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Experimental considerations for the assessment of in vivo and in vitro opioid pharmacology

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Abstract

Morphine and other mu-opioid receptor (MOR) agonists remain the mainstay treatment of acute and prolonged pain states worldwide. The major limiting factor for continued use of these current opioids is the high incidence of side effects that result in loss of life and loss of quality of life. The development of novel opioids bereft, or much less potent, at inducing these side effects remains an intensive area of research, with multiple pharmacological strategies being explored. However, as with many G protein-coupled receptors (GPCRs), translation of promising candidates from in vitro characterisation to successful clinical candidates still represents a major challenge and attrition point. This review summarises the preclinical animal models used to evaluate the key opioid-induced behaviours of antinociception, respiratory depression, constipation and opioid-induced hyperalgesia and tolerance. We highlight the influence of distinct variables in the experimental protocols, as well as the potential implications for differences in receptor reserve in each system. Finally, we discuss how methods to assess opioid action in vivo and in vitro relate to each other in the context of bridging the translational gap in opioid drug discovery.

Keywords

Opioids; Antinociception; Respiratory depression; Constipation; Opioid-induced hyperalgesia; Tolerance

1 Introduction

Opioids are the gold standard for analgesic medications, both for acute severe pain and chronic pain management. Chronic pain in particular is a major health issue across the world today. Estimates of the prevalence of chronic pain range from 20 to 40% of the population

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in both developing (Sá et al., 2019) and developed (Dahlhamer et al., 2018) countries, representing a significant societal and economic cost.

Opioids still remain mainstay analgesics despite the suite of on-target side effects mediated by activation of the μ-opioid receptor (MOR), the primary target of most opioid pain medications (Matthes et al., 1996). Two major side effects include respiratory depression and addiction. These side effects are, in many ways, the “face” of opioids (Okie, 2010), with respiratory depression being the cause of fatality in an overdose situation and opioid addiction being present in most, if not all, countries worldwide. In 2017, fatal opioid overdoses totalled over 60,000 in the USA (Hedegaard & Warner, 2017) and the number and rate of increase in overdose deaths was the highest in the UK since records began (Office of National Statistics, UK, 2019). Additional side effects include constipation, due to opioid-induced reduction in gut motility, and hyperalgesia, a condition where opioid use paradoxically induces a heightened sensitivity to pain (Benyamin et al., 2008). These are still significant side effects that affect the quality of life and decrease patient compliance (Camilleri, 2011; Katz, 2002; Panchal, Mueller-Schwefe, & Wurzelmann, 2007).

Intense research is currently addressing the urgent need for improved and safer analgesics, either through the discovery and validation of new targets such as other cell surface receptors e.g. cannabinoid receptors (Vučković, Srebro, Vujovic, Vučetić, & Prostran, 2018), voltagegated sodium channels (Bennett, Clark, Huang, Waxman, & Dib-Hajj, 2019), targeted toxins (Yaksh, Woller, Ramachandran, & Sorkin, 2015) or through the development of new opioid receptor ligands with new mechanisms of action (Crombie et al., 2015; Dekan et al., 2019; DeWire et al., 2013; Gassaway, Rives, Kruegel, Javitch, & Sames, 2014; Koblish et al., 2017; Schmid et al., 2017).

Early data from arrestin-3 KO mice suggested that with the removal of arrestin-3 morphine induced enhanced antinociception, with a reduced degree of respiratory depression (Raehal, Walker, & Bohn, 2005). Since then, focus has been centred in the development of G protein-biased agonists to potentially retain the antinociceptive properties while avoiding the respiratory depressant effects (Dekan et al., 2019; DeWire et al., 2013; Gillis et al., 2020; Manglik et al., 2016; Schmid et al., 2017; Varadi et al., 2016). Some novel opioids generated based on this hypothesis have resulted in marginally improved separation between the antinociceptive and respiratory depressant dose response curves (DeWire et al., 2013; Gillis, Gondin, et al., 2020; Schmid et al., 2017). However, the mechanisms responsible for this wider therapeutic windows are still unclear (Azevedo Neto et al., 2020; Bachmutsky et al., 2021; Haouzi, McCann, & Tubbs, 2021; Kliewer et al., 2019; Kliewer et al., 2020).

