PET represented a major breakthrough in the field of KOR brain imaging, some challenges remained. An examination of KOR-selectivity in rhesus monkey discovered that LY2795050 KOR in vivo selectivity over MOR was only modest, ~7:1 (Kim et al. 2013). In addition, kinetic modeling of PET data with tissue reference (cerebellum) and arterial blood sampling provided estimates of binding potential (BP_{ND}) that were relatively low compared to previous KOR radiotracer [^{11}C]GR103545. The low BP_{ND} was attributed to a higher degree of nonspecific binding (off-target binding).

2.3.2 Discovery and Preclinical Evaluation of – [^{11}C]/[^{18}F]LY2459989

Toward the discovery of a KOR antagonist PET radiotracer with improved binding potential and specificity for KORs, LY2459989 (fluorine analog of LY2795050) was radiolabeled (carbon-11) using similar labeling conditions as [^{11}C]LY2795050 (Zheng et al. 2014). In vitro, LY2459989 has higher KOR affinity ($K_i = 0.18$ nM) and selectivity (43:1 KOR:MOR), compared to LY2795050 (KOR $K_i = 0.72$ nM; 35:1 KOR:MOR). In nonhuman primates, [^{11}C]LY2459989 has high brain uptake (C_{max} ~3.0 SUV), similar to [^{11}C]LY2795050, fast tissue kinetics (t_{max} 10–20 min), and improved specific binding (twofold higher) in KOR-rich brain regions compared to [^{11}C]LY2795050. As a result, it was determined that [^{11}C]LY2459989 was a superior radiotracer to [^{11}C]LY2795050 for in vivo brain PET imaging.

The arylfluorine on LY2459989 allowed for synthesis of its isotopologue (F-18) from the corresponding hypervalent-iodine precursor (Fig. 5). The synthesis and evaluation of [^{18}F]LY2459989 represents the first successful F-18 KOR brain PET radiotracer (Zheng et al. 2014) and allows more widespread use and distribution due to the extended half-life (F-18 $t_{1/2} = 110$ min; C-11 $t_{1/2} = 20$ min). In rhesus monkey, [^{18}F]LY2459989 had similar brain uptake and kinetics compared to its C-11 isotopologue. Time activity curves were considerably less noisy at later time points due to the high signal from F-18 with its longer half-life. Rate of metabolism measured from plasma samples indicated good stability and no indication of F-18 accumulation in bone (defluorination can be problematic for some F-18 radiotracers) (Li et al. 2018).

2.3.3 First-in-Human Evaluation [^{11}C]LY2795050 and [^{11}C], [^{18}F] LY2459989

Both first and second generation KOR antagonist PET radiotracers have been evaluated in humans for PET neuroimaging. [^{11}C]LY2795050 and [^{18}F] LY2459989 performed well in human brain PET studies, demonstrating high brain uptake in KOR dense brain regions, fast tissue kinetics, acceptable rate of metabolism, good plasma free fraction, low test-retest variability (Naganawa et al. 2014b, 2019). While [^{11}C]LY2459989 human PET has yet to be reported, its isotopologue [^{18}F]LY2459989 outperformed [^{11}C]LY2795050. In humans, compared to [^{11}C] LY2795050, [^{18}F]LY2459989 has a higher free fraction in plasma (% $f_{u,p} = 3.2\%$

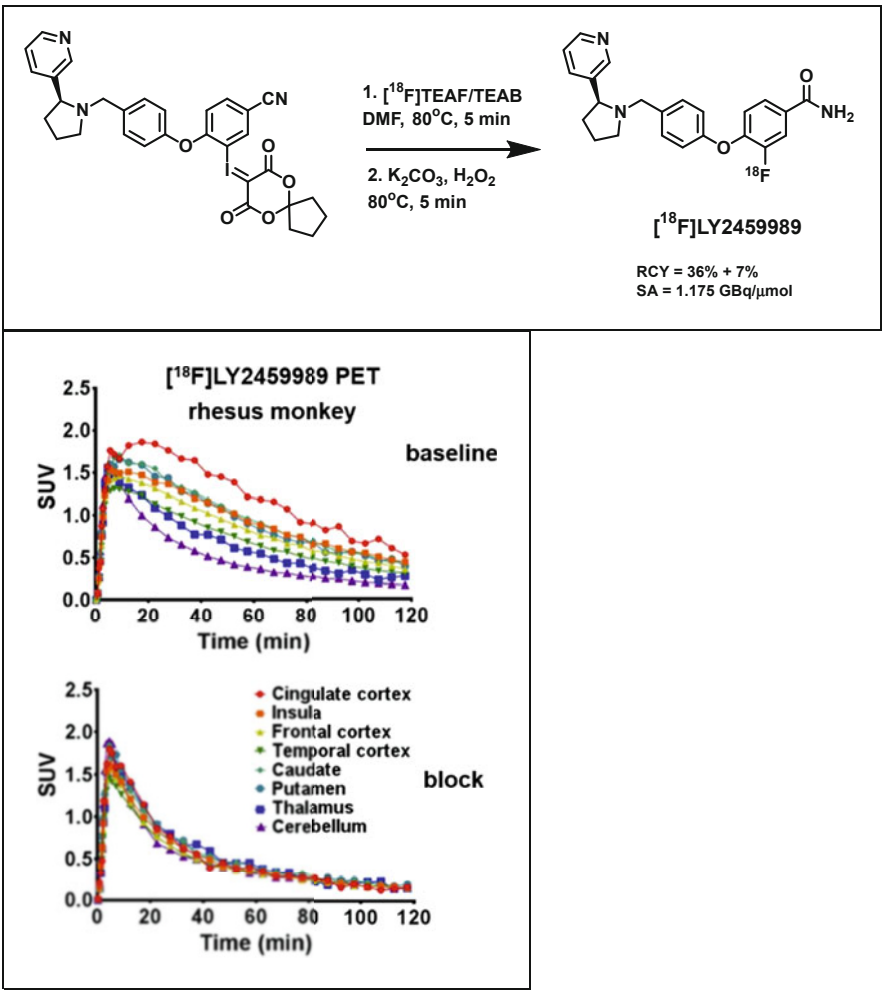


Fig. 5 (**a**) Synthesis of the 1st F-18 KOR PET radiotracer, [^{18}F]LY2459989. Development of this labeling method allows wider distribution of KOR PET radiotracers due to the longer half-life of F-18 ($t_{1/2}$ ~110 min) relative to C-11 ($t_{1/2}$ ~20 min). (**b**) [^{18}F]LY2459989 PET in monkey (bolus, i.v.) demonstrated high brain uptake and regional distribution consistent with previous KOR PET radiotracers. Binding was reduced significantly in animals pretreated with a selective KOR antagonist, LY2456302 (1 mg/kg, i.v.). Figures adapted from Li et al. (2018)

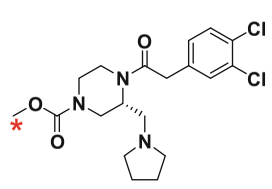
vs 0.77%), higher radiotracer binding (V_T) (MA1 V_T: 2.8 vs 1.95 in cerebellum; 8.3 vs 3.95 in amygdala), and improved test-retest variability compared (MA1 V_T aTRV: 7% vs 10%). Overall, given the improvements in key radiotracer criteria, it is recommended that [^{18}F]LY2459989 be used instead of [^{11}C]LY27959050 for KOR PET studies using an antagonist radiotracer.

