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TITLE PAGE

Dynamic Opioid Receptor Regulation in the Periphery

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Non-standard Abbreviations:

AA: arachidonic acid, AKAP 79/150: A-kinase anchoring protein 79/150, BK: bradykinin, cAMP, cyclic adenosine monophosphate, CFA: complete Freund's adjuvant, CNS: central nervous system, COX: cyclooxygenase, DAMGO: D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin, DRG: dorsal root ganglia, ERK: extracellular signal-regulated kinase, GIRK: G-protein inward rectifying potassium channel, GPCR: G-protein coupled receptor, GRK: G-protein receptor kinase, LOX: lipoxygenase, MAPK: mitogen-activated protein kinase, NGF: nerve growth factor, PKA: protein kinase A, PKC: protein kinase C, RGS: regulator of G-protein signaling, RKIP: raf kinase inhibitory protein, TrkA: tropomysin receptor kinase A, TRPA1: transient receptor potential family A1, TRPV1: transient receptor potential family V1, VGCC: voltage-gated calcium channel, μ: mu opioid receptor isoform, δ: delta opioid receptor isoform, κ: kappa opioid receptor isoform,

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ABSTRACT

Opioids serve a vital role in the current analgesic array of treatment options. They are useful in acute instances involving severe pain associated with trauma, surgery, and in terminal diseases such as cancer. In the past three decades, multiple receptor isoforms and conformations have been reported throughout literature. Most of these studies conducted systemic analyses of opioid receptor function, often generalizing findings from receptor systems in central nervous tissue or exogenously-expressing immortalized cell lines as common mechanisms throughout physiology. However, a culmination of innovative experimental data indicate that opioid receptor systems are differentially modulated depending on their anatomical expression profile. Importantly, opioid receptors expressed in the peripheral nervous system undergo regulation uncommon to similar receptors expressed in central nervous system tissues. This distinctive characteristic begs one to question whether peripheral opioid receptors maintain anatomically unique roles, and whether they may serve an analgesic advantage in providing pain relief without promoting addiction.

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INTRODUCTION

Opioid receptor systems belong to one of three isoform families: μ (mu, MOP), δ (delta, DOP), or κ (kappa, KOP) (Lord et al., 1977). Each isoform is composed of a 7-transmembrane spanning G Protein-coupled Receptor (GPCR) that signals through Gαi/o subunits to inhibit adenylyl cyclase activity, reducing the production of cAMP and subsequent PKA activity. Activation of the accompanying Gβγ subunits results in activation of inward rectifying K^+ channels (GIRK, (Christie et al., 1987)) and inhibition of voltage-gated Ca^{+2} channels (VGCC, (Gross and Macdonald, 1987)), thereby hyperpolarizing neurons in which opioid receptors are activated. In several cell models, opioid receptor activation stimulates Gβ displacement from Gαi/o subunits and subsequent ERK/MAPK phosphorylation (Belcheva et al., 2003). However, it is well accepted that all three isoforms produce similar effects across physiology, resulting in outcome measurements appropriately reproducible between research groups and models.

Opioid receptors were originally and widely considered susceptible to canonical desensitization mechanisms, in which agonist activation of the GPCR stimulates β-arrestin-dependent internalization (Bohn et al., 2000; Bohn et al., 1999; Gainetdinov et al., 2004). Within this model, opioid receptor activation triggers G protein receptor kinase 2/3 (GRK 2/3) to phosphorylate residues on the intracellular face of opioid receptors to uncouple the receptor from G-proteins and attract β-arrestin association with the receptor (Zhang et al., 1998). Clathrin-mediated internalization then occurs, directing vesicles to degradation or recycling to the plasma membrane. This cycle represents a common desensitization mechanism for many

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GPCRs, including μ, δ and κ. However, recent work has discovered anatomical differences in opioid receptor desensitization, as well as responsiveness.

CENTRAL OPIOID RECEPTOR REGULATION

Opioid receptor function *in vivo* was initially characterized utilizing CNS tissue, including brain and spinal cord. These studies, including work from the labs of Macdonald Christie, John Traynor, Mark Von Zastrow, and Chris Evans, among many others, found receptor behavior similar to studies in immortalized cell lines. All three isoforms of opioid receptors are constitutively sensitive to agonist activation, and undergo canonical, clathrin-mediated GPCR desensitization. Opioid receptor regulation in central nervous tissue involves dynamic Protein Kinase A phosphorylation events that dictate receptor function (Bernstein and Welch, 1998; Chakrabarti et al., 1998), G-protein Receptor Kinase events that mediate receptor desensitization (Chakrabarti et al., 2001; Hasbi et al., 1998), β-arrestin association that can dictate downstream signaling function (Appleyard et al., 1999; Bohn et al., 2000; Bohn et al., 1999; Zhang et al., 1999), and modulation by Regulators of G-protein Signaling (RGS, (Dripps et al., 2017; Huang et al., 2015)). Importantly, these regulatory processes occur similarly throughout the central nervous system as well as in exogenously over-expressing immortalized cell lines. However, new and innovative studies have discovered unique differences in the regulation of opioid receptors in peripheral nervous tissue that could bear pharmacological utility.

