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THE DELTA OPIOID RECEPTOR TOOL BOX

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Abstract

In recent years, the delta opioid receptor has attracted Increasing Interest as a target for the treatment of chronic pain and emotional disorders. Due to their therapeutic potential, numerous tools have been developed to study the delta opioid receptor from both a molecular and a functional perspective. This review summarizes the most commonly available tools, with an emphasis on their use and limitations. Here, we describe (1) the cell-based assays used to study the delta opioid receptor. (2) The features of several delta opioid receptor ligands, including peptide and non-peptide drugs. (3) The existing approaches to detect delta opioid receptors in fixed tissue, and debates that surround these techniques. (4) Behavioral assays used to study the *in vivo* effects of delta opioid receptor agonists; including locomotor stimulation and convulsions that are induced by some ligands, but not others. (5) The characterization of genetically modified mice used specifically to study the delta opioid receptor. Overall, this review aims to provide a guideline for the use of these tools with the final goal of increasing our understanding of delta opioid receptor physiology.

Keywords

pain; cell lines; mutant mice; G protein-coupled receptor

INTRODUCTION

Opioids have been used for centuries, during which time they have been alternately hailed as both a panacea of man's ills, and cursed as a scourge of civilization. The vast majority of opioids used clinically (eg. morphine, hydrocodone, oxycodone) or recreationally (eg. heroin) produce their behavioral effects primarily through activation of the mu opioid receptor. Although mu agonists are incredibly potent analgesics, they also produce deleterious adverse effects which severely limit their therapeutic use. Activation of the mu opioid receptor can produce constipation, respiratory depression, and sedation. Furthermore, tolerance and physical dependence is observed after chronic use, and the abuse liability of mu agonists is very high.

Another member of the opioid receptor family - the delta opioid receptor - may offer a potential alternative to mu-based therapies. In acute pain states, activation of the delta opioid receptor is relatively ineffective compared to their mu counterparts (Gallant and Meert,

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2005). However, they are highly effective in chronic pain states (Hurley and Hammond, 2000; Fraser et al., 2000a; Cahill et al., 2003; Nadal et al., 2006; Gaveriaux-Ruff et al., 2008; Pradhan et al., 2009, 2010), and delta agonists were also recently shown to be effective for the treatment of migraine (Pradhan et al., 2014). Further, activation of the delta opioid receptor appears to have low abuse liability, as delta agonists are not self-administered (Negus et al., 1998; Stevenson et al., 2005) nor do they cause physical dependence (Brandt et al., 2001). Interestingly, delta opioid receptors also positively modulate emotional tone. Genetic deletion of either the delta opioid receptor or its endogenous ligand, enkephalin, results in anxiogenic and depressive-like behaviors (Konig et al., 1996; Filliol et al., 2000). In contrast, delta agonists can produce anxiolytic and antidepressant effects (Saitoh et al., 2004; Perrine et al., 2006). This emotional regulation may be particularly important for the treatment of chronic pain states, as there is a high comorbidity with depression and anxiety (Yalcin and Barrot, 2014). The potential of targeting this receptor has not gone unnoticed, and there are ongoing efforts to develop delta opioid receptor agonists for the treatment of a number of psychiatric and neurological conditions (Pradhan et al., 2011a).

During the past 5 years, there have been many reviews published on the delta opioid receptor, which have highlighted their biological function, therapeutic utility, and signaling mechanisms; and we direct the reader to these excellent resources (Pradhan et al., 2011a; Chu Sin Chung and Kieffer, 2013; van Rijn et al., 2013; Gendron et al., 2014; Charfi et al., 2015; Klenowski et al., 2015). The aim of this review, however, is to provide a more practical guide on how to study the delta opioid receptor. Our intention is to discuss the tools that are available to probe delta opioid receptor signaling, distribution, and function; and to highlight some of the pros and cons associated with each method. We hope that this review will be a useful “how to” guide for those interested in working on the delta opioid receptor.

How to examine delta opioid receptors in cells

The expression of delta opioid receptors in non-neuronal cell lines has served as a powerful tool to delineate delta opioid receptor trafficking and regulation (Whistler et al., 2002; Puthenveedu et al., 2010; Henry et al., 2011), signaling complexes (Audet et al., 2008, 2012; Charfi et al., 2014), and a multitude of other mechanisms. Delta opioid receptors have been transfected into several non-neuronal cell lines, such as the Chinese Hamster Ovary (CHO), the Human Embryonic Kidney (HEK) 293, and the African Green Monkey Kidney (COS-7) (Chan et al., 2003; Hong et al., 2009; Tudashki et al., 2014; Nagi et al., 2015a). Cells are transfected with cDNA encoding the delta opioid receptor, modified to express a peptide epitope or tag within either the N-terminal extracellular region or the C-terminus. Widely used epitope tags include hemagglutinin (HA), *c-myc*, and FLAG (Qiu et al., 2007; Tudashki et al., 2014; Nagi et al., 2015a). Epitope tagging facilitates the detection of the receptor by high-affinity antibodies, and overcomes many of the difficulties posed by using delta opioid receptor specific antibodies (see section below). In addition, delta opioid receptors can also be modified for FRET/BRET- (fluorescence/bioluminescence resonance energy transfer) based technologies, thus allowing for the direct visualization of specific protein-protein interactions (Audet et al., 2008, 2012; Richard-Lalonde et al., 2013; Charfi et al., 2014; Nagi et al., 2015b; Pradhan et al., 2016).