3 Small Animal Neuroimaging of KORs with PET

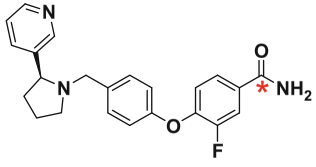
Small animal brain PET imaging of saturable receptor systems such as KORs can present challenges (Hume and Jones 1998; Jagoda et al. 2004; Herfert et al. 2020), but has great potential for use in reverse-translational studies given the number of rodent genetic models available. For radiotracer concentration to stay below tracer levels in vivo, receptor occupancy by cold mass should not exceed 5% by definition; although in small rodents ~10% baseline occupancy from cold mass is acceptable (Kung and Kung 2005). PET radiotracer specific activity can range from 0.5 to 2 mCi/nmol at the time of injection (TOI) depending on the isotope and radiosynthesis method. Therefore, strict limits on injection volume should be set to maximize signal-to-noise while avoiding cold mass effects that compromise signal dynamic range. In rat brain, KOR density ranges from 100 to 150 fmol/mg protein (10–15 nM) in receptor-rich regions (nucleus accumbens, claustrum, amygdala, hypothalamus) (Nock et al. 1988). Given the potency of KOR PET radiotracers (subnanomolar), it is advisable to keep the injected cold mass from KOR PET radiotracers <2 nmol/kg). This will avoid considerable occupancy from radiotracer cold mass and subsequent pharmacological effects from potent KOR agonist radiotracers (e.g., GR103545). In our lab, we administer 0.3–0.5 mCi of C-11 KOR PET radiotracer for rat imaging (~0.3 kg rat). This typically corresponds to 0.5–1.5 nmol/kg cold mass injected.

3.1 Evaluation of KOR Radiotracers and PET Analysis in Rodents

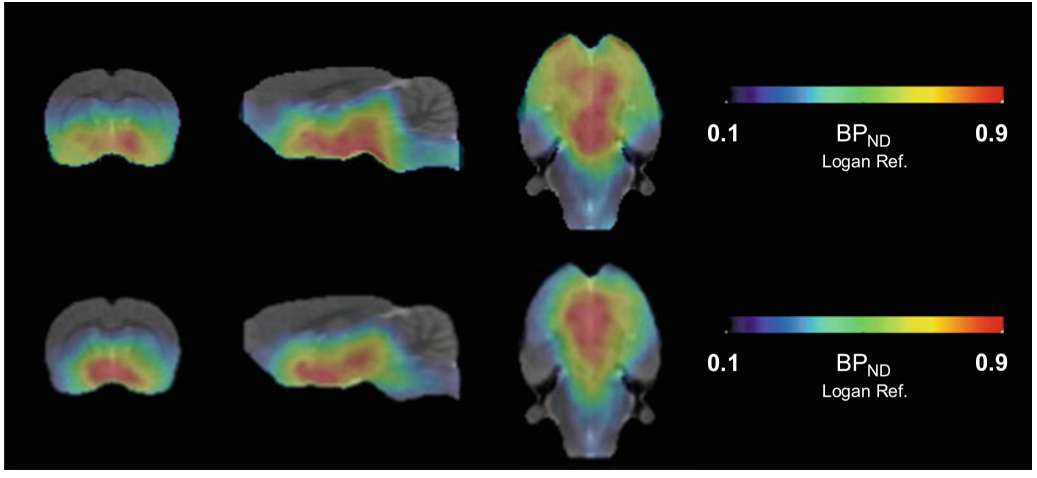
Given the diverse ligands available for KOR brain PET imaging, our lab conducted head-to-head comparisons of both kappa antagonist and kappa agonist radiotracers. In Sprague Dawley rats, we compared PET imaging results from [^{11}C]GR103545, [^{11}C]LY2795050, [^{11}C]LY2459989 at baseline conditions, and following pharmacological treatment with a number of different KOR ligands (Placzek et al. 2019). At baseline, both [^{11}C]GR103545 and [^{11}C]LY2459989 had high brain uptake and well-correlated regional distribution in the rat brain with highest uptake in the hypothalamus, ventral tegmental area, periaqueductal gray, nucleus accumbens, midbrain, amygdala, olfactory; moderate uptake in the thalamus, caudate putamen; and lowest uptake in the cerebellum (Fig. 6). In pharmacological PET studies, measuring salvinorin A occupancy at KORs in rat brain with [^{11}C]GR103545 PET proved reliable. Rats were pretreated with salvinorin A at 1 of 10 doses (0.010–1.8 mg/kg; i.v.) immediately prior to [^{11}C]GR103545 bolus and dynamic PET. Specific binding (BP_{ND}) in the brain was plotted against administered dose, to determine salvinorin A occupancy. These results show that salvinorin A (i.v.) achieves 50% occupancy at KORs in the rat brain at 0.36 mg/kg (Placzek et al. 2015). While a dose response relationship was observed for salvinorin A and [^{11}C] GR103545, this was not observed when the same study was repeated with either [^{11}C[LY2795050 or [^{11}C]LY2459989 (Placzek et al. 2019).



