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In Vivo Techniques to Investigate the Internalization Profile of Opioid Receptors

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Abstract

G-protein-coupled receptors (GPCRs) regulate a remarkable diversity of biological functions, and are thus often targeted for drug therapies. Receptor internalization is commonly observed following agonist binding and activation. Receptor trafficking events have been well characterized in cell systems, but the in vivo significance of GPCR internalization is still poorly understood. To address this issue, we have developed an innovative knock-in mouse model, where an opioid receptor is directly visible in vivo. These knockin mice express functional fluorescent delta opioid receptors (DOR-eGFP) in place of the endogenous receptor, and these receptors are expressed at physiological levels within their native environment. DOR-eGFP mice have proven to be an extraordinary tool in studying receptor neuroanatomy, real-time receptor trafficking in live neurons, and in vivo receptor internalization. We have used this animal model to determine the relationship between receptor trafficking in neurons and receptor function at a behavioral level. Here, we describe in detail the construction and characterization of this knockin mouse. We also outline how to use these mice to examine the behavioral consequences of agonist-specific trafficking at the delta opioid receptor. These techniques are potentially applicable to any GPCR, and highlight the powerful nature of this imaging tool.

Keywords

Behavior; Delta opioid receptor; G-protein-coupled receptor; Immunohistochemistry; Ligand-directed signaling; Mouse; Pain; Receptor trafficking

1 Introduction

G-protein-coupled receptors (GPCRs) form the most abundant receptor class in the human genome [1]. A variety of biological functions are regulated by GPCRs, and this receptor class is most commonly targeted for pharmacological therapies. Activation of a GPCR by endogenous or synthetic agonists elicits receptor signaling via heterotrimeric G proteins. This process is highly regulated and receptor activation is often accompanied by receptor internalization, a key process in the modulation of receptor signaling. Internalization is a complex regulatory process which terminates receptor cell surface signaling, but can also initiate different signaling cascades intracellularly (for review see [2]). In addition, receptor internalization and subsequent receptor trafficking also serves to recycle refreshed receptors back to the cell surface, or designate activated receptors for degradation [3]. The specific trafficking events that occur following receptor internalization are dependent on a number of factors, including the specific receptor, the agonist, duration of exposure, and cell type. Ligand-induced receptor internalization serves as an important modulator of receptor signaling.

Importantly, not all agonists elicit the same signaling and trafficking events. GPCRs can exist in multiple conformations, and different agonists can stabilize different activation states [4]. Each receptor conformation in turn produces distinct receptor–effector complexes, which can initiate differing signaling and receptor trafficking events [5, 6]. Therefore, not all agonists produce receptor internalization, and ligand-specific differences in receptor trafficking have acute and long-term consequences. This concept, referred to as ligand-directed signaling/trafficking, functional selectivity, or biased agonism [7] has important biological and pharmacological implications.

1.1 Cell Trafficking of Opioid Receptors

The opioid receptor family regulates a number of important physiological processes, including pain, reward, mood, and stress [8, 9]. The most clinically effective analgesics are mu opioid receptor (MOR) agonists (e.g. morphine and fentanyl). These are limited in their clinical applications as they can produce a number of adverse effects, including addiction. Delta opioid receptors (DOR) offer a promising alternative to MOR ligands. Stimulation of delta opioid receptors does not result in any of the severe adverse effects associated with MOR agonists, including euphoria (for review see [10]). DOR agonists are also effective in chronic inflammatory and neuropathic pain models [1–16]. In addition, DORs also modulate emotional state. Genetic deletion of DOR or its endogenous ligand, enkephalin, results in anxiogenic and depressive-like behaviors [17, 18]. Further, DOR agonists also produce anxiolytic and antidepressant effects [19–21]. Considering these preclinical findings, DOR agonists are currently being developed for the treatments of pain, and emotional disorders; and a greater understanding of the consequences of DOR activation and internalization is imperative.

The majority of studies characterizing receptor internalization have been performed in transfected cellular systems. These *in vitro* systems are highly useful in identifying specific receptor trafficking and signaling events that occur within the cell. However, these *in vitro*

models do not always reflect in vivo systems in terms of receptor density, protein content of receptor-expressing cells, or even receptor localization within subcellular compartments, as must be considered for neurons [22]. In addition, cellular models cannot provide insight into how receptor trafficking influences integrated responses in the living organism. Compared to our cellular understanding of receptor trafficking, the in vivo significance of this highly regulated process is still in its infancy.