Other strategies to develop improved opioid analgesics target other opioid receptor subtypes. κ-Opioid receptor (KOR) agonists have been shown to provide effective antinociception without inducing respiratory depression. However, the dysphoria associated with KOR agonism limits their clinical utility (Chavkin & Koob, 2016). Several reports suggest that dysphoria is induced predominantly by activation of an arrestin-mediated signalling pathway (Brust et al., 2017; Lovell et al., 2015) suggesting the development of G-protein biased KOR agonists as a potential strategy to develop new analgesics. Unfortunately, attempts to obviate KOR-induced dysphoria whilst producing an appreciable level of antinociception have not

translated into therapies as yet (Mores, Cummins, Cassell, & van Rijn, 2019). There has also been an increasing body of evidence suggesting that the δ-opioid receptor (DOR) may be an attractive target for specific modalities of pain such as chronic inflammatory or neuropathic pain (Pradhan, Befort, Nozaki, Gavériaux-Ruff, & Kieffer, 2011). However, interest in DOR therapeutics has been limited due to pro-convulsive activity exhibited by certain DOR agonists (Broom et al., 2002), as well as an apparent greater liability for tolerance (Pradhan et al., 2010). Attempts to alleviate these side effects are ongoing (Audet et al., 2012; Charfi, Audet, Bagheri Tudashki, & Pineyro, 2015; Conibear et al., 2020). Additionally, beyond the three classical MOR, KOR and DOR subtypes, the nociceptin opioid peptide receptor (NOP) is actively pursued as a potential target for novel analgesic therapeutics (Zaveri, 2016); the widespread expression of NOP and its upregulation in chronic pain states suggest a potential role for NOP antagonists in pain therapy (Calo & Lambert, 2018; Zaveri, 2016). Indeed, molecules that combine a MOR and NOP partial agonist mode of action have been shown to provide antinociception in the absence of side effects in nonhuman primates (Ding et al., 2018).

The paucity of novel opioid-based analgesics reaching the bedside reflects a general attrition in G protein-coupled receptors (GPCR) drug discovery programmes. Translation of candidate molecules from in vitro pharmacological characterisation into pre-clinical and clinical settings still represents a significant hurdle. Differences in experimental conditions, both in vitro and in animal models, are likely to underlie this translational challenge.

This review examines the preclinical animal models used to evaluate key opioid-induced behaviours. We consider the influence of distinct variables in the experimental protocols, as well as the potential implications for differences in receptor reserve in each system. Finally, we discuss how the methods to assess opioid action in vivo and in vitro relate to each other in the context of bridging the translational gap in opioid drug discovery (Fig. 1). In particular, we focus on the methods used to assess opioid-induced antinociception, respiratory depression, constipation, hyperalgesia and tolerance in the drug discovery setting. While the rewarding, addictive and consequent withdrawal effects associated with opioid use are major side effects, their complex and multi-faceted nature as well as the myriad of approaches used to investigate them are outside the scope of this review. The mechanisms underlying these behaviours, as well as the methods used to study them in preclinical models are elegantly reviewed elsewhere (Banks & Negus, 2017; Charbogne, Kieffer, & Befort, 2014; Kreek et al., 2012; Negus & Moerke, 2019; Rodríguez-Arias, Aguilar, Manzanedo, & Miñarro, 2010; Sadee, Oberdick, & Wang, 2020; Swain, Gewirtz, & Harris, 2021).

2 Assessment of opioid-induced antinociception

Antinociception refers to the inhibition of the detection of a painful stimulus by nociceptive (pain) neurons. In contrast, the term analgesia is used when referring to the alleviation of the experience of pain, which includes not only the inhibition of nociceptive signalling but also a subjective component that cannot be assessed in animal models. As such, the term antinociception is used to indicate the reductions in pain state observed in nonhuman animals, where an assessment of the subjective component is not possible. The most

widely used in vivo models of antinociception involve a pain stimulus (physical activation of nociceptive fibre) being delivered to the animal to elicit a pain behaviour and the subsequent measurement of the reduction of such pain behaviours by analgesic agents. Thermal nociception tests are commonly used in the discovery and characterisation of opioid compounds with the hot plate assay and tail flick (or tail withdrawal) tests being regularly used as the first in vivo measurement of antinociceptive efficacy (Table 1). Other mechanical (e.g Von Frey, Hargreaves) and chemical (e.g formalin, complete Freund's adjuvant) nociception assays are also used to evaluate antinociceptive efficacy of opioids in conditions such as inflammatory and neuropathic pain.