[^{11}C]GR103545



[^{11}C]LY2459989



		HTH	VTA	PAG	NACC	MB	AG	OLF	INS	THA	CPU	HC	CIN	FRT	MTR	CBL	High Binding Avg
[^{11}C]GR103545	BP_{ND}	0.868	0.691	0.626	0.637	0.551	0.504	0.593	0.527	0.440	0.474	0.271	0.068	0.072	0.047	-	0.598
baseline	Logan Ref.	±	±	±	±	±	±	±	±	±	±	±	±	±	±		±
n = 11		0.176	0.170	0.187	0.150	0.160	0.156	0.145	0.166	0.149	0.147	0.111	0.121	0.152	0.097		0.130
[^{11}C]LY2459989	BP_{ND}	0.884	0.645	0.622	0.586	0.609	0.447	0.436	0.240	0.460	0.354	0.297	-0.021	-0.145	-0.127	-	0.560
baseline	Logan Ref.	±	±	±	±	±	±	±	±	±	±	±	±	±	±		±
n = 5		0.146	0.040	0.105	0.046	0.055	0.053	0.097	0.097	0.056	0.055	0.038	0.046	0.089	0.059		0.046

High Binding Average Regions = HTH, VTA, PAG, NACC, MB, AG, OLF, THA, CPU

Fig. 6 Regional binding comparison between KOR agonist [^{11}C]GR103545 and [^{11}C]LY2459989 with PET in rats. Both radiotracers demonstrated high specific binding and strong correlation with KOR-rich brain regions. BP_{ND} for each region was calculated using Logan Reference Analysis with cerebellum as a reference region. Figure adapted from Placzek et al. (2019)

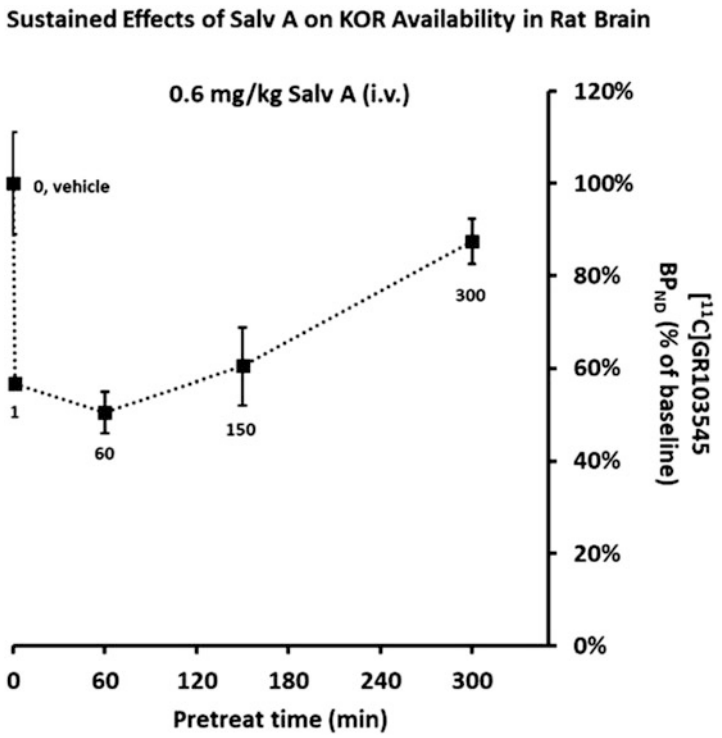


Fig. 7 Salvinorin A effects on KOR availability after drug clearance, measured with [^{11}C]GR103545 PET. Animals were treated with vehicle or salvinorin A (0.6 mg/kg, i.v.) followed by [^{11}C]GR103545 brain PET. [^{11}C]GR103545 PET was initiated at 1 of 4 time points (1 min, 60 min, 150 min, or 300 min after salvinorin A treatment). Figure adapted from Placzek et al. (2015)

As KOR agonists have been shown to cause receptor desensitization and internalization, we conducted PET studies to determine if [^{11}C]GR103545 was capable of probing this mechanism. Given the rapid pharmacokinetics of intravenous salvinorin A (rat brain $t_{1/2} = 8$ min), and its ability to internalize KORs in vitro (Wang et al. 2008), we pretreated rats with salvinorin A at 1 of 4 time points (1 min, 1 h, 2.5 h, 5 h) prior to [^{11}C]GR103545 bolus and dynamic brain PET. At later time points (i.e., 1–5 h), salvinorin A is expected to clear from the brain, allowing measurements of KOR availability and avoiding competitive binding from exogenous drug (Fig. 7). These data showed that KORs in the rat brain are unavailable for [^{11}C]GR103545 binding following KOR agonist exposure after the drug has cleared from tissue, and binding returns to baseline availability after several hours (5 h was the latest time point examined in this study).

The pursuit of KOR-targeting therapeutics spans several decades resulting in an array of pharmacological tools to study KOR function. To better interrogate pharmacological KOR PET studies, we measured both KOR agonist and antagonist radiotracer binding in vivo with PET in rats following administration of several

KOR ligands (Placzek et al. 2019). These data show both classes of agonist and antagonist radiotracers were sensitive to KOR antagonist blockade (naltrexone, naloxone, LY2795050). When KOR agonist drugs were administered prior to radiotracer bolus and PET, some differences in sensitivity were observed. Animals pretreated with U50,488H (1 mg/kg, i.v.) blocked 80% of [^{11}C]GR103545 binding, but did not block [^{11}C]LY2459989 binding. In addition, animals pretreated with salvinorin A (0.6 mg/kg or 1.8 mg/kg, i.v.) blocked 37% and 74% of [^{11}C] GR103545 binding but only blocked 10% and 25% of [^{11}C]LY2459989 binding.

To understand this variation in binding, in vitro competition binding studies with KOR wild-type and KORD mutant cells (D138N hKOR) were conducted with [^{3}H] diprenorphine and GR103545 and LY2459989. D138N mutations substitute a negatively charged amino acid (aspartic acid – D) for a polar residue (asparagine – N). When D138 is mutated, dynorphin A affinity for hKOR is abolished, while interestingly, salvinorin A binding is enhanced (Vardy et al. 2015; Che et al. 2018). In our studies we found that when D138 is mutated, LY2459989 potency was reduced ~500-fold (K_i 0.54 ± 0.04 nM vs 287 ± 11 nM). In comparison GR103545 potency was reduced only 40-fold ($K_i = 0.07$ nM vs 2.9 nM), indicating this contact is not as critical for binding (Placzek et al. 2019).

Recently, ligand-specific KOR binding states were described, using nanobody stabilization, providing further evidence of multiple KOR binding sites (Che et al. 2020). By locking the receptor in a particular conformational state (i.e., inactive, intermediate, and active states) and studying changes in ligand binding, it is evident that distinct KOR binding sites exist. Relevant to the in vivo discrepancies observed through pharmacological PET experiments, KOR-nanobody investigations showed LY2459989 binds to a unique inactive state of the receptor (i.e., neutral antagonist). This low affinity receptor state, or inactive state, showed dramatically reduced U69,593 and dynorphin A binding affinity (KOR agonists) but increased LY2459989 binding affinity (KOR antagonist). While GR103545 was not evaluated in this report, it is an analog of U69,593 (a phenylacetamide), so similar results could be expected. These X-ray crystallography data on KOR binding states, as well as our own in vivo PET data, highlight the importance of selecting the appropriate PET radiotracer for a particular study paradigm (i.e., measurement of KOR density, measurement of agonist or antagonist drug occupancy, or endogenous ligand occupancy) (Placzek et al. 2016).