However, it is important to keep in mind some of the limitations of transfected cell systems. There is the possibility that tagging the receptor may affect receptor function. For example, tags can interfere with post-translational processing (Jiang et al., 2012), oligomerization, ligand binding (N-terminal tags), and receptor regulatory and signaling events (C terminus). In addition, some tags may also affect receptor distribution within the cell itself ((Wang et al., 2008) and see detection section below). Furthermore, each cell line will express different types and levels of G proteins and regulatory proteins that will influence the outcome of the study. For example, delta opioid receptor internalization was found to be regulated by different proteins in rat primary neuronal cultures versus HEK 293 cells (Charfi et al., 2014). Also, microarray analysis has revealed several differences in gene expression of GPCRs and different signaling molecules between HEK 293 cells, AtT20, BV2 and N18 cell lines (Atwood et al., 2011). Moreover, transfection can often result in overexpression of the receptor, which also alters receptor regulation and function. Expression level may also vary across passages or batches of cells and across labs.

As an alternative to recombinant cells, there are neuronal lines that endogenously express the delta opioid receptor that may be considered closer to an endogenous environment. However, the lack of a tag means that receptor detection is more restricted. A further caveat of the use of immortalized hybrid cell lines is that they tend to lose chromosomes as they divide (Heumann et al., 1977; Geraghty et al., 2014). Therefore, it is recommended to create a large stock of frozen cells of low passage number, strictly track the number of passages, use the hybrid cells in a defined bracket of passage numbers, and report which passages were used for publication. The genetic instability of these cells lines can lead to batch variability and could contribute to variations in results between different laboratories. Some of the most commonly used cells lines that endogenously express the delta opioid receptor are the following:

NG108-15 cell line—These cells were obtained by inactivated Sendai virus-induced fusion of mouse | N18TG2 neuroblastoma cells with rat C6-BU-1 glioma cells (Klee and Nirenberg, 1974). They served as a source for the initial cloning of the delta opioid receptor (Evans et al., 1992; Kieffer et al., 1992), and have long been used as a cellular model for this receptor (Law et al., 1982, 1983; Hsia et al., 1984; Moses and Snell, 1984; Cone et al., 1991; Roerig et al., 1992). They carry a homogenous population of endogenous delta opioid receptor, and do not express mu or kappa opioid receptors (Chang and Cuatrecasas, 1979). However, they do express the opioid receptor-like receptor (ORL1 (Ma et al., 1997)). Importantly, NG108-15 cells rely to a large extent on anaerobic glycolysis for energy production. This gives rise to high concentrations of lactic acid, producing cell dissociation and loss of viability (Hamprecht et al., 1985). A further important issue to keep in mind is the instability of delta opioid receptor expression in this line; where early passages have higher probability of expressing the delta opioid receptor (passage number <30).

F11—This is a hybrid cell line created from the fusion of embryonic rat dorsal root ganglia (DRG) neurons with cells from mouse neuroblastoma N18TG2 (Platika et al., 1985). These cells exhibit properties characteristic of DRG neurons including neuronal morphology, expression of cell-surface gangliosides and excitable membranes (Platika et al., 1985). They

express mu and delta opioid receptors that inhibit adenylate cyclase through pertussis toxin-sensitive Gi/o proteins (Francel et al., 1987; Fan et al., 1992). Overall, F11 cells are a useful tool to study delta opioid receptors in a nociceptor-like cell model that displays properties similar to sensory DRGs.

SK-N-BE cell line—This is a human neuroblastoma cell line established from a bone marrow biopsy, which endogenously expresses the human delta opioid receptor (Polastron et al., 1994). It has been proposed that this cell line expresses two delta opioid receptor subtypes, $\delta 1$ and $\delta 2$ (Polastron et al., 1994; Allouche et al., 2000). However, there is still controversy on the existence of delta opioid receptor subtypes since the different abilities of delta agonists to stimulate the receptor can also be explained by other means (different ligand-binding sites at the same receptor, biased-agonism, heterodimers etc). SK-N-BE cells may also express mu opioid receptors at a similar ratio to delta (Palazzi et al., 1996). These cells have been used extensively to study ligand directed signaling at the delta opioid receptor (Allouche et al., 1999a,b; Marie et al., 2003b, 2008).

SK-N-SH cell line—This is a human neuroblastoma cell line established from a bone marrow biopsy, which contains both mu and delta opioid receptors in a μ/δ ratio of $\sim 5:1$ (Yu et al., 1986). However, phenotypic heterogeneity in this range has been described due to differences in culture conditions (Baumhaker et al., 1993). This cell line has been used extensively to study interactions between the mu and delta opioid receptor (Baumhaker et al., 1993), and the formation of mu-delta heterodimers specifically (Gomes et al., 2000, 2004). Work with this cell line has also revealed a stimulatory effect of opioid peptides on adenylate cyclase (Same et al., 1998; Rubovitch et al., 2003). In addition to mu and delta opioid receptors, SK-N-SH cells also express ORL-1 receptors (Cheng et al., 1997).