2.1 Tail flick assay

The tail flick assay measures the latency required for an animal to remove or flick its tail from a heat source. It is typically used in rodents that are usually restrained (by scruffing, cupping, wrapping, or placed in a tube) when a heat stimulus is applied to the distal portion of the tail (D'Amour & Smith, 1941; Hardy, 1953; Hardy, Stoll, Cunningham, Benson, & Greene, 1957; Hardy, Wolff, & Goodell, 1940). The noxious heat stimulus can be immersion of the tail in warm water or application of radiant heat to a smaller section of the tail with greater heat intensity (i.e. non radiant heat) resulting in shorter reaction times (Carroll, 1959; Carstens & Wilson, 1993; Granat & Saelens, 1973; Levine, Murphy, Seidenwurm, Cortez, & Fields, 1980). Baseline tail-flick latency is typically 2–4 s (Raffa et al., 1992). Importantly, this assay measures the lower cervical reflex arc responsible for the tail flick. It therefore monitors the response to a spinal reflex, rather than an indication of pain behaviours involving higher brain centres.

2.2 Hot plate assay

The hot plate assay measures behaviours that require central processing and are considered to integrate supraspinal pathways. It consists of the application of a thermal stimulus to the paws, tail and the dorsal flank of the animal and measures the latency for the animal to elicit a pain behaviour (Eddy & Leimbach, 1953; Ocallaghan & Holtzman, 1975; Woolfe & Macdonald, 1944). Typically unrestrained rodents are placed in a metal surface maintained at a constant temperature and manifestations of pain are seen as fluttering of the feet, rearing and licking forepaws/hindpaws, squirming to dissipate heat, and jumping (Carter, 1991).

While both the tail flick and the hot plate assay provide useful information on the antinociceptive actions of opioids, there are obvious ethical considerations integral to the experiment that prevent unnecessary animal suffering, and yet ensure that the data remain valid and useable. Limits in the maximum temperature of the noxious stimulus (the thermal limit - typically 48-55 °C) and maximum latency cut-offs (i.e maximum time until response is observed, typically 10–30 s) prevent damage to the tissues of the experimental animal (Table 1). It is also important to note that increased temperatures are associated with not just decreased latencies (Carroll, 1959; Carstens & Wilson, 1993; Ren & Han, 1979) but also associated reduced antinociceptive efficacy, namely, analgesics are less efficacious the higher the temperature (Ankier, 1974; Hunskaar, Berge, & Hole, 1986; Zimet, Wynn, Ford, & Rudo, 1986). In addition, ethical considerations also limit the frequency at which the test can be repeated in the same animal. This usually implies that data is acquired in 15–30 min

bins, which impacts the temporal resolution of the assay and can affect determinations of peak effects and of pharmacokinetic profiles.

The necessary cut-offs in antinociceptive assays have significant implications in estimations of in vivo agonist efficacy. Antinociception is calculated as a percentage of the maximum possible effect (%MPE) which is calculated as follows:

$$\%MPE = \frac{test\ latency - control\ latency}{Max\ .\ latency - control\ latency} \times 100$$

where “test latency” is the latency measured for the test drug, “control latency” is the baseline latency and “Max latency” is the upper temporal limit established to prevent tissue damage. It follows that when the latency measured for the test drug equals or exceeds the maximum latency cut-off, the test compound will display 100% MPE. Thus, detection of differences of opioid antinociceptive efficacy beyond a certain threshold will be prevented.

The above-mentioned cut-offs also have an impact on the determinations of antinociceptive potency, which in turn are important in the determination of therapeutic windows, where ED50s (the effective dose eliciting a 50% of the maximal response) between antinociception and other side effects are compared. Similarly, they should be taken into account when different efficacies are observed between measurements of antinociception and of other opioid-induced side effects. Thus, different temperatures and latency cut-offs being used can impact interpretations of effects across different studies (Table 1).

There is significant accumulated evidence that shows that inbred strains of mice can vary significantly in their nociceptive sensitivity (Table 2) (Homanics, Quinlan, & Firestone, 1999; Mogil et al., 1996). Differences across strains have been generally observed with regards to the baseline latencies and the antinociceptive responses of classical opioids such as morphine (Crain & Shen, 2000; Kest, Hopkins, Palmese, Adler, & Mogil, 2002; Mogil & Wilson, 1997). While such differences can be related to the different pharmacokinetics of opioid drugs in different strains, this remains to be systematically investigated. Thus, when comparing reports of antinociceptive efficacy of novel opioid drugs as well as when designing new compound evaluation strategies, the choice of mouse strain should also be taken into account.