3.2 Application of KOR PET to Study Negative Affect in Rat

Rodent neuroimaging of KORs was used to study negative affect and dynorphin levels in a rat model of inflammatory pain (Massaly et al. 2019). [^{11}C]LY2795050 PET was conducted 48 h following injection of Complete Freund′s Adjuvant (CFA) solution in the right hind-paw, to drive pain-induced negative affective states thereby activating dynorphin-containing neurons. Distribution volume ratios (DVR) were estimated using Logan Reference tissue model (Logan 2000) with extracerebral reference region (muscle). In CFA-treated animals, [^{11}C]LY2795050 whole brain

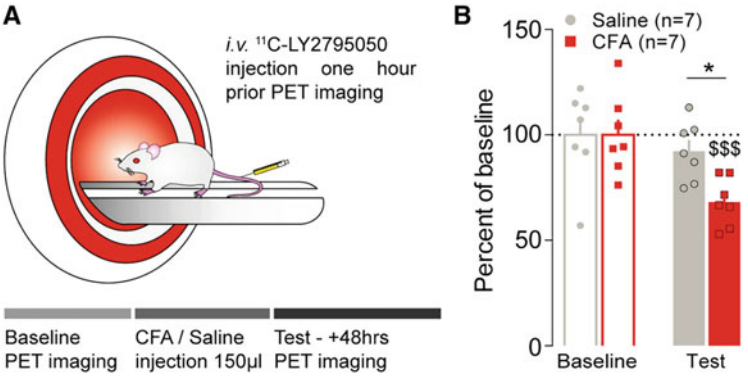


Fig. 8 [^{11}C]LY2795050 brain PET in inflammatory pain. (**a**) Rats were treated with CFA in the right hind paw and imaged 48 h after with [^{11}C]LY2795050. (**b**) Whole brain DVR was calculated and compared to baseline (sham pain control animals). [^{11}C]LY2795050 DVR was reduced ~25% in CFA animals. Figure adapted from Massaly et al. (2019)

DVR was significantly decreased compared to baseline (Fig. 8). This was attributed to elevated dynorphin levels that occupy KOR sites and block [^{11}C]LY2795050 binding. These results raise interesting questions given the use of extracerebral tissue reference for kinetic modeling and changes in whole brain binding. Future preclinical KOR PET studies with agonist radiotracers to measure dynorphin levels will shed further light on KOR active states and ligand-specific binding sites (Che et al. 2020).

4 Human KOR PET

Given the importance of the KOR/dynorphins system in a variety of mood disorders and psychiatric illnesses, neuroscientists are now well-poised to examine the role of this system in the living human brain with neuroimaging. These imaging tools can be used to measure KOR availability, changes in receptor density, altered dynorphin levels caused by cognitive function or brain disorders, or to measure drug occupancy by KOR-targeting therapeutics. In this section we have highlighted human brain PET studies used to interrogate KOR function in human brain disease.

As we interpret results from human imaging studies, it is important to point out that a direct measure of receptor availability (BP_{ND}) is not yet possible with KOR PET, due to the lack of a suitable reference region in the brain (brain region devoid of KORs). As a result, V_T (amount of radioligand in a volume of tissue relative to plasma) has been used as the outcome measure for receptor availability. It is also important to note that V_T is radiotracer dependent, so comparisons across studies that employed different KOR radiotracers can be challenging. When differences in V_T are discovered within a particular study, it is important to examine whether V_{ND} levels

were different across groups (amount of nondisplaceable radiotracer in tissue relative to plasma; typically determined from a blocking study with sufficient receptor occupancy, e.g., imaging post-naloxone). If V_{ND} is measured, and remains steady across subject groups, then V_T estimates can be inferred as a more direct measurement of receptor availability.

4.1 Alcohol Use Disorder (AUD)

In a neuroimaging study with [^{11}C]LY2795050 examining KOR availability between alcohol-dependent (AD) participants and age-matched healthy controls (HC), AD subjects had lower KOR availability compared to HC across all brain regions and was statistically significant in amygdala, caudate, front cortex, insular cortex, ventral pallidum, parietal cortex, putamen, and temporal cortex (Vijay et al. 2018). V_{ND} was calculated from additional scans in a subset of subjects following pretreatment with naltrexone and was found to be stable across groups (AD vs HC). Therefore, V_T in this study can be viewed as an indirect measure of specific binding, V_S (i.e., $V_T = V_S$). Lower V_T in AD subjects was attributed to lower KOR expression (downregulation), which has also been supported in preclinical research (Rosin et al. 1999; Lindholm et al. 2000). Lastly, age-related changes in KOR availability were not observed, indicating that KOR density is conserved in age, contrary to other neuroreceptor systems (Volkow et al. 2000; Karrer et al. 2017).

In a separate report, a group of 44 alcohol-dependent participants completed a study examining the efficacy of naltrexone in an alcohol drinking paradigm with naltrexone occupancy at KORs as assessed by [^{11}C]LY2795050 PET (de Laat et al. 2020). Subjects received a daily dose of naltrexone (100 mg, p.o.) for seven days with the last dose 2 h prior to [^{11}C]LY2795050 PET, and V_T was calculated and averaged from six bilateral brain regions (amygdala, pallidum, striatum, hippocampus, frontal cortex, and cingulate cortex). Naltrexone occupancy at KORs was 92.4 $\pm$ 1.3%, and consistent in both men and women. Interestingly, this 7-day high dose naltrexone (100 mg/day) was negatively correlated with reduced drinking. It has been shown that lower doses of naltrexone (25 or 50 mg/day) can be more effective than high dose naltrexone (100 mg/day) at reducing hazardous drinking (O'Malley et al. 2009). In a simulation study on naltrexone occupancy, a lower dose of naltrexone (25 mg) would still occupy ~60% of KORs. Lower efficacy in AUD with high dose naltrexone could be attributed to high MOR and KOR occupancy, especially since MOR density in the human brain is lower than KORs (Peckys and Landwehrmeyer 1999), and mixed-affinity of naltrexone for MORs and KORs (KOR $K_i = 0.92$ nM; MOR $K_i = 0.63$ nM) (Grimwood et al. 2011a). Significantly, high accuracy predictions on reduced consumption were found to be feasible based on an individual's KOR occupancy, years on drinking, and family history. Taken together, titrating naltrexone occupancy in humans with brain PET could maximize treatment efficacy for AUD.