In vivo trafficking of mu opioid receptors has been previously investigated using immunohistochemical techniques [23–25]. However, a lack of specific antibodies has limited this strategy for characterization of the delta opioid receptor [26]. To address this issue, we have created knockin mice expressing a fully functional fluorescent delta opioid receptor (DOR-eGFP) in place of the endogenous DOR [27]. In these animals, DOR-eGFP receptors are expressed at physiological levels within their native environment. Furthermore, these receptors are directly visible in vivo. These mutant mice have proven to be an exceptional tool for studying receptor neuroanatomy, real-time receptor trafficking in live neurons, and receptor movements in vivo [26–31].

1.2 Future

Applications of In Vivo Analysis of Opioid Receptor Trafficking

The DOR-eGFP mice have proven to be a unique tool to directly monitor in vivo delta opioid receptor trafficking events. These mice are particularly valuable considering the paucity of specific antibodies for immunohistochemical detection of DOR, and we have shown that they provide a dynamic, noninvasive way to monitor receptor internalization. There are a number of exciting uses for these knockin mice that are only just being explored. In this chapter, we focused on receptor internalization following exogenous agonist administration; however, these mice can also be used to track release of endogenous opioids. Faget et al. [32] recently found that in a behavioral model of context-dependent withdrawal, DOR-eGFP internalization was transiently detected in a subset of hippocampal neurons. This receptor trafficking revealed regionally restricted endogenous opioid peptide release. Importantly, internalization to endogenous opioid release consistently showed a pool of receptors remaining on the cell surface, which was in marked contrast to internalization following stimulation with SNC80 [32]. These studies show that, in vivo, physiological and pharmacological stimulation produces distinct delta receptor regulation.

DOR-eGFP mice can also be used to examine receptor trans-location to the cell surface. Several lines of evidence indicate that the cell surface population of DORs may be pliable within specific cell types, and is increased following certain stimuli. Peripheral injury [11, 33, 34], morphine treatment [35–37], ablation of MOR [38], and chronic ethanol exposure [39] are a few of the events that have been proposed to increase functional DORs on the cell surface. Recently, DOR-eGFP mice were used to show learning-related plasticity of delta opioid receptors. Increased predictive learning was directly associated with increased translocation of DOR-eGFP to the cell surface in cholinergic interneurons of the nucleus accumbens shell [28]. Receptor trafficking is an active process of translocation of receptor to the cell membrane, and subsequent activation and internalization of the receptor away from the surface; and future studies will focus on clarifying this in vivo dynamic.

G-protein-coupled receptor trafficking is regulated by a number of modulatory proteins, and interactions of this nature have primarily been studied in vitro. Along with several other modulatory proteins, DORs have been shown to interact with β -arrestins [40–42], regulator of G protein signaling 4 [43–45], and G-protein-coupled receptor-associated sorting proteins [46, 47]. Mutant mouse lines exist for these proteins, and there is the opportunity to cross DOR-eGFP knockin mice with these lines. Such crosses would provide insight on the role of these regulatory proteins on in vivo DOR trafficking.

More broadly, the DOR-eGFP knockin strategy highlights the potential of labeled receptors in vivo. Brain receptor imaging is a major focus of research in neuroscience. Standard imaging techniques such as positron emission tomography and magnetic resonance imaging provide a wealth of information on anatomy and receptor occupancy, but they have limited resolution in the small animal. At the other end of the spectrum, receptor internalization and trafficking have important functional implications, but work in this field has primarily been restricted to cell systems. Mouse models where proteins of interest are replaced by labeled versions provide a powerful tool to observe specific molecular events within the complex circuitry of in vivo physiological processes.

In this chapter we outline how to construct and characterize a DOR-eGFP knockin mouse, and how to use this knockin mouse model to specifically examine in vivo receptor internalization in a pain-based behavioral assay.

2 Materials

2.1 In Vivo Trafficking of DOR-eGFP

1. Manual von Frey hairs (weight in grams: 0.008, 0.04, 0.07, 0.16, 0.4, 0.6, 1, 2).
2. Wire testing rack (IITC or made in house).
3. Complete Freund's Adjuvant (CFA, Sigma).
4. 25 μ l Hamilton syringe with 27 gauge beveled needle.
5. Rodent anesthesia machine with isoflurane and O₂.
6. SNC80, ARM390 (Tocris), distilled H₂O, saline.
7. Syringes (1 cc), needles (27 gauge), and feeding needles for oral gavage.