PERIPHERAL OPIOID RECEPTOR "PRIMING"

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In 2011, members of the Tesmer group found that GRK2/3 inhibition in locus coeruleus neurons only partially blocks agonist-induce μ desensitization, indicating that other desensitization mechanisms may participate, or that GRK2/3 may play another role (Thal et al., 2011). Although this was one of the first reports identifying anatomical distinctions in opioid receptor desensitization, it was almost a decade earlier in which Stein and Schafer discovered anatomical differentiation in opioid receptor agonist responsiveness. In 2003, Zollner *et al* identified μ responsiveness in peripheral sensory neurons isolated from the dorsal root ganglia as significantly greater following painful inflammation of the innervated tissue (Zollner et al., 2003). These results were later confirmed by Clarke and colleagues across all three isoforms in both *in vivo* and *in vitro* models (Berg et al., 2007; Berg et al., 2011; Patwardhan et al., 2005). In these studies, the application of an inflammatory mediator, bradykinin, stimulated increased responsiveness of opioid receptor signaling in peripheral nervous tissues. Importantly, basal opioid receptor responsiveness was considerably low, relative to other studies conducted with central nervous tissues (Arttamangkul et al., 2008; Dang et al., 2009; Whistler and von Zastrow, 1998). This disparity intimated that a type of "braking mechanism" was unique to peripheral nervous tissues expressing opioid receptors and naively repressing receptor responsiveness. However, the molecular players of this mechanism remained undiscovered, prompting additional studies to highlight potential targets to increase peripheral opioid efficacy.

The unique physiology and phenotype of peripheral nervous system tissues, including dorsal root and trigeminal ganglia neurons, serve as an important starting point for examining potential players in the mechanism that supports naïve desensitization of opioid receptors. Peripheral

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sensory neurons have a bipolar phenotype, with afferent innervations that can extend over a meter in length from the cell body. Due to this length, many effects seen on opioid receptors in the periphery are likely due to temporally-discriminative changes in protein-protein interactions and receptor coupling, in contrast to more time-extensive modifications in receptor translation and insertion (Jung et al., 2012). Therefore, investigations centered around post-translational mechanisms that would either stimulate or inhibit protein associations within short time frames appropriately represent previously published *in vivo* findings. In 2015, work by the Clarke group identified that δ functional competence in peripheral trigeminal sensory neurons was positively regulated by arachidonic acid (AA) and bradykinin (BK) pre-treatment (Sullivan et al., 2015). Additional work revealed various sensitivities to cyclooxygenase (COX) or lipoxygenase (LOX) inhibition, suggesting that COX and LOX metabolites of AA also stimulate OR functional competence. Importantly, BK and AA both activate protein kinase C (PKC, (Burgess et al., 1989; O'Flaherty and Nishihira, 1987)), as do COX and LOX metabolites (Abayasekara et al., 1993; Castrillo et al., 2003; Liu et al., 1991; Shearman et al., 1989), suggesting that PKC may play an important role in reversing naive opioid receptor desensitization in peripheral afferent sensory neurons (**Figure 1**).

PKC is an important kinase and signaling molecule in neurons, including peripheral sensory neurons. As a post-translational modifier, it reacts to changes in lipid composition, intracellular Ca^{+2}, and numerous upstream regulators to target ion channels, GPCRs, and other proteins to significantly change activities and downstream functions. Indeed, Brackley et al discovered that PKC activation by bradykinin was necessary to reverse functional incompetence of δ in peripheral

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dorsal root ganglia neurons (Brackley et al., 2016). Results from this work demonstrated that application of the inflammatory mediator BK stimulated PKC to phosphorylate Raf Kinase Inhibitory Protein (RKIP). Phosphorylation of this scaffolding protein stimulates dimerization and sequestration of GRK2, which was found to be naively associated with δ in sensory neurons (**Figure 1**). Importantly, expression of the kinase-dead mutant GRK2-K220R (Freedman et al., 1995) in sensory neurons failed to increase δ competence, suggesting that kinase activity of GRK2 was not the limiting factor for δ functional incompetence, but rather steric blockade of signaling molecules that would associate with the receptor. In this model, GRK2 occupation of G-protein binding sites would prevent functional activation of pathways downstream from δ, thereby rendering the receptor system incompetent. Additional work corroborates the importance of basal participation by GPCR desensitization machinery as β-arrestin plays a role similar to GRK2 in μ and δ functional incompetence in trigeminal sensory neurons (Sullivan et al., 2016). Importantly, recent findings agree in that opioid receptors expressed in peripheral sensory neurons exist in a functionally-desensitized state, unlike in neurons from central nervous tissues. Furthermore, inflammatory activation of PKC "primes" the opioid receptors for full responsiveness to agonist stimulation, providing a mechanism for this unique level of anatomically-distinct receptor regulation.