4.2 Cocaine Use Disorder (CUD)

A study in CUD was conducted to investigate if changes in KOR availability, as a marker of KOR/dynorphins function, could be detected with [^{11}C]GR103545 PET (Martinez et al. 2019). Twelve CUD subjects completed the 3-scan imaging study, consisting of (1) a baseline scan, (2) post-naltrexone scan, (3) after cocaine binge scan. Data from these subjects were compared to data from fourteen HC who completed a 2-scan study with [^{11}C]GR103545 PET (scan 1 = baseline; scan 2 = post-naltrexone). Baseline comparison between CUD and HC showed no difference in [^{11}C]GR103545 V_T. These results are in contrast with previous post-mortem studies that show increased KOR density in caudate, nucleus accumbens, putamen, amygdala, cingulate cortex, and orbitofrontal cortex (Hurd and Herkenham 1993; Staley et al. 1997; Mash and Staley 1999). Baseline V_T correlated with choice to self-administer cocaine following cold pressor test, a stress (subjects chose cocaine dose or money after completing cold pressor task in cold water), indicating stress-induced vulnerability and higher KOR expression in the striatum are associated. Comparing CUD subjects at baseline and post 3-day cocaine binge, [^{11}C]GR103455 V_T was reduced 14.4% across all brain regions (Fig. 9). This global change was attributed to increases in dynorphin, lowering the number of available KOR binding sites, and subsequently, V_T.

4.3 Social Stress

It is well understood that the KOR/dynorphins system is critically involved in mood regulation including stress (Land et al. 2008, 2009; Bruchas et al. 2010; Nygard et al. 2016). To better understand the KOR/dynorphins system in relation to social status in humans, a PET study was conducted to determine correlations between social status [as assessed by the Barratt Simplified Measure of Social Status (BSMSS) – a measure of socio-economic status] (Barratt 2006), and KOR availability measured with PET (Matuskey et al. 2019). The second-generation KOR agonist radiotracer

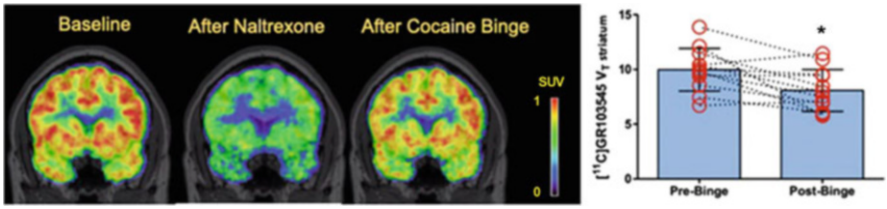


Fig. 9 [^{11}C]GR103545 V_T in CUD subjects. Data from this study shows significantly lower V_T in CUD subjects post-cocaine binge (3-day cocaine binge; 2 doses per day). V_T was lower in all brain regions examined and averaged 14% across the whole brain. For clarity, only striatum is shown here. Comparison performed as a linear model implemented in the mixed model framework. Mean comparison with a two-group *t*-test. Pre-binge $V_T = 9.98 \pm 1.95$; Post-Binge $V_T = 8.08 \pm 1.91$ (mean ± SD). %Change $= -17.7 \pm 18.3$ ($p = 0.01$). Figure adapted from Martinez et al. (2019)

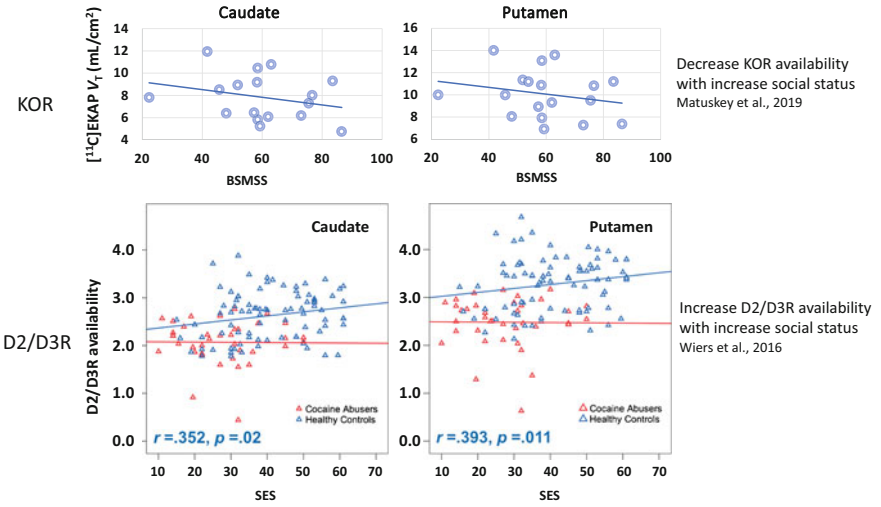


Fig. 10 [^{11}C]EKAP PET in humans and the effect of social status on the KOR system. Regional V_T and BSMSS were negatively correlated in brain regions involved in reward/aversion. Lower social status was associated with increased KOR availability. This indicates a possible role for dynorphin in regulating social stressor. Figure adapted from Matuskey et al. (2019). As a comparison, a similar study on correlating socioeconomic status (SES) with PET outcome measures was conducted on D2/3R with [^{11}C]raclopride in humans. This work shows a positive correlation between social status and D2/D3R availability. Figure adapted from Wiers et al. (2016)

[^{11}C]EKAP was used to study KOR availability in eighteen health participants (9 male, 9 female).

Results from this study show social status (stressor) was inversely correlated with [^{11}C]EKAP binding in brain areas associated with reward/aversion (amygdala, anterior cingulate cortex, caudate, frontal cortex, hippocampus, pallidum, putamen, and ventral striatum). This indicates that subjects with higher social status had lower V_T or KOR availability compared to low social status individuals (Fig. 10). While these results are certainly interesting and highlight the importance of the KOR/dynorphins system in social phenomena, the study results are confounded by an inability to decouple KOR availability from changes in dynorphin levels. A decrease in KOR availability could be the result from either (1) decreased KOR levels, (2) increased dynorphin levels, or (3) a combination of both. As a comparison, a similar study on correlating social status with PET outcome measures was conducted on D2/3R with [^{11}C]raclopride in humans (Wiers et al. 2016), and showed D2/3R availability was positively correlated with increased social status (Fig. 10). It is known that the KOR activation by dynorphin on presynaptic DA neurons inhibits dopamine release (Bruchas and Chavkin 2010; Kivell et al. 2014; Al-Hasani et al. 2015). If KOR density is conserved across social status, and dynorphin tone is decreased (i.e., decreased inhibition = activation), an elevated mood state could be expected. Further studies should be conducted for mechanistic interpretation of these observations.