3 Assessment of opioid-induced respiratory depression

Opioid-induced respiratory depression is a significant, potentially lethal, adverse effect of opioid agonists. MOR is expressed throughout the respiratory network of the brainstem, and recent studies have demonstrated a key role of MOR expressed in nuclei including; the preBötzinger complex (preBötC), Kölliker-Fuse (KF), post-inspiratory complex, ventral respiratory column and the retrotrapezoid/parafacial nucleus (Bachmutsky et al., 2021; Ramirez et al., 2021; Varga, Reid, Kieffer, & Levitt, 2020). The relative importance of each respiratory brain nuclei to respiratory rhythmogenesis as well as their role in the response to exogenous opioids is currently debated (Ramirez et al., 2021). While it is clear that the preBötC is an important site of action for opioid depression of respiration (Bachmutsky, Wei, Kish, & Yackle, 2020; Ramirez et al., 2021), reports suggest both, an essential and

a non-essential role of the preBötC for breathing rhythmogenesis (Montandon & Horner, 2014) (Lalley, Pilowsky, Forster, & Zuperku, 2014). Further investigation is required to elucidate the importance of each nuclei both in the persistent control of respiration as well as in the effect of opioids on respiration.

Due to its acute onset and relative ease of measurement, respiratory depression is one of the first adverse effects to be assessed in the characterisation of novel opioid agonists (Gillis et al., 2020a; Gillis, Gondin, et al., 2020; Hil let al., 2018; Manglik et al., 2016; Schmid et al., 2017). Several non-invasive methods can be used to monitor respiration in rodents and determine respiratory parameters (for extended review of these methods see (Hoymann, 2007).

3.1 Whole body plethysmography

In whole body plethysmography (WBP), freely moving animals are placed in a closed chamber and the pressure fluctuations that occur during the breathing cycle are recorded. A pressure transducer monitors the pressure differences between the experimental chamber of the ple-thysmograph where the animal is placed and a reference chamber. Both chambers have a regulated flow of room air or a controlled combination of gas and the system is calibrated with known air volume changes. The three primary parameters derived from this technique are tidal volume (TV), the volume of each breath; respiratory frequency, the number of breaths per minute (BPM); and minute volume (MV), the composite of breath volume and frequency (Hill et al., 2016). It is important to consider all three parameters as changes in MV may be due to a decrease in either BPM, TV or both. For example, fentanyl-like drugs are thought to induce a decrease in TV through a separate action of muscle stiffness not commonly seen in other opioids (Hill, Santhakumar, Dewey, Kelly, & Henderson, 2020), which may contribute to their lethality.

As respiration can be altered by stressed and quiescent states, measurements of respiration in WBP systems need to minimise the effect of these variables. Stress can induce a heightened respiratory baseline, and therefore, changes in respiration may indirectly be assessing the anxiogenesis and anxiolytic effects of the compounds tested (Lynch 3rd et al., 2019). Minimising stress can be achieved with sufficient habituation of the animal to the WBP chamber (ideally the day prior to the experiment) (Hill et al., 2016). As animals are unrestrained in the WBP chamber, it is possible for them to curl up and either sleep or enter a quiescent state. This can often be detected in vehicle administered groups (e.g. saline) when a significant reduction in respiration is observed while the treatment is known to have no effect on respiration. This effect appears to be mitigated by using larger WBP chambers such as those in comprehensive lab animal monitoring systems (CLAMS) (Reilley et al., 2010) which provide a greater area for normal locomotor behaviour.

Timing of experiments and housing conditions that maximise the activity of rodents can also help mitigate these confounds in smaller WBP chambers. These usually involve conducting measurements in the most active phases of the night cycle (Bains etal., 2018) and housing the rodents on a reverse lit day-night cycle that allows experimentation during the night cycle of the animal, when they are naturally more active. Additionally, a mild hypercapnic stimulus can help maintain steady respiratory rates of mice without a significant induction of

stress (as measured by corticosterone release (Hill et al., 2016) without apparent changes in the ability or sensitivity of opioids to induce respiratory depression (Hill et al., 2018).