GRK BIOCHEMISTRY

Transient GRK isoform association with the opioid receptor has been detailed previously in multiple cell models. Homologous desensitization of opioid receptors is most often initiated by kinase phosphorylation of amino acids along the C-terminus of the receptor. For μ, GRK2/3

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phosphorylation predominantly occurs following high-efficacy opioid agonist administration, while GRK5 serves to phosphorylate the receptor following morphine (low-desensitizing agonist) (Doll et al., 2012; Schulz et al., 2004). Importantly, PKC has also demonstrated a role in receptor phosphorylation, although this typically occurs following activation of a co-expressing Gαq-coupled GPCR (Bailey et al., 2004; Doll et al., 2011; Feng et al., 2011). GRK2 is also responsible for post-agonist phosphorylation and internalization of δ (Guo et al., 2000; Kouhen et al., 2000) and κ (Appleyard et al., 1997; McLaughlin et al., 2003) in both immortalized and central nervous system cell models. In 2017, Brackley *et al* found that PKA phosphorylation of GRK2 drives plasma membrane targeting and constitutive association with δ in peripheral dorsal root ganglia neurons (Brackley et al., 2017). GRK2 phosphorylation in this neuronal model was mediated by A-Kinase Anchoring Protein 79/150 (AKAP 79/150), thereby utilizing a scaffolding protein necessary for post-translational upregulation of several pain-sensing TRP channels, including TRPV1 (Jeske et al., 2008; Jeske et al., 2009; Schnizler et al., 2008; Zhang et al., 2008) and TRPA1 (Brackley et al., 2017). Importantly, the expression of an AKAP-mutant with a deleted PKA binding domain resulted in a functionally competent δ in sensory neurons, indicating an important scaffolding role for AKAP in the naïve and inhibitory association of GRK2 with δ in peripheral nervous tissue.

Previous studies in peripheral sensory neurons primarily examined initial opioid receptor responsiveness, and not desensitized responses, so little is known concerning the potential for anatomical distinction in receptor desensitization. Given that GRK2 constitutively associates with δ, is it possible that receptor desensitization follows a non-GRK2 mechanism? Phosphorylation

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at T394 of μ has been reported to be critical to acute DAMGO desensitization and not morphine but has no effect on chronic desensitization to either (Li et al., 2013). This is also the case for CHO cells (Pak et al., 1997), and animals (Wang et al., 2016), suggesting similar desensitization mechanisms for μ in peripheral and central nervous tissues. Furthermore, phosphorylation of T394 μ appears to be GRK2-dependent (Zhang et al., 1998), suggesting that the kinase potentially governs both pre-agonist and post-agonist functional competence of peripheral opioid receptors. Less information is available for δ and κ in peripheral sensory neurons, however given their similarities in mechanisms of agonist-dependent desensitization, it can be presumed that all three isoforms follow similar patterns of desensitization, with similar dependencies on GRKs. Importantly, can the anatomical distinctions of opioid receptors between peripheral and central nervous tissues be targeted pharmacologically to increase receptor responsiveness in the periphery and reduce systemic side effects?

On the intracellular side, blocking constitutive GRK2 association with an opioid receptor would induce functional receptor competence and the possibility for analgesia. Tesmer and colleagues have conducted a number of studies on the efficacies of various GRK inhibitors on GPCR function and physiology (Bouley et al., 2017; Homan and Tesmer, 2015; Tesmer et al., 2010; Thal et al., 2012). However, as Brackley *et al* found, GRK2 kinase activity was not a rate-limiting step in maintaining δ in a desensitized state (Brackley et al., 2016). Rather, GRK2 provides steric hindrance to G-protein activation by δ. Given the large number of proteins and enzymes that constitute the GRK "interactome", it becomes difficult to speculate whether other intracellular pathways would be affected, thereby modifying tissue physiology beyond an opioid receptor.

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However, current and unpublished studies stretch the treatment possibilities for GRK inhibitors to unconsidered realms, providing hope for multiple diseases.