4.4 Depression

While the KOR system is well known to arbitrate pathological changes to stress (Land et al. 2008; Bruchas et al. 2010; Massaly et al. 2016), the relationship between KORs and depression in humans is understudied. KOR tone in major depressive disorder (MDD) was examined in vivo with [^{11}C]GR103545 PET in 10 MDD and 13 healthy volunteers (Miller et al. 2018). They investigated the correlation between [^{11}C]GR103545 V_T in specific brain regions (amygdala, hippocampus, ventral striatum, raphe nuclei) with diagnoses, childhood traumas, newer life stresses and salivary cortisol levels using a modified Trier Social Stress Test (mTSST). The results showed there was no difference in [^{11}C]GR103545 V_T for the effect of diagnosis, severity of depression, and recent life stress in MDD compared to HC in all four *a priori* regions of interests (ROIs). No correlation was observed between [^{11}C]GR103545 V_T and covariates including sex and age. In their discussion they reflect that the study was made to detect larger effects in a small population which could lead to their observation of no difference between the MDD and healthy volunteer groups. The investigators also indicate that future studies may need to restrain the study group to a more stress-responsive subgroup of MDD.

The study did observe a trend-level inverse relationship between the total cortisol output during the mTSST and [^{11}C]GR103545 V_T (Fig. 11). Further, they suggest an explanation that the hypothalamic-pituitary-adrenal (HPA) axis tone may adjust dynorphin levels in humans which would potentially cause a change in KOR binding and other downstream pathways. Because of the small sample group, the investigators explained that further studies with larger sample sizes would be necessary with groups that are of higher risk for KOR abnormality and MDD volunteers that have psychotic features. The relationship between depression and the KOR system remains to be investigated further.

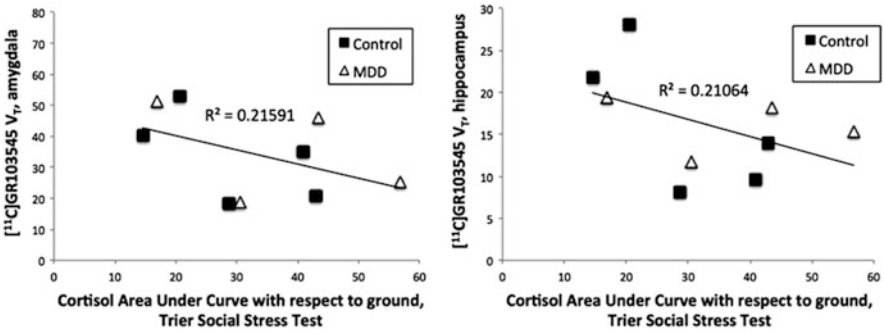


Fig. 11 [^{11}C]GR103545 V_T inversely correlates with total cortisol output. Total cortisol output during the Trier Social Stress Test was found to have a trend-level inverse relationship with [^{11}C]GR103545 V_T in all regions of interest including the amygdala, hippocampus, raphe nuclei, and ventral striatum ($p = 0.081$). Figure adapted from Miller et al. (2018)

4.5 Sex Differences

PET imaging has shown differences in [^{11}C]LY2795050 regional binding in male ($n = 18$) and female ($n = 9$) subjects, with females displaying lower V_T in all brain regions examined (Vijay et al. 2016). In nine of the brain regions, differences in V_T reached statistical significance (Fig. 11). Of the 27 participants in this study, approximately half completed an additional [^{11}C]LY2795050 PET scan following naltrexone (150 mg p.o., 75 min prior) to determine V_{ND} for this group (Fig. 12). Results from these naltrexone scans showed V_{ND} was consistent across groups. Therefore, V_T represents specific binding in this study ($V_T \approx V_S$). These differences are not surprising given that others have shown differences in pain perception, as well as analgesic response in men compared to women (Chartoff and Mavrikaki 2015).

4.6 Drug Occupancy

In KOR drug occupancy studies measured with PET, a valid tissue reference region is not available for PET kinetic modeling, so V_T and V_{ND} are used to estimate specific binding (V_S). This requires arterial blood sampling to measure radiotracer levels in plasma and subsequent modeling of PET data. To calculate drug occupancy, V_T is calculated following baseline scans and postdose scans, within subject. Radiotracer nonspecific binding, or nondisplaceable uptake (V_{ND}), must also be calculated and is typically estimated across subjects within a study from multiple drug doses, using a modified "Lassen Plot" (Cunningham et al. 2010). With V_{ND} calculated, a relationship between V_T within a brain region, $\boldsymbol{i}$, can be formulated to estimate receptor occupancy (RO) with a theoretical max value of 100%.

$$\frac{V_{T,i}^{\text{Baseline}} - V_{T,i}^{\text{Drug}}}{V_{T,i}^{\text{Baseline}} - V_{\text{ND}}} = \text{Receptor Occupancy (RO)}$$

Lastly, guidelines on measuring drug occupancy in the CNS with PET have recently been summarized (Takano et al. 2016).

PF-04455242 is a high-affinity selective KOR antagonist (KOR $K_i = 3$ nM; MOR $K_i = 65$ nM; DOR $K_i > 4{,}000$ nM) developed by Pfizer as a potential treatment for depression and addiction disorders (Grimwood et al. 2011b). To titrate optimal dosing in humans while avoiding off-target effects PF-04455242 KOR occupancy was examined with [^{11}C]GR103545 PET (Naganawa et al. 2014a). Subjects participated in three PET scans with [^{11}C]GR103545 at (1) baseline, (2) 1.5 h post-drug, (3) 8 h post-drug (PF-04455242 p.o., 15 mg $n = 1$; 30 mg $n = 5$). PF-04455242 concentration in plasma was also measured at 8 time points (1.5–10.5 h postdose) and correlated with [^{11}C]GR103545 V_T. V_T was calculated for baseline and post-drug scans across 14 brain regions and grouped to estimate drug occupancy at KORs. The relationship between plasma concentration (ng/mL)