3.2 Head-out plethysmography

In contrast to WPB, head-out plethysmography restrains the experimental animal in a plethysmograph chamber supplied with a controlled flow of air (Hoymann, 2007) but leaves the head free through a sealed neck ring. As movement restraint is a significant stressor (Lynch 3rd et al., 2019), it is likely that the anxiolytic effect of opioids, in addition to their respiratory depressant effects, contribute significantly to the measurements using this approach. However, it may be the method of choice when additional more invasive methods are used for the study of lung and airway pathology.

3.3 Pulse-oximetry

Unlike plethysmography, pulse-oximetry does not measure the mechanical actions of breathing in rodents but instead measures changes in blood oxygen levels. A decrease in blood oxygen saturation is used as a proxy for respiratory depression. The non-invasiveness and nonstressful properties of this method are advantages for the use of this approach to characterise the respiratory depressant effects of opioids in rodents (DeWire et al., 2013; Faouzi, Varga, & Majumdar, 2020; Schmid et al., 2017). It is however unclear how the respiratory parameters obtained using pulse oximetry relate to those obtained with the more established systems of whole body plethysmography and head out plethysmography. As mentioned above, minute volume, tidal volume as well as breathing frequency can be altered to differing extents by different opioids (Hill et al., 2020).

It is important to note that experimental assessments of respiratory depression do not have the same cut-offs as antinociception assays. This allows for estimates of efficacy and potency that are not subject to a defined maximal effect. Moreover, it allows the assessment of the respiratory depressant potential of all novel opioids at higher doses than those providing effective antinociception, which defines the therapeutic window. Assessing the potential to induce respiratory depression is of relevance as this side effect underlies most opioid-overdose fatalities in humans.

The impact of strain sensitivity on the respiratory depressant effects of opioids has recently been described (Table 2) (Bubier et al., 2020; Young et al., 2018). Of note, the reduced sensitivity of the 129Sv1J mouse line to opioidergic responses has been suggested to underlie the arrestin-3 knock-out data (Bubier et al., 2020; Kliewer et al., 2020).

4 Assessment of opioid-induced constipation

Opioid-induced constipation (OIC) is one of the most common side effects of opioid use in the clinic (McNicol et al., 2003). Severe OIC has been reported in 40-95% of patients following the onset of a prescribed opioid regime (Pappagallo, 2001; Prichard & Bharucha, 2015; Swegle & Logemann, 2006), impacting the patient’s quality of life, decreasing compliance and often resulting in heightened pain (Hjalte, Berggren, Bergendahl, & Hjortsberg, 2010; Katz, 2002; Trescot et al., 2008).

Opioids act to inhibit acetylcholinergic neurons preventing the release of neurotransmitters and increasing longitudinal smooth muscle tone while decreasing propulsive activity (Brock et al., 2012; Wood & Galligan, 2004). These effects are thought to be primarily mediated by the MOR as the effects are absent in MOR knock-out mice (Roy, Liu, & Loh, 1998). Importantly, both in animals and in humans, tolerance does not seem to develop within the GI tract actions of opioids, unlike antinociceptive tolerance, that requires increasing doses of opioids to provide the same level of analgesia (Müller-Lissner et al., 2017; Prichard & Bharucha, 2015). This was first identified in dogs (Plant & Miller, 1926) and has since been replicated across different species (Ling, Paul, Simantov, & Pasternak, 1989; Matsumoto et al., 2016;). Lack of tolerance to morphine-induced constipation has been shown from short (≤72 h) (Ross, Gabra, Dewey, & Akbarali, 2008) to longer (up to 10 days) administration periods. The use of peripherally restricted opioid antagonists, such as methylnaltrexone (Anissian et al., 2012; Bader, Durk, & Becker, 2013; Michna et al., 2011) or combination therapies of oxycodone and naloxone can be used to effectively treat OIC whilst retaining pain relief (Lowenstein et al., 2009; Simpson et al., 2008). Pre-clinically, the most common methods to measure OIC are faecal boli accumulation and glass bead expulsion.

Faecal boli accumulation measures the overall production of faecal matter over time (typically 2-6 h) following administration of opioids. This is usually done in under controlled feeding in order to measure food consumption against expulsion, and additionally a dye (e.g blue ink, charcoal) may be introduced into the diet to aid tracking of the bolus (Anand et al., 2018).