OPIOID RECEPTOR TRANSLOCATION

Opioid receptor function is dependent on sub-cellular localization (Stoeber et al., 2018). Similar to many CNS models, δ function in dorsal root ganglia neurons is dependent upon plasma membrane expression (Scherrer et al., 2006). However, several studies have discovered mechanisms unique to primary afferent neurons that regulate opioid receptor function based on receptor translocation. For example, subcellular localization of δ to the plasma membrane was increased in DRG neurons following ipsilateral exposure to CFA (Gendron et al., 2006). Bradykinin, an inflammatory mediator increased *in vivo* following CFA administration, was reported to increase the number of DRG neurons positively expressing δ on its cell surface (Pettinger et al., 2013). Additionally, NGF-activation of TrkA in DRG increases μ agonist efficacy by increasing receptor translocation to peripheral nerve terminals (Mousa et al., 2007). Pradhan *et al* reported in 2013 that inflammation increases N-type Ca^{+2} channel contribution to δ-mediated analgesia in DRG neurons in a β-arrestin 2-dependent manner (Pradhan et al., 2013). This intimates that β-arrestin 2 affects subcellular localization of the δ receptor isoform and its functional conjugation to Gβγ-mediated inhibition of VGCC, a primary mechanism that supports hyperpolarization of primary afferent neurons. Interestingly, research from the same group found that δ receptor responses were increased in DRG neurons with β-arrestin 1 knocked out (Mittal et al., 2013). However, this mechanism was tied to ROCK- and LIMK-dependent

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translocation mechanisms to the plasma membrane, such that exposure to δ-specific agonists resulted in increased δ receptor expression on the plasma membrane.

Multiple sources indicate varying amounts of δ/μ co-expression in primary afferent neurons, including values as low as 5% total (Scherrer et al., 2009) to approximately 30% (Bardoni et al., 2014). Despite their functional similarities/differences, co-expression of the two isoforms appear to regulate function in the periphery. Reduced δ expression concomitantly reduces cell surface expression and function of μ in DRG neurons (Walwyn et al., 2009). Unique to this pairing, others have demonstrated functional heterodimerization of μ and δ isoforms in peripheral afferents (Wang et al., 2010), even demonstrating dynamic changes in δ, μ, and κ isoform expression in DRG afferents following inflammation (Ji et al., 1995). Together, these reports indicate a unique interplay of cross-functionalization between opioid receptor isoforms that maintain unique characteristics in the peripheral nervous system.

PERIPHERAL OPIOID TARGETS

In 2017, Spahn *et al* designed a μ-specific agonist uniquely selective for receptor association in inflamed tissues only (Spahn et al., 2017). Utilizing computer modeling of μ at low pH values, thereby attempting to re-create inflammatory conditions for agonist association, they designed a new agonist that only binds to μ when the pH is low and in a state of inflammatory flux. Importantly, this compound was capable of stimulating Gαi signaling when applied following inflammatory injury without affecting central autonomic centers, thereby avoiding depressed respiration, sedation or addiction. This aspect was utilized by Jamshidi et al while reporting on

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the functional selectivity of the peripherally-restrictive κ opioid agonist U50488 (Jamshidi et al., 2015), which avoids CNS modulation of mood, and is especially important in reference to recent work on κ as a peripheral pain target (Snyder et al., 2018). Taken together, recent findings are important for understanding the anatomical complexity and differential possibilities of peripheral opioid receptors when designing safer and newer opioids for the treatment of pain.

CONCLUSIONS AND FUTURE DIRECTIONS

A considerable amount of peripheral opioid receptor research has focused on the δ and μ receptor isoforms. However, previous work on the κ receptor system identifies that specific kappa agonists may hold analgesic properties isoforms in peripheral nervous tissue (Berg et al., 2011; Cunha et al., 2012; Labuz et al., 2006; Su et al., 1998), despite little biochemical evidence supporting whether kappa opioid receptors are regulated similarly to δ and μ. Given recent breakthroughs associated with receptor dimerism and allosteric agonists, multiple research groups are consistently identifying new mechanisms unique to peripheral opioid receptor systems that can be manipulated to provide accurate analgesia without central side effects. This remains an important goal in patient care in the generational combat against the worldwide opioid crisis.

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AUTHORSHIP CONTRIBUTIONS

N. Jeske wrote the manuscript.

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LEGENDS FOR FIGURES

Figure 1: Illustration of inflammation stimulating a conversion of the opioid receptor system from a state of incompetence to that of competence for activation on a peripheral nerve ending. Abbreviations include AA: arachidonic acid, βArr2: β-Arrestin 2, GRK2: G-protein receptor kinase 2, PKC: protein kinase C.

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FIGURE 1