2.2 Transcardial Perfusion and Tissue Preparation

0.2 M phosphate buffer stock: Na₂PO₄ 122 g, NaH₂PO₄ 21 g, ddH₂O to 4 l.

0.1 M phosphate buffered saline: 0.2 M PB 500 ml, ddH₂O 500 ml, NaCl 9 g.

30 % (w/v) sucrose: Sucrose 150 g, 0.1 M PB to 500 ml.

Glycerol cryoprotectant: Ethylene glycol 150 ml, glycerol 150 ml, 0.2 M PB 50 ml, ddH₂O 150 ml.

4 % Formaldehyde: 0.1 M PBS 27 ml, formaldehyde 3 ml.

2.3 Immunohistochemistry

1. 0.1 M PBS-0.3 % Triton X-100 (PBST): 0.1 M PBS 500 ml, Triton X-100 1.5 ml.
2. 5 % NDST block: PBST 19 ml, normal donkey (or goat) serum 1 ml.
3. 1 % NDST block: PBST 99 ml, normal donkey (or goat) serum 1 ml.

2.3.1 Antibodies

1. Rabbit anti-green fluorescent protein, Life technologies, 1:1,000.
2. Donkey anti-rabbit Alexa Fluor 488-conjugated, Life technologies, 1:1,000.

3 Methods

3.1 Construction and Characterization of the DOR-eGFP Mouse

3.1.1 DOR-eGFP Receptor—The key to generating a knockin mouse expressing a fluorescently labeled GPCR is to ensure that the fusion protein maintains its endogenous properties and function. When designing the knockin strategy, there are three main parameters that can be modified: (1) the nature of the tag (fluorescent protein, FLAG-tag, HA-tag, etc.); (2) the position of the tag in the native protein (C-terminus, loop, N-terminus); and (3) the composition of the linker, amino acid identity and length, between the GPCR and the tag. We elected to use the enhanced green fluorescent protein (eGFP) as a tag because of its brightness, photostability and functionality as a monomer, compared to other fluorescent proteins available at the time (CFP, YFP, RFP). Numerous other fluorescent proteins are now available and it is essential to select the fluorescent tag based on future applications [48]. Previous work indicated that C-terminal fusions allow correct receptor folding, insertion, and export to the cell surface with minimal alterations to ligand binding or receptor signaling [49]. We therefore tested multiple constructs in vitro and determined that linking the eGFP protein to the DOR C-terminus via a six amino-acid linker (GSIAT) resulted in retained receptor function. This was confirmed in radioligand binding experiments on HEK293 cell lines that stably expressed either the native DOR or the DOR-eGFP fusion. Our results demonstrated that a variety of opioid ligands (endogenous opioid peptides, agonists, antagonists) all displayed similar affinities for the two receptors (native and fluorescent) [27], suggesting that the addition of the eGFP tag did not alter receptor conformation.

Next, we determined whether DOR signaling through Gi/o protein coupling was intact, given the possibility of steric hindrance from the fluorescent tag. We treated membrane preparations expressing either native or DOR-eGFP receptors with the agonist deltorphin II and found that the potency and maximal effect of this agonist was identical for the two receptors [50]. Following receptor activation, binding of multiple molecules to the DOR intracellular domains has been shown to lead to internalization and subsequent lysosomal or proteasomal degradation [41, 51–54]. To confirm that the DOR-eGFP fusion protein maintained native internalization and desensitization we used the stable DOR-eGFP HEK293 cell line and found that under basal conditions the fluorescent signal was primarily at the cell surface, however, following the application of DOR agonists (met-enkephalin, deltorphin II or SNC80), internalization of the fusion protein occurred as expected. In

agreement with previous reports [55, 56], and with native receptor action, DOR-eGFP was downregulated following prolonged exposure to deltorphin II. In summary, we demonstrated that addition of the eGFP tag did not alter DOR conformation, G protein binding, interaction with adaptor proteins or internalization, and we therefore selected this construct to create the knockin mouse line.