SH-SY5Y cell line—This cell line was subcloned from the SK-N-SH line, and express mu and delta opioid receptors (Kazmi and Mishra, 1986; Yu and Sadee, 1988). Like its parent line, SH-SY5Y cells express delta opioid receptors to a lesser extent than mu (Kazmi and Mishra, 1987; Yu and Sadee, 1988). SH-SY5Y cells also express ORL-1 receptors (Cheng et al., 1995), and signaling proteins like the Regulator of G protein signaling 4 (RGS4) (Lambert et al., 1989; McDonald et al., 1994; Wang et al., 2009; Levitt et al., 2011; Wang and Traynor, 2011). Given its repertoire of signaling proteins, SH-SY5Y cells have been widely used for the study of mu and delta opioid receptor activity, and receptor desensitization (Elliott et al., 1997; Nowoczyn et al., 2013). In addition, it has been reported that mu and delta opioid receptors share a common pool of G proteins and adenylate cyclase in SH-SY5Y cells (Alt et al., 2002; Levitt et al., 2011), indicating the close proximity of both receptors in these cells; however, no direct evidences of mu-delta heterodimerization have been found in this cell line. In addition, when these cells are used in a differentiated state they show a more mature neuronal phenotype and upregulated expression of opioid receptors (Yu and Sadee, 1988).

What ligands bind to the delta opioid receptor

There are many ligands that bind to the delta opioid receptor, and the aim of this section is to cover only those that are most commonly used and commercially available (Table 1). A wide

range of peptide and small molecules have been developed that target the delta opioid receptor. Among the peptide delta opioid receptor agonists, the most popular are [D-Ala², D-Leu⁵]-enkephalin (DADLE), [D-Pen^{2,5}]-enkephalin (DPDPE), deltorphin I and deltorphin II. DADLE and DPDPE were obtained through chemical modification of the endogenous peptides met- and leu-enkephalin (Beddell et al., 1977; Mosberg et al., 1983). DADLE and DPDPE possess higher delta opioid receptor selectivity and stability than their prototypes, although DADLE appears to have less selectivity to the delta opioid receptor (Goldstein and Naidu, 1989), and both agonists have affinity for the mu opioid receptor as well (Fraser et al., 2000b; Chan et al., 2003). Deltorphan I and II were isolated from frog skin extracts (Erspamer et al., 1989). In comparison with the enkephalin analogs (DADLE and DPDPE), deltorphan I and II have higher biostability and delta opioid receptor selectivity, and comparatively deltorphan II has greater selectivity for the delta opioid receptor over mu (Payza, 2004). Peptide antagonists have also been developed against the delta opioid receptor, specifically TIPP, TIPP-NH₂ and its analogs (Schiller et al., 1992a,b). Among them, TIPP[ψ] has shown subnanomolar affinity and extraordinary selectivity for the delta opioid receptor (Schiller et al., 1993).

Although peptide agonists have been extensively used to characterize the delta opioid receptor, they generally have low blood-brain permeability and poor metabolic stability. There are some reports that DPDPE and deltorphins can cross the blood-brain barrier (Williams et al., 1996; Fiori et al., 1997); and there has also been some effort to make more stable peptidomimetics (Rochon et al., 2013). In addition, considerable effort has been expended in developing small molecule (non-peptide) agonists to study the delta opioid receptor. Among these small molecule agonists SNC80, ARM390 and TAN67 are commonly used.

SNC80 was derived from the highly potent delta agonist (+)BW373U86 (Calderon et al., 1994), and is the most widely used systemically available small molecule delta agonist. *In vitro*, the K_i of SNC80 is in the low nM range, and it has similar selectivity to deltorphin II, as compared to mu (~200 fold) and kappa (~2500 fold) opioid receptors (Bilsky et al., 1995; Payza, 2004). SNC80 is usually administered subcutaneously or intraperitoneally, and is not highly effective when given *per os* (AstraZeneca personal communication). Unfortunately, SNC80 is difficult to dilute, and requires an acidic vehicle (Table 1). In our opinion, SNC80 acts as a bench-mark delta agonist, as it has a high selectivity for the delta opioid receptor and produces the widest array of receptor regulatory and signaling events, and behavioral effects.

ARM390 (Wei et al., 2000) is a highly selective delta opioid receptor agonist, with similar receptor binding and G protein activation properties to SNC80 (Wei et al., 2000; Marie et al., 2003a; Payza, 2004; Pradhan et al., 2009). It has a greater selectivity for the delta opioid receptor than SNC80, and affinity for mu and kappa ~4000 and ~6000 fold greater. Differences in the internalization properties of SNC80 and ARM390 have been found; while SNC80 produces robust receptor internalization, ARM390 does not induce substantial delta opioid receptor sequestration (Marie et al., 2003a; Pradhan et al., 2009); and this can have an impact on short- and long-term tolerance following chronic use (Pradhan et al., 2009, 2010). Although ARM390 is a low-internalizing agonist, it does appear to recruit

arrestin 3; and this interaction is limited to the cell membrane (Pradhan et al., 2016). Importantly, ARM390 is only effective when administered *per os* by gavage (AstraZeneca, personal communication). Indeed, no analgesic effects to ARM390 were observed in the lactic acid-induced stretching assay in rats after its intraperitoneal administration (Negus et al., 2012).