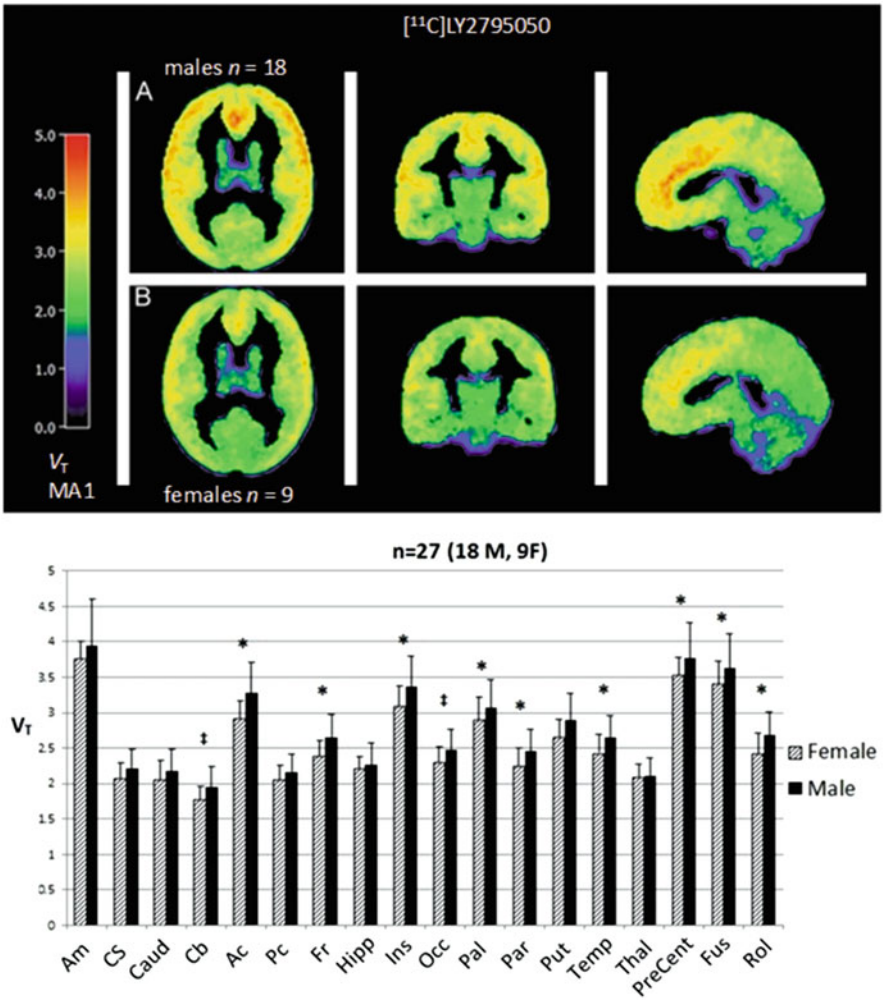


Fig. 12 Sex differences in KOR availability as measured with [^{11}C]LY2795050 PET in humans. Males $n = 18$; females $n = 9$. V_T (MA1) was significantly lower in females in nine brain regions: anterior cingulate cortex, AC; frontal cortex, Fr; insula, Ins; ventral pallidum, Pal; parietal cortex, Par; temporal cortex; precentral gyrus, PreCent; fusiform gyrus, Fus; Rolandic operator, Rol. Data are expressed as mean +/− SD, $^*p < 0.05$, $‡p < 0.10$ by unpaired *t*-test. Figure adapted from Vijay et al. (2016)

and receptor occupancy (r) showed PF-0445242 plasma concentration was 55.3 ± 4.9 ng/mL at 50% KOR occupancy (IC_{50}). Unfortunately, the PF-0445242 dosing was limited because of preclinical toxicology findings. As a result, KOR occupancy did not exceed 60% which presented challenges when modeling the data using 2-parameter fits, although a previous estimate supports a similar relationship between plasma levels and occupancy (IC_{50} = ~48.6 ± 7.2 ng/mL) (Jacobsen

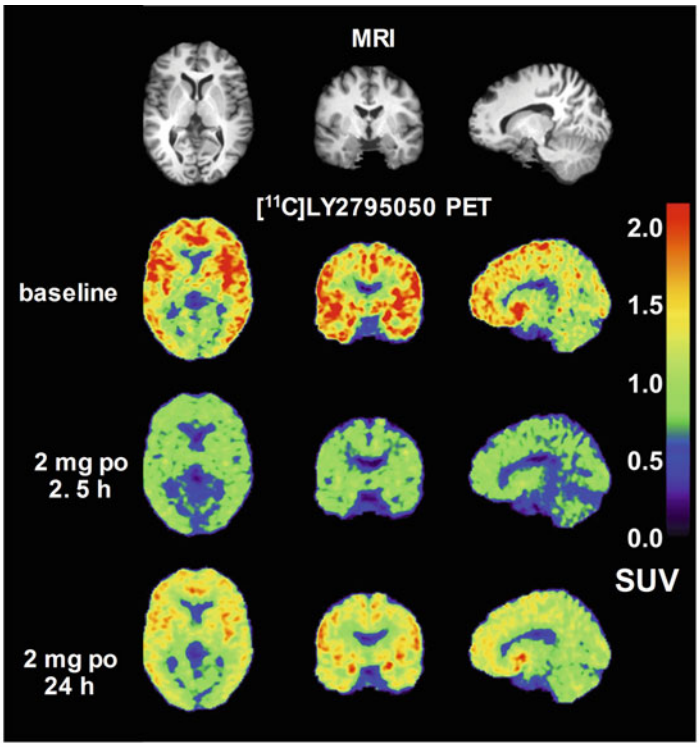
et al. 2010). The clinical trial with PF-0445242 was later terminated, so it is still unclear whether 50% occupancy is associated with antidepressant efficacy in humans.

JNJ-67953964 (formerly LY2456302 and CERC-501, the current name aticaprant) is a short-acting KOR-selective antagonist (K_i (nM): $\kappa = 0.72$; $\mu = 25.8$; $\delta = 153$), analogous to KOR PET radiotracers [^{11}C]LY295050 and [^{11}C]/[^{18}F]LY2459989 (aminobenzyloxyarylamides) (Mitch et al. 2011). JNJ-67953964 is currently being evaluated in several clinical trials for major depressive disorder and substance use disorder (Jacobson et al. 2020). To guide clinical administration, drug occupancy studies with [^{11}C]LY2795050 PET were conducted in 13 healthy human subjects (Naganawa et al. 2016). Each subject underwent 3 PET scans with [^{11}C]LY2795050 at (1) baseline, (2) 2.5 h postdose, and (3) 24 h postdose JNJ-67953964. Subjects received 1 of 5 doses of JNJ-67953964 (0.5 mg, 2 mg, 4 mg, 10 mg, 25 mg, po). JNJ-67953964 plasma levels reached 0.53–0.90 ng/mL (C_{max}) for the lowest dose and 15.2 ng/mL at the highest dose. PET kinetic modeling was used to calculate regional V_T (MA-1) across 13 ROIs. [^{11}C]LY2795050 V_T decreased in a dose-dependent manner and considerable occupancy KORs was observed with 4 mg dose (83% at 2.5 h; 49% at 24 postdose) (Fig. 13). KOR sites reached saturation at 2.5 h postdose with 10 mg (94% occupancy) or 25 mg (93% occupancy). At these doses, high target engagement was still observed at 24 h postdose (10 mg, 72% occupancy; 25 mg 82% occupancy). When the relationship between JNJ-67953964 plasma concentration and KOR occupancy was modeled, an IC_{50} was estimated at 0.6–0.8 ng/mL. (Fig. 13).