3.1.2 Targeting Strategy—Our goal when designing the knockin targeting strategy was to minimize modifications to the genome that might disrupt gene transcription or RNA stability. The targeting vector was constructed by inserting the sequence encoding the GSIAT-eGFP tag to replace the STOP codon in exon 3 of the DOR gene (*Oprd1*; Fig. 1). In this construct, all the *Oprd1* gene sequences regulating gene transcription, mRNA stability and protein translation were unchanged and therefore intact in the final recombined allele. To select for recombined alleles in ES cells, we inserted a floxed hygromycin resistance gene as close as possible (less than 300 base pairs) to the GSIAT-eGFP sequence to ensure selection of clones that had integrated both the resistance gene and the tagged sequence. Once the eGFP-expressing clones were selected, we removed the hygromycin cassette by transfecting ES cells with a Cre recombinase-expressing vector and further selecting an appropriate ES clone in the absence of hygromycin. The final mutant DOR-eGFP allele contained the desired GSIAT-eGFP vector and one remaining loxP sequence of 34 base pairs.

We confirmed the expression of the *Oprd1* gene in the DOR- eGFP mice and found a slight alteration in processing that led to an approximate twofold increase in DOR-eGFP mRNA levels in the knockin mouse compared to wild-type controls. It is possible that the exogenous GSIAT-eGFP sequence increases mRNA stability as we also found a parallel increase in receptor density (320 vs. 160 fmol/mg brain membrane protein), and maximal level of G protein activation (E_{max}) to deltorphin II and SNC80, but not met-enkephalin. In transfected systems, overexpression levels are in the picomolar range suggesting that our observed increase is subtle. In addition, this increase in receptor number did not impact receptor distribution throughout the nervous system (see below), suggesting the existence of a receptor reserve in wild-type animals. Overall, our data suggest that the DOR-eGFP mice recapitulate the DOR expression found in physiological conditions and therefore can serve as a tool for probing the distribution of the DOR in the central nervous system (CNS).

3.1.3 Receptor Expression and Function in DOR-eGFP Knockin Mouse Line—

Once the DOR-eGFP knockin mouse line was established, we first tested whether the DOR-eGFP fusion protein was functional in mutant animals. We utilized membrane protein preparations from DOR-eGFP mice and control littermates to perform a series of biochemical and pharmacological assays. Western blot analysis demonstrated an 80 kDa eGFP immunoreactive band, corresponding to the predicted size of the fusion protein, and found only in homozygous and heterozygous mutant mice, but not in wild-type controls. Furthermore, ligand-binding experiments confirmed that ligand affinity for the DOR-eGFP receptor was identical to that of the wild-type receptor, regardless of the nature (partial or full agonists, antagonists) or structure (peptides or alkaloids) of the ligand tested. Finally, using a G protein activation assay, we found that potencies for prototypical DOR agonists,

SNC80, deltorphin II and met-enkephalin were similar in knockin and wild-type mice. Together, these results indicate that DOR-eGFP is expressed and functional in tissues from DOR-eGFP knockin mice.

We next needed to confirm that the fusion protein was expressed in the correct anatomic distribution and remained functional. To do this we used fluorescence microscopy on CNS tissue and directly compared the pattern of eGFP fluorescence to the known distribution of wild-type DOR as determined by autoradio-graphic binding [57–59]. Fluorescence microscopy demonstrated that the DOR-eGFP was expressed at particularly high levels in the striatum, cortex, basolateral nucleus of the amygdala, similar to previously published results [57]. The agreement in expression pattern between the eGFP-labeled and native receptor indicates that the eGFP fusion protein is found in the same neuronal population as the native receptor and that it is correctly transported to subcellular compartments such as dendrites and axon terminals. Finally, in agreement with previous data using transfected cells [41, 52, 54], DOR-eGFP was internalized upon exposure to an agonist either in striatal or hippocampal primary cultures or in vivo (see below, and [27, 30]). Taken together, these data strongly support the notion that DOR-eGFP is expressed and traffics as the native DOR does in wild-type mice.

Next, we tested whether DOR-eGFP mice exhibited the known behavioral responses to DOR agonists using the compound SNC80 for its well-documented stimulant effect. We found a dose- dependent increase in locomotor activity that was comparable in DOR-eGFP mice and their wild-type littermates, but absent in DOR knockout mice [27]. Furthermore, in a model of inflammatory pain, we determined that the analgesic effects of SNC80 (the prototypical nonpeptide DOR agonist) and AR-M100390 (ARM390) were identical in DOR-eGFP mutant mice and wild- type controls (see below and [60]).