TAN-67 is a morphinan derivative with high selectivity for the delta opioid receptor (Knapp et al., 1995). Although the racemic (+/-)-TAN-67 showed no analgesic activity, its enantiomer (-)-TAN-67 is highly effective (Nagase et al., 2001). TAN-67 has also been shown to be a low-internalizing delta agonist (Bradbury et al., 2009). Like most small molecule delta opioid receptor agonists, TAN-67 is systemically active and has been shown to be effective by icv, sc, it, and ip routes in different *in vivo* studies (Refs. in Table 1).

The extensive research carried out to discover non-peptide ligands for the delta opioid receptor has also provided highly selective antagonists. Naltrindole is the most commonly used delta opioid receptor antagonist (Portoghese et al., 1988). It has a high affinity for the delta opioid receptor, with a K_i of 0.18 nM for the human delta opioid receptor. However, naltrindole also has a low nanomolar affinity for the mu opioid receptor; and is only ~25-fold selective for delta over mu (Payza, 2004). Thus, at high concentration naltrindole will also bind to the mu opioid receptor. Other non-peptide antagonists are nal-triben (NTB), naltrindole 5'-isothiocyanate (5'-NTII) and 7-benzylidenenaltrexone (BNTX), which have been used to study the putative delta opioid receptor subtypes ($\delta 1$ and $\delta 2$). Most of the pharmacological evidences for the existence of delta opioid receptor subtypes comes from pharmacological studies (Mattia et al., 1991; Sofuoglu et al., 1991; Maslov et al., 2009; Sugiyama et al., 2014; Beaudry et al., 2015). However, only one clone has been identified for the delta opioid receptor with no polymorphisms or viable splice variants (Gaveriaux-Ruff et al., 1997; Wei and Loh, 2002).

How to detect delta opioid receptors

Although there is a general consensus on the broad anatomical localization of delta opioid receptors (Mansour et al., 1988; Le Merrer et al., 2009), there has been much debate about the specific cell types that express these receptors (Bao et al., 2003; Guan et al., 2005; Cahill et al., 2007; Scherrer et al., 2009; Wang et al., 2010; Bardoni et al., 2014; Gendron et al., 2014). The main arguments are focused on which primary afferents delta opioid receptors are expressed on – small, peptidergic fibers that co-express mu opioid receptors (Bao et al., 2003; Zhang et al., 2006; Overland et al., 2009; Riedl et al., 2009; Wang et al., 2010; Chabot-Dore et al., 2015; Huang et al., 2015), versus medium/large fibers that are non-peptidergic with low co-expression with mu (Scherrer et al., 2009; Bardoni et al., 2014). This argument further extends to whether endogenous delta opioid receptors are located on intracellular vesicles (Svingos et al., 1998; Cahill et al., 2001, 2003, 2007; Wang and Pickel, 2001; Bao et al., 2003; Morinville et al., 2003, 2004; Gendron et al., 2006; Zhang et al., 2006; Kabli and Cahill, 2007; Wang et al., 2010; Huang et al., 2015) or are expressed on the cell membrane (Scherrer et al., 2009; Bardoni et al., 2014). The main discussion has centered on the best way to examine these receptors in fixed tissue. One issue that has plagued the field is the specificity of the antibodies used for traditional

immunohistochemical detection of delta opioid receptors. Many of the commercially available antibodies that were cited in previous publications continued to show staining in delta opioid receptor knockout mice (Scherrer et al., 2009). Even in earlier studies examining the distribution of delta opioid receptor, a commonly used antibody (Dado et al., 1993; Arvidsson et al., 1995; Tao et al., 1998) was also found to be blocked by substance P (Arvidsson et al., 1995). Wang et al. have showed that two commercially available rabbit antibodies, used at very dilute concentrations, did not show staining in the exon 1 targeted delta opioid receptor knockout mouse (Wang et al., 2010). Confirmation of these results by other labs is still ongoing. To add additional complexity, there is batch variation in antibody production, so an antibody that was specific in one lot may no longer be in subsequent lots. Furthermore, variation in species, strain, and how the tissue is processed also contributes to the variability across labs. Antibodies to the delta opioid receptor may be used in immunoblotting protocol (Billa et al., 2010); and there are also antibodies targeting the serine 363 phosphorylated form of the delta opioid receptor (Pradhan et al., 2009), which may also be adapted for immunostaining (Faget et al., 2012). In addition, subtractive immunization techniques have been used to generate antibodies specific to the mu-delta heterodimer (Gomes et al., 2014). Unfortunately, the lack of an unequivocal delta opioid receptor specific antibody has limited our ability to definitively characterize the physiology and expression of this receptor, especially in the translation of preclinical rodent data to human post-mortem tissue.