5 Conclusions

In the human brain, KORs have higher expression compared to mu and delta ORs. In addition, there are significant neuroanatomical differences across species, with the lowest expression observed in mouse and rat, and the highest observed in humans (Mansour et al. 1988; Peckys and Landwehrmeyer 1999). This highlights the importance for understanding the KOR/dynorphins system in the human brain, particularly in living subjects. Through advances in medicinal chemistry focused on KOR-targeting ligands along with CNS radiotracer development, the field now has KOR-specific PET radiotracers available for use in all species. These advances in KOR PET offer molecular insight into KOR and dynorphin tone in brain disease, most evident in psychiatric disorders and substance use disorders. In addition, KOR PET has been successful at measuring KOR drug occupancy in the human brain. These studies can be used to guide dosing in clinical trials to maximize efficacy while avoiding off-target effects. Pharmacological PET experiments can also validate target engagement and measure OR-subtype specificity when employed in combination with MOR neuroimaging (Kim et al. 2013).

One area of KOR neuroimaging that has not been well characterized is the contribution of endogenous dynorphins toward changes in PET radioligand binding. While in theory, occupancy at KORs by dynorphins should alter radioligand binding



LY2456302 Dose	2.5 h Postdose		24 h Postdose	
	Occupancy	V_{ND}	Occupancy	V_{ND}
	%	ml/cm^3	%	ml/cm^3
0.5 mg (n = 2)	35 ± 4	1.23 ± 0.06	19 ± 3	1.92 ± 0.21
2 mg (n = 4)	71 ± 10	1.32 ± 0.19	43 ± 14	1.41 ± 0.25
4 mg (n = 2)	79 ± 6	1.43 ± 0.10	48 ± 1	1.37 ± 0.03
10 mg (n = 3)	94 ± 0.5	1.43 ± 0.10	72 ± 4	1.51 ± 0.10
25 mg (n = 1)	93	1.50	82	1.40

Data are mean ± S.D. Individual parameters are reported where n = 1.

Fig. 13 JNJ-67953964 (formerly, LY2456302) occupancy at KORs in human brain measured with [^{11}C]LY2795050 PET. PET images represent total radioactivity, SUV, summed from 30 to 90 min after [^{11}C]LY2795050 bolus at baseline, 2.5 h postdose, or 24 h postdose, 2 mg po. As shown in data table, kinetic modeling of PET data across 14 brain ROIs showed receptor occupancy (RO) was >90% from 10 mg at 2.5 h postdose. This estimated an IC_{50} = 0.6–0.8 ng/mL. Figure adapted from Naganawa et al. (2016)

of orthosteric ligands, it is still unclear which radioligands are sensitive to changes in extracellular dynorphin concentration. In D2/D3R PET, the contribution of endogenous dopamine to basal occupancy has been characterized with simultaneous

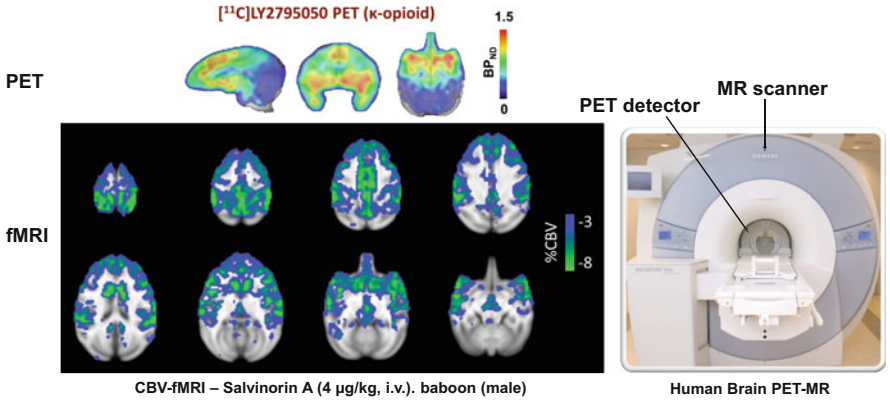


Fig. 14 Simultaneous PET-MR in baboon to study KOR-mediated neural activation. Salvinorin A (4 µg/kg, i.v.) induced robust bilateral changes in cerebral blood volume (CBV) throughout the cortices and basal ganglia (preliminary results, $N = 1$)

microdialysis and PET in rodents (Schiffer et al. 2005). Unfortunately, microdialysis measurements of dynorphins have been challenging historically, due in part to neuropeptide sample stability and detection with mass spectrometry (Karkhanis and Al-Hasani 2020). Recent technological advances in microdialysis probe design and neuropeptide detection methods could improve the reliability and adaptability of this technique for measuring dynorphin release in vivo. In addition, advances in optogenetics and chemogenetics have shown ability to stimulate dynorphin release (Al-Hasani et al. 2018; Anderson et al. 2019). Coupled with PET, these techniques likely allow for estimations of dynorphin basal occupancy and radiotracer sensitivity to dynorphin release.

While PET has excellent spatial resolution, MRI has greater temporal resolution and can be used to map hemodynamic changes in the brain associated with neural activity. Given the advancements in multimodal imaging such as simultaneous PET-MR (Sander et al. 2020), further opportunities exist to study KOR activation with functional MRI (fMRI). Both task-based and pharmacological MRI (phMRI) have potential to help understand KORs and dynorphin tone in brain disorders, especially mood and psychiatric disorders. A pilot study on OR-mediated neural activation with fMRI compared effects of U69,593 and fentanyl in awake cynomolgus monkeys (Kaufman et al. 2013). Results demonstrate neural activation and deactivation of OR-related neural circuits caused by either KOR agonists (aversive) or rewarding MOR-agonists (rewarding). In our lab, we have conducted pilot experiments using fMRI to study KOR-mediated neural activation patterns from salvinorin A in baboon. Utilizing simultaneous PET-MR neuroimaging, we measured receptor occupancy from salvinorin A with [^{11}C]LY2795050, and agonist-induced changes in cerebral blood volume (CBV) with fMRI. Salvinorin A produces rapid decreases in %CBV in the cortices and basal ganglia (Fig. 14). Given the array of KOR pharmacological tools and recent advances in characterizing intracellular

signaling bias for KOR ligands, further studies measuring neural signaling with fMRI are warranted. This approach would provide another translational tool for studying KOR/dynorphins tone in the living human brain.

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