Taken together, our biochemical, pharmacological, histological, and behavioral studies have demonstrated that the DOR-eGFP fusion protein is a fully functional receptor with unchanged distribution and biology. Importantly, in spite of a slightly higher receptor number in DOR-eGFP mice, DOR-mediated responses in vivo are unchanged in the knockin mouse line. These data support the use of the DOR-eGFP mouse as a unique tool to gain insights into several aspects of DOR function at a resolution that was previously not possible.

3.1.4 Anatomical Characterization of DOR Receptor Distribution—As described above, the DOR-eGFP mouse allows direct visualization of receptor distribution in all CNS tissue and allows for immunohistochemical colocalization experiments that can discern subpopulations of neurons expressing DOR (Fig. 2). For example, the distribution of *Oprd1* (DOR gene) and choline acetyl- transferase mRNAs was investigated in adjacent sections by in situ hybridization and a large overlap was noted between the two populations [50, 51]. In agreement with this work, immunostaining of striatal sections from DOR-eGFP knockin mice showed that 76 % of cholinergic neurons expressed the DOR [27]. Recently, we used DOR-eGFP mice to resolve the molecular identity of DOR- expressing hippocampal neurons. These studies indicated that DOR is predominantly expressed in GABAergic inhibitory interneurons in the oriens and principal layers [52]; further, colocalization

experiments in many brain areas are currently underway to provide a more complete description of DOR anatomy within neural circuits.

The DOR-eGFP mouse was also used to study receptor distribution in the spinal cord and peripheral nervous system. Molecularly diverse primary afferent neurons of the dorsal root ganglia (DRG) detect a wide range of stimulus modalities and intensities to encode a variety of somatosensory effects such as heat pain or light touch [61]. In situ hybridization studies have demonstrated DOR mRNA in large diameter DRG neurons with myelinated axons, and in a restricted population of small-diameter DRG neurons [59, 62]. The molecular identity of these DOR-expressing cells is key to understanding the function of the receptor and we took advantage of the DOR-eGFP mice to perform colocalization studies with markers for the various neuronal populations. Consistent with previous in situ hybridization studies, we found that about 60 % of DOR-eGFP-expressing neurons were large-diameter DRG neurons that immunostain for neurofilament 200, a marker of myelinated neurons (A fibers) [26]. Some of these cells transmit mechanical pain and touch information, and play a role in the development of chronic mechanical hypersensitivity after tissue or nerve injury (inflammatory or neuropathic pain, respectively). In addition, DOR-expressing small-diameter DRG neurons were identified as the nonpeptidergic population that detects noxious stimuli as indicated by colocalization with the lectin IB4. In contrast, DOR-eGFP was found to only rarely colocalize with markers of the peptidergic small-diameter population of neurons: only about 2 % of DOR-eGFP DRG neurons expressed substance P and only 5 % of DOR-eGFP DRG neurons expressed the heat sensor transient receptor potential vanilloid 1 (TRPV1) [26].

In the spinal cord, in situ hybridization and autoradiographic studies have demonstrated *Oprd1* mRNA and DOR binding in neurons throughout the gray matter (laminae I-X) of the ventral and dorsal horns [59, 63]. Our studies with the DOR-eGFP mice confirm this expression pattern with preliminary work suggesting co-labeling with PKC γ , a marker of interneurons critical for neuropathic pain [64] in the ventral part of lamina II. Identification of the DOR-eGFP expressing neurons throughout the rest of the spinal cord is currently underway.

Taken together, these data show that the knockin of DOR- eGFP does not adversely affect receptor binding or expression. In addition, these mice are viable and behave similar to wild-type controls. We next used these mice to correlate receptor trafficking with in vivo behavioral effects.

3.2 In Vivo Trafficking of Delta Opioid Receptors

As described above, the DOR-eGFP mouse allows direct visualization of the receptor, which we then used to characterize in vivo receptor trafficking events. Evidence from a number of in vitro cellular studies has revealed functional selectivity at the delta opioid receptor (for review see [65]). We used DOR-eGFP knockin mice to explore the relationship between agonist-induced receptor internalization and receptor function further. We compared two structurally related DOR agonists, SNC80 [66], and ARM390 [67] in several in vivo and ex vivo assays. These two ligands show similar binding, and G protein coupling [26], and like other systemically administered DOR agonists [68, 69], both SNC80 and ARM390 produce

analgesia in a model of inflammatory pain, and have anxiolytic effects [30, 31]. However, in primary striatal and hippocampal cultures made from DOR-eGFP mice, SNC80 and ARM390 had profoundly different internalization profiles [30]. SNC80 produced internalization at concentrations as low as 10 nM [27, 30]. In contrast, internalization with ARM390 was only observed in cultures at a 100× higher concentration (1,000 nM). These results show biased agonism at the delta opioid receptor, where the binding of two different agonists induces highly divergent receptor trafficking events.