Another approach to visualizing the delta opioid receptor in fixed tissue has been to use a knockin mouse strategy in which the endogenous delta opioid receptor has been replaced with a fluorescent tagged receptor (DOR-eGFP), where eGFP is linked to the C-terminal tail ((Scherrer et al., 2006; Pradhan et al., 2015), see below and Table 2). These mice show fidelity to wildtype animals in terms of overall anatomical expression of delta opioid receptors as determined by *in situ* hybridization and radioligand binding (Bardoni et al., 2014). In addition, these mice have similar behavioral responses to C57BL/6 mice when treated with delta agonists (Pradhan et al., 2010). They are also an excellent tool to study the *in vivo* trafficking of the delta opioid receptor (Scherrer et al., 2006; Pradhan et al., 2009, 2010; Faget et al., 2012; Bertran-Gonzalez et al., 2013). However, there has been much debate about whether the addition of the eGFP tag alters the cellular distribution and subcellular compartmentalization of delta opioid receptors compared to the endogenous form (Gendron et al., 2014; Zhang et al., 2015). Controversy in this regard is due to the differences in the localization of delta opioid receptors found using DOR-eGFP mice versus delta opioid receptor antibodies. In dorsal root ganglia (DRG) and lumbar spinal cord, very few DOR-eGFP receptors were co-localized with mu opioid receptors (Scherrer et al., 2009), which is in contrast to studies performed with antibodies that indicate a physical and functional interaction between both receptors (see (Gendron et al., 2014) for a review). In addition, DOR-eGFP receptors are expressed on the cell membrane, in opposition to the findings that delta opioid receptors are on intracellular vesicles which are trafficked to the cell surface by release of substance P (Bao et al., 2003; Guan et al., 2005). Further investigation into these contentious issues is ongoing, and it is likely that under certain conditions different aspects of all of these findings are possible. For instance, multiple lines of evidence indicate that the delta opioid receptor is dynamic, and increased functionality is

observed following chronic stimuli such as pain (Cahill et al., 2003; Kabli and Cahill, 2007; Pradhan et al., 2013), morphine (Cahill et al., 2001), and ethanol exposure (van Rijn et al., 2012). This receptor plasticity may also enhance mu-delta interactions. More importantly, a greater emphasis must be placed on the translational implications of these findings, especially considering that there are major differences in the expression of delta opioid receptor in the DRG and spinal cord of rodents versus primates and humans (Mennicken et al., 2003).

There are many alternative approaches to visualizing the delta opioid receptor, although these approaches do not generally allow for co-localization or cellular characterization. Autoradiography was used early on to determine the central and spinal regions that express delta opioid receptors (Mansour et al., 1988; Le Merrer et al., 2009). Commercially available radioligands include the agonists: [^3H] or [^{125}I]Deltorphan II, [^3H]DADLE, and [^3H]DPDPE; and the antagonist [^3H]naltrindole. The radiolabeled delta agonists may also bind to the mu opioid receptor (Fraser et al., 2000b; Payza, 2004; Scherrer et al., 2004), but can be overcome with the addition of the mu antagonist CTOP to the binding buffer (Pradhan and Clarke, 2005a).

Another approach which can minimize non-specific binding, and allows for electron microscopy is the use of photoaffinity radioligands. Photoaffinity labels can be covalently bound to the receptor by UV irradiation after washing away ligand that is free or weakly bound to non-specific targets. However, these ligands usually render a very low percentage of covalent incorporation (5–20%). The photoaffinity radioligands [^3H]azido-DTLET and [^{25}I]azido-DTLET have been used to study the autoradiographic localization of the delta opioid receptor in rat brain sections and membrane homogenates (Zajac et al., 1987; Bochet et al., 1988; Pasquini et al., 1992, 1993); and at the [^{25}I]azido-DTLET has been used to detect delta opioid receptors at the ultrastructural level (Pasquini et al., 1992).

One of the more effective ways to examine delta opioid receptor expression and trafficking is the use of fluorescently labeled delta ligands. In comparison with radioligands, fluorescent probes are safer and allow receptor localization with enhanced temporal and spatial resolution (Handl et al., 2005; Emami-Nemini et al., 2013). However, fluorophores are bulky and can influence the binding properties of the ligand; moreover, they have hydrophobic properties that can affect their biological activity and selectivity (Kolb et al., 1983; Balboni et al., 2004). Nevertheless, a wide range of fluorescent conjugates of delta agonists and antagonists have been synthesized and are commercially available (Cell Aura Technologies Ltd. and Cisbio Bioassays). Fluorescent antagonists have proved useful to examine delta opioid receptor distribution in living cells (Kshirsagar et al., 1998; Balboni et al., 2004; Cohen et al., 2016); while agonists have been used to study delta opioid receptor internalization and trafficking *in vivo* (Arttamangkul et al., 2000; Cahill et al., 2001; Lee et al., 2002).

Functional imaging techniques have also been used to study delta opioid receptor distribution, and specific radioligands for positron emission tomography (PET) are available. A potent and selective delta opioid receptor antagonist coupled to [^{11}C]iodomethane, [^{11}C]-N1'-methylnaltrindole, has been successfully used in several PET studies in rodents and

humans (Lever et al., 1992; Madar et al., 2007; Weerts et al., 2008, 2011; Wand et al., 2013). The non-selective agonist [¹¹C]diprenorphine that labels mu, kappa and delta opioid receptors has also been used to examine the distribution of opioid receptors in a variety of clinical conditions, including epilepsy (Hammers et al., 2007; McGinnity et al., 2013), pain (Jones et al., 2004; Willoch et al., 2004), and opioid addiction (Melichar et al., 2005; Williams et al., 2009). Taking into consideration the potential of PET to advance our understanding of the opioid system in the normal and diseased brain *in vivo*, considerable efforts are being made to increase the number of radiotracers designed to study the delta opioid receptor (Clayson et al., 2001; Tyacke et al., 2002).

THINGS TO CONSIDER WHEN EXAMINING THE BEHAVIORAL EFFECTS OF DELTA AGONISTS

Treatment of chronic pain, anxiety and depression are some of the primary behaviors in which delta agonists have been shown to be effective. However, they are also involved in a number of other behavioral effects including regulation of other drugs of abuse (Bosse et al., 2008; van Rijn et al., 2010; Pradhan et al., 2011a). Delta opioid agonists are not very effective in acute pain models (Gallantine and Meert, 2005), although they do work in the paw pressure assay (Fraser et al., 2000b). Delta agonists are effective in chemically-induced pain such as the formalin (for a review see (Gaveriaux-Ruff and Kieffer, 2011)), and acetic acid writhing tests (Gallantine and Meert, 2005). Animal models of chronic pain is where delta agonists have shown the most promise. They are highly effective in the Complete Freund's Adjuvant (CFA) model of inflammatory pain (Gendron et al., 2006; Pradhan et al., 2009), peripheral neuropathy models (Kabli and Cahill, 2007; Nozaki et al., 2014), and bone cancer-induced pain models (Otis et al., 2011). In addition, they have recently been shown to work in a model of migraine pain and aura (Pradhan et al., 2014; Charles and Pradhan, 2016).

The effectiveness of delta opioid receptor agonists in models of anxiety and depression has led to the development of these compounds for the treatment of emotional disorders. They have been highly effective in the elevated plus maze, forced swim test, tail suspension test, and social interaction tests (Broom et al., 2002a; Saitoh et al., 2005; Perrine et al., 2006). One caveat to consider when testing delta opioid receptor agonists is that some of them can cause locomotor stimulation, which can interfere with assays such as the elevated plus maze. For this reason, agonists such as SNC80, are often given subcutaneously in these paradigms and testing occurs at 1 h post-drug, after the loco-motor stimulant effect has diminished. There is very little expression of delta opioid receptors in brain stem regions that regulate nociception, and a high expression in regions that regulate emotional responses to pain (Pradhan and Clarke, 2005b; Pradhan et al., 2011b). Thus, the true potential for the therapeutic use of delta agonists may lie in the treatment of chronic pain conditions that have a significant co-morbidity with emotional dysregulation, such as fibromyalgia and migraine.

A further adverse effect of delta agonists is that some of them can also produce convulsions, and this has limited their development for clinical use. The pro-convulsant effect of delta agonists is ligand specific, and is a clear example of ligand-directed signaling or functional

selectivity at the delta opioid receptor (Pradhan et al., 2012; Chu Sin Chung et al., 2014). SNC80 and (+)BW373U86 produce convulsions, however, most other systemically available agonists do not (Pradhan et al., 2011a). This deleterious effect is dependent on the activation of the delta opioid receptor (Comer et al., 1993; Broom et al., 2002b), and has been observed in mice, rats, and squirrel and rhesus monkeys (Dykstra et al., 1993; Broom et al., 2002a; Danielsson et al., 2006; Jutkiewicz et al., 2006). The convulsions produced by delta agonists like SNC80 or (+)BW373U86 are behaviorally similar, but weaker than those produced by 4 the epileptogenic drug pentylenetetrazol (Comer et al., 1993). Delta agonist-induced convulsions may be related to absence seizures in humans (Jutkiewicz et al., 2006). Convulsions produced by delta agonists generally occur within 2–5 min of subcutaneous injection, and are characterized by clonic movements of the head, face and fore-paws and potentially extension of the body (Comer et al., 1993). The convulsant period is of very short duration, and can be completed in less than 5–10 min post-injection. Therefore, it is imperative to pay close attention to animals following administration of delta agonist as these convulsions are easy to miss. Convulsions are followed by catalepsy, which is a brief period lasting 10–30 min post-injection of delta agonist, where the animal shows muscular rigidity and lack of reaction to external stimuli. Catalepsy can be determined using the bar test, which consists on placing the forepaws of the animals on a bar suspended approximately 1 inch above the ground. Animals are considered cataleptic when they fail to remove their forepaws within 15 s (Comer et al., 1993). Following delta agonist-induced convulsion and catalepsy, rodents circumambulate normally and are indistinguishable from naive controls (Comer et al., 1993; Jutkiewicz et al., 2006).

Genetic tools to study the delta opioid receptor

Over the past 30 years, genetic mouse models have provided a novel platform in which to explore the role of delta opioid receptor *in vivo*. Here we discuss genetic mouse models that serve as suitable tools to further investigate the delta opioid receptor (Table 2).

Delta opioid receptor-KO_{exon 2}

There are four different delta opioid receptor knockout animals (Zhu et al., 1999; Filliol et al., 2000). Although they show similar phenotypes, there are some important differences between them. In general, delta opioid receptor knockout mice show no apparent developmental deficits, are similar in size to their wild-type littermates, and both sexes are reproductively fertile. John Pintar's group developed a knockout of the delta opioid receptor by deleting exon 2 of the *Oprd1* gene in 1999 (Zhu et al., 1999) (Table 2). These mice showed no binding to both [³H]DPDPE or [³H]Deltorphin II, and neither agonist produced pain-relieving effects when injected intrathecally. However, intracerebroventricular injection of DPDPE and deltorphin II to these knockouts continued to produce an antinociceptive effect, which may be due to activation of non-delta opioid receptors (Zhu et al., 1999). In 2009, Jennifer Whistler's group also produced an exon 2 knockout of the delta opioid receptor using a bacterial artificial chromosome (BAC) transgenic approach (van Rijn and Whistler, 2009a). In the process, this group also obtained “floxed” DOR mice, in which exon 2 is flanked by 2 loxP sites; which can be used to generate conditional knockouts ((van Rijn and Whistler, 2009a), and see below).