We next examined the effect of ligand-directed trafficking in vivo, and we compared these two DOR agonists in the Complete Freund's Adjuvant (CFA) model of inflammatory pain. Following repeat injection, SNC80, but not ARM390, produced acute behavioral desensitization. Upon examination of receptor trafficking, we showed that inhibition of agonist-induced behavior and decreased DOR-G protein coupling were directly correlated with receptor internalization [30]. In addition, we also examined the long-term effects of treatment with these two different DOR agonists. We found that distinct forms of tolerance were observed depending on the internalization property of the drug [31]. Chronic treatment with the high-internalizing agonist SNC80 produced widespread receptor downregulation and a generalized behavioral tolerance. In contrast, the low-internalizing agonist ARM390 produced no detectable change in receptor localization, number, or binding. Only an analgesic tolerance was observed following chronic ARM390, which corresponded to an uncoupling of DOR from voltage- dependent Ca^{2+} channels in DRG neurons (Fig. 3). Taken together, these results show that ligand-specific internalization of DORs in vivo needs to be considered during the development of these agonists as novel drug therapies.

3.2.1 In Vivo Observation of DOR-eGFP Trafficking

1. Habituate DOR-eGFP mice to testing rack for 20 min for 2 days (*see Note 1*).
2. Determine baseline mechanical responses in the plantar surface of either the left or right hindpaw. Mechanical responses are determined by using von Frey hairs. The 50 % threshold for response to these punctate mechanical stimuli is determined according to the up-and-down method [70]. In this case, the plantar surface of the hindpaw is stimulated with a series of eight von Frey filaments. The first filament tested is 0.4 g. In the absence of a response a heavier filament (up) is tried, and in the presence of a response a lighter filament (down) is tested. This pattern is followed for a maximum of four filaments following the first response. A response is defined as a lifting, shaking, or licking of the paw upon stimulation.
3. Inflammatory pain is induced by injecting 13 μl of CFA into the plantar surface of the hindpaw. Inject the same hindpaw in which mechanical responses were determined. Lightly anesthetize the mice with isoflurane (5 % to induce anesthesia, and 0.8–1.5 % for maintenance). Gently insert the needle (bevel side up) of the filled Hamilton syringe subcutaneously into the center of the plantar surface of the paw. Pull the plunger up 1–2 μl to ensure that the needle is not in an artery. Slowly

¹For pain testing, we have observed less variability when we test under low light conditions, preferably at the beginning of the light cycle.

inject CFA, remove needle, and apply mild pressure to the wound opening for 3–5 s (*see Note 2*).

4. On the test day, inject mice with SNC80 (10 mg/kg IP), ARM390 (10 mg/kg PO), or vehicle (*see Note 3*). To ensure that all animals are treated equally, all mice receive an intraperitoneal (SNC80 or saline) and a per os (ARM390 or distilled H₂O) injection. Immediately place them on the testing rack.
5. Mechanical responses for each mouse are determined 45 min later. As above, use mechanical von Frey filaments on the ipsi-lateral paw.
6. Mice may be euthanized immediately after testing or up to 24 h later. Animals may also be tested further, see below.

3.3 Acute Behavioral Desensitization and Chronic Tolerance

3.3.1 Analysis of Receptor Internalization State at the Time of Behavioral Desensitization

1. 4–12 h after the first injection of vehicle, SNC80 or ARM390, mice are re-injected with the same drug, placed on the testing rack; and tested 45 min later.
2. Another cohort of animals are euthanized/perfused at the time that the tested group receives the second injection.

3.3.2 Analysis of Receptor Internalization Following Chronic Drug Treatment

1. Mice are treated as on the test day outlined above (numbers 4 and 5), every day for 5 days.
2. Mice are euthanized/perfused 24 h after the final injection (day 6) (*see Note 4*).

3.4 Transcardial Perfusion and Tissue Preparation

1. Using standard protocol for transcardial perfusion, infuse:
 - (a) 10 ml 0.1 M PBS.
 - (b) 30 ml 4 % formaldehyde.
2. Remove tissue of interest by dissection.
3. Postfix tissue in 4 % formaldehyde for 4 h at 4 °C.
4. Transfer to 30 % sucrose and store at 4 °C overnight.
5. Freeze tissue in OCT.