Delta opioid receptor-KO_{exon 1}

Another delta opioid receptor knockout mouse was generated by Brigitte Kieffer's lab, and in this case exon 1 of the *Oprdl* gene was targeted (Filliol et al., 2000) (Table 2). Similar to the delta opioid receptor-KO_{exon 2} mice, delta opioid receptor-KO_{exon 1} showed no binding to [³H]Naltrindole, [³H]DPDPE, and [³H]Deltorphan in the brain or periphery. Interestingly, quantitative receptor autoradiography shows compensatory down-regulation of mu and changes in kappa opioid receptor expression in the homozygous mutant mice, but not heterozygous controls (Goody et al., 2002). These mice were used to demonstrate that delta opioid receptors regulate emotional tone, and they show higher responses in tests of anxiety and depression as well as increase locomotor activity and impaired learning (Filliol et al., 2000; Kieffer and Gaveriaux-Ruff, 2002; Le Merrer et al., 2011). In addition, these mice show enhanced sensitivity in models of chronic pain (Gaveriaux-Ruff et al., 2011). For a summary of the changes that occur in delta opioid receptor knockout animals we refer the reader to the following reviews (Kieffer and Gaveriaux-Ruff, 2002; Le Merrer et al., 2009).

DOR-eGFP knockin mice

DOR-eGFP mice are knockin mice in which the endogenous delta opioid receptor is replaced by delta opioid receptor fused at the C terminus with enhanced green fluorescent protein (eGFP) (Scherrer et al., 2006) (Table 2). Although delta opioid receptor transcription remains functional in DOR-eGFP mice, quantitative mRNA analyses show a ~50% increase of *Oprdl* transcription when compared to wild-type littermates (Scherrer et al., 2006). DOR-eGFP fluorescence can be detected as early as postnatal day 3, reaching maximal intensity throughout the entire brain, spinal cord, and dorsal root ganglia by postnatal day 15 (Scherrer et al., 2006). These mice have been used to determine receptor expression (Scherrer et al., 2009; Poole et al., 2011; Erbs et al., 2012; Bardoni et al., 2014), and *in vivo* receptor trafficking to both exogenous (Pradhan et al., 2009, 2010) and endogenous (Poole et al., 2011; Faget et al., 2012; Bertran-Gonzalez et al., 2013) stimuli. These mice have also been crossed with the fluorescent mu-Cherry knockin mice to examine co-expression of these two receptors (Erbs et al., 2015). Several considerations are associated with these mice, as discussed in the detection section.

CONDITIONAL KNOCKOUT MOUSE MODELS

DOR^{CMV} cKO

Conditional knockouts (cKOs) of the delta opioid receptor have been generated using the Cre-loxP system. Floxed delta opioid receptor (*Oprdl*^{fl/fl}) mice were generated by introducing two loxP sites into exon 2 (Gaveriaux-Ruff et al., 2011). In order to assess the functionality of the loxP sites in *Oprdl*^{fl/fl} mice they were crossed with a constitutively expressing Cre line ((cytomegalovirus) CMV-Cre) (Gaveriaux-Ruff et al., 2011). These DOR^{CMV} cKO mice are thus a fourth delta opioid receptor knockout mouse, in which there is a complete deletion of delta opioid receptor from brain and periphery, as well as an enhanced anxiety phenotype (Gaveriaux-Ruff et al., 2011; Chu Sin Chung et al., 2015).

DOR^{Nav1.8} cKO

Oprd1^{fl/fl} mice were crossed with a Nav1.8-Cre line to generate a peripherally restricted knockout of delta opioid receptors (Gaveriaux-Ruff et al., 2011). DOR^{Nav1.8} cKO show a ~60–70% decrease in delta opioid receptors in the small and medium dorsal root ganglia, but otherwise brain and spinal delta opioid receptor expression remains intact (Table 2). These mice show no change in responses to acute noxious heat and mechanical stimuli. However in a model of chronic inflammatory pain; mechanical, but not heat, hyperalgesia is altered in DOR^{Nav1.8} cKO interestingly, the anti-hyperalgesic effects of SNC80 are lost in inflammatory and neuropathic pain conditions, but this agonist continues to inhibit formalin-induced pain (Gaveriaux-Ruff et al., 2011). One caution with these cKO mice, is that the Nav1.8 Cre line may not be restricted to nociceptors, and may also delete delta opioid receptors in ganglia involved with mechanosensation (Shields et al., 2012).

DOR^{Dlx5/6} cKO

Floxed delta opioid receptor mice have also been bred with the Dlx5/6-Cre line (Chu Sin Chung et al., 2014, 2015), a line in which Cre is expressed in forebrain GABAergic neurons (Ruest et al., 2003; Monory et al., 2006). DOR^{Dlx5/6} cKO show a near total loss of delta opioid receptors in the olfactory bulb, nucleus accumbens and caudate putamen, as well as a ~50% loss in the hippocampus. Delta opioid receptor levels remain unchanged in the midbrain, brain stem, and spinal cord (Chu Sin Chung et al., 2014, 2015) (Table 2). Interestingly, DOR^{Dlx5/6} cKO display reduced levels of anxiety and depression in contrast to the constitutive delta opioid receptor knockout (Chu Sin Chung et al., 2015). These mice have also been used to show that SNC80 produces convulsions through disinhibition of forebrain GABAergic neurons (Chu Sin Chung et al., 2014). One caveat when generating these mice is to breed male Dlx5/6-Cre mice with female floxed mice, as breeding with the female Cre will result in constitutive knockout.

Future work with currently available mutant mice, and novel genetic tools will continue to shed light on the role of delta opioid receptors in diverse biological conditions.

CONCLUSIONS

The delta opioid receptor, since it's cloning in the early 1990s, has been pursued as a therapeutic target for the treatment of chronic pain. Unfortunately, clinical trials of delta agonists for this indication have not yielded positive outcomes (<https://clinicaltrials.gov/ct2/show/results/NCT00979953>). Although a number of factors can account for this lack of success, these disappointing results have dampened enthusiasm for this receptor in the treatment of pain disorders. The phenotype of delta opioid receptor knockouts (Filliol et al., 2000), and subsequent discovery that delta agonists have anxiolytic and anti-depressant effects (Broom et al., 2002a; Saitoh et al., 2005; Perrine et al., 2006) moved the development of delta agonists toward the treatment of emotional disorders. Recent phase I and IIa clinical trial tested the delta agonists AZD2327 (Richards et al., 2016) and AZD7268 (<https://clinicaltrials.gov/ct2/results?term=AZD7268>) in anxious major depressive disorder. Although neither study reached primary endpoints for depression or anxiety, in both cases, analysis of secondary endpoints indicated potential benefit for anxiety. In addition, recent

preclinical work indicates that delta agonists may be effective for the treatment of migraine (Pradhan et al., 2014), which opens yet another avenue to the therapeutic benefit of this receptor. Considering that the delta opioid receptor is less studied than the mu opioid receptor, there are an incredible number of well-validated tools available to probe delta opioid receptor function. The ongoing development of additional tools, and encouragement of scientists from other fields to examine the delta opioid receptor in their models, will enhance the clinical potential of this receptor.

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Highlights

- Tools available to study the delta opioid receptor are reviewed.
- Cell-based systems to study delta opioid receptor are discussed.
- Behavioral effects and limitations of delta agonists are reviewed.
- Mutant mouse models to study *in vivo* delta opioid receptor action are described.

Table 1

Peptide and non-peptide delta opioid receptor ligands

Compound	Structure	Route	Dilution	References
SNC80		ip, sc, it, icv	saline with 2.5 µL/mg of 1 N HCl plus probe sonication	Bilsky et al. (1995), Fraser et al. (2000b), Perrine et al. (2006), Pradhan et al. (2009), Chiang et al. (2015)
AR-M100390		po	dH ₂ O	Pradhan et al. (2009, 2010), Chiang et al. (2015)
(-)-TAN-67		icv, sc, it, ip	saline	Suzuki et al. (1995), Tseng et al. (1997), Matsuzawa et al. (1998), Chiang et al. (2015)
DADLE DPDPE	Tyr-D-Ala-Gly-Phe-D-Leu Tyr-c(D-Pen-Gly-Phe-D-Pen)	icv icv, it	saline saline	Sanchez-Blazquez and Garzon (1994), Tseng et al. (1997) Sanchez-Blazquez and Garzon (1994), Tseng et al. (1997), Scherrer et al. (2004), Mika et al. (2014)
Deltorphan I [D-Ala ²]-Deltorphan II	Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH ₂ Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH ₂	icv, it icv, it	saline saline	Bilsky et al. (2000), Schuster et al. (2015)
Naltrindole		sc, ip	dH ₂ O or saline	Saitoh et al. (2005), Codd et al. (2009)
Naltriben (NTB)		sc	dH ₂ O or saline or 5% DMSO	Saitoh et al. (2005), van Rijn and Whistler (2009b), Sugiyama et al. (2014)
BNTX		sc	dH ₂ O or saline or 5% DMSO	Saitoh et al. (2005), van Rijn and Whistler (2009b), Sugiyama et al. (2014)
TIPP[ψ]	H-Tyr-Ticψ-[CH ₂ NH]-Phe-Phe-OH	icv	saline	Fundyus et al. (1995)

Table 2

Mutant mouse models to study the delta opioid receptor

Mouse model	Genetic mutation	References
Wild-type		Evans et al. (1992), Kieffer et al. (1992)
DOR-KO _{exon 2}		Zhu et al. (1999), van Rijn and Whistler (2009a,b)
DOR-KO _{exon 1}		Filliol et al. (2000)
DOR ^{CMV} KO		Gaveriaux-Ruff et al. (2011), Chu Sin Chung et al. (2014, 2015)
DOR-eGFP		Scherrer et al. (2006)
DOR ^{Nav1.8} cKO		(Gaveriaux-Ruff et al. (2011)
DOR ^{Dlx5/6} cKO		Chu Sin Chung et al. (2014, 2015)