²Animals are subsequently tested 72 h post-CFA administration, to ensure increased functionality of delta opioid receptors in vivo [11, 71].

³In rodents, the original manufacturer of ARM390 (AstraZeneca) found that the bioavailability of this compound was greatest when given per os. Intraperitoneal administration of ARM390 does not appear to have pain-relieving effects when tested by us or by others [72].

⁴To ensure that all of the behavioral results are generalizable to wild-type mice, all behavioral experiments should also be performed in commercially available C57Bl/6 mice, which we have shown to have a similar dose response to SNC80 and ARM390 [30].

6. Cut sections on cryostat (40 μ m thickness for standard immunohistochemistry) into PBS.
7. Transfer free floating sections into glycerol cryoprotectant and store at -20°C until use.

3.5 Immunohistochemistry

1. Block tissue with 5 % NDST for 1 h at room temperature.
2. Prepare primary antibody solution by diluting in 1 % NDST buffer.
3. Remove blocking solution and apply primary antibody solution (no washes). Incubate overnight at room temperature.
4. Wash 3×10 min with 1 % NDST buffer.
5. Meanwhile, prepare secondary antibody solution by diluting in 1 % NDST buffer.
6. Apply secondary antibody solution and incubate for 2 h at room temperature (*see Note 5*).
7. Wash 3×10 min with 0.1 M PB.
8. Mount sections on slides using a paintbrush.
9. Coverslip with Fluoromount G and allow slides to dry prior to imaging.

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⁵To avoid photobleaching of the fluorophore, it is best to perform secondary antibody incubation and subsequent steps with as little exposure to light as possible.

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Fig. 1.

Tagging delta opioid receptors in vivo with eGFP by homologous recombination in mice. **(a)** Schematic structure of the DOR-eGFP receptor. The eGFP tag is fused to the C-terminus of the receptor by means of a five amino acid linker (GSIAT). **(b)** Strategy used to insert the eGFP cDNA in place of the stop codon within the *Oprd1* gene. *Oprd1* exons, eGFP cDNA, and the floxed hygromycin cassette are displayed as empty box, gray box and black triangles, respectively. Homologous recombination (HR) was followed by Cre recombinase treatment (Cre) in ES cells

Fig. 2. Identification of neurons expressing the delta opioid receptor using DOR-eGFP mice. (a) Cholinergic (ChAT) neurons (NeuN) in the striatum express the delta opioid receptor (DOR-eGFP). DOR-eGFP mice were treated with SNC80 to cause receptor internalization. (b) In the hippocampus of SNC80-treated mice DOR-eGFP is concentrated in inhibitory interneurons, marked with an antibody against glutamate acid decarboxylase (GAD). (c) In the spinal cord, DOR-eGFP fluorescence is present throughout the grey matter (GM) and enriched in lamina I and in the ventral portion of inner lamina II (IIi) of the dorsal horn

(DH). *WM* white matter. *VH* ventral horn. **(d)** In the dorsal root ganglia, DOR-eGFP is expressed in subsets of small (*arrowhead*) and large (*arrow*) diameter neurons. **(e)** Labeling of dorsal root ganglion (DRG) neurons with the marker isolectin B4 (IB4) reveals that small-diameter neurons expressing DOR-eGFP belong mainly to the nonpeptidergic subset of DRG neurons (IB4 positive). *Arrow*, large-diameter DRG neuron expressing DOR-eGFP

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Fig. 3.

Chronic ligand-directed trafficking at the delta opioid receptor results in two distinct forms of tolerance. SNC80 and ARM390 (ARM) have comparable selectivity and potencies for the delta opioid receptor, but result in highly distinct receptor trafficking events. Systemic SNC80, but not ARM390, produces clear receptor internalization in vivo as shown in slices from DOR-eGFP knockin mice (representative images from the hippocampus and dorsal root ganglia) [30]. Chronic administration of either agonist produces two distinct forms of tolerance. Repeated administration of SNC80 produces widespread receptor downregulation, thus resulting in a generalized tolerance where all DOR agonist-induced behaviors are inhibited. In contrast, chronic administration of the low-internalizing agonist, ARM390, affects DOR coupling to Ca^{2+} channels in dorsal root ganglia, thus producing tolerance only at the level of pain processing [31]