The *glass bead expulsion assay* requires brief anaesthesia of the animal in order to insert a small glass bead into a defined distance in the colon (Matsumoto et al., 2016). Following recovery from the anaesthetic, the time taken for the bead expulsion is measured. In this assay the opioid treatment can be administered before or after insertion of the glass bead. Whilst this assay is reliable and reproduceable, it is limited in its assessment of overall gut activity, as it only measures colonic transit effects. Comparatively, the faecal boli accumulation assay measures the effect of opioid inhibition throughout the entire gut, does not require the use of anaesthetics and is less invasive. However, it must also be considered that opioids can induce tolerance to differing degrees in colonic versus small and large intestinal transit in rodents (Matsumoto et al., 2016; Mori et al., 2013). Given these differences, the use of both assays in parallel may provide valuable information into regional differences in OIC.

While the literature surrounding mouse strain differences in relation to OIC is sparse, a recent study compared two inbred mouse strains (A/J and C57BL/6J) and found that C57BL/6 J mice were significantly more constipated following doses of morphine exceeding 40 mg/kg (Table 2)(Young et al., 2018).

5 Assessment of opioid-induced hyperalgesia and antinociceptive tolerance

Chronic opioid use can lead to opioid-induced antinociceptive tolerance and hyperalgesia. Analgesic or antinociceptive tolerance corresponds to a progressive decrease of analgesia

produced by a given opioid dose. This results in the need to increase the dose of opioids to provide a similar analgesic effect. Cellular and molecular mechanisms that underlie MOR regulation have been comprehensively reviewed (Williams et al., 2013). Whilst some mechanisms of MOR regulation such as phosphorylation (Doll et al., 2011) and desensitization (Bailey et al., 2009) have been thoroughly studied, there are still significant gaps in our understanding of the molecular processes responsible for loss of MOR function after chronic exposure to opioids. In particular the role of arrestins, whilst clearly important in the development of opioid tolerance through MOR desensitization, is still not fully understood (Bohn, Lefkowitz, & Caron, 2002; Kliewer et al., 2019; Williams et al., 2013).

Opioid induced hyperalgesia (OIH) is characterised by a paradoxical increase in pain perception following the onset of opioid medication (Lee, Silverman, Hansen, Patel, & Manchikanti, 2011; Roeckel, Le Coz, Gaveriaux-Ruff, & Simonin, 2016). While most opioid drugs appear to induce OIH regardless of their intrinsic efficacy and mechanism of action (Angst & Clark, 2006; Araldi, Ferrari, & Levine, 2018; Compton, Canamar, Hillhouse, & Ling, 2012), the molecular mechanisms underlying this state of nociceptive sensitisation are complex and still not fully understood. The most important proposed mechanisms for OIH have been reviewed elsewhere (Lee et al., 2011; Roeckel et al., 2016). These mechanisms involve the NMDA-glutamatergic system, transient receptor potential channels V1 and M8 (TRPV1 and TRPM8), and are influenced by several factors including genetic background and sex differences of experimental animals (Roeckel et al., 2016).

Experimentally, OIH and antinociceptive tolerance are measured through antinociception assays upon chronic administration of opioids, either by repeated administration or through osmotic minipumps (Hill et al., 2016; Hill et al., 2018; Koblish et al., 2017). The most common antinociception assays used in this context are thermal stimulation (hot plate and tail flick) for antinociceptive tolerance and OIH, and mechanical stimulation for OIH assessment only (Koblish et al., 2017; D.-Y. Liang et al., 2006; Roeckel et al., 2016). These allow the detection of significant changes in opioid-induced responses, and baseline latencies differences of naïve versus chronically treated animals are readily observed (in particular for OIH). However, when assessing for hyperalgesia, higher temperatures or applied forces are an impediment to obtaining clear, meaningful data; the baseline nociceptive latency of the animal decreases as the thermal or physical intensity increases (Carroll, 1959; Carstens & Wilson, 1993; Ren & Han, 1979), therefore the ability to detect a decrease in baseline threshold is also reduced at higher temperatures.

The same inherent ethical limitations apply for assessment of hyperalgesia and tolerance as they do for antinociception assays. Moreover, another limiting factor is the amplitude of the baseline nociceptive latency as an increase in the severity of the noxious stimulus will compromise the detection window even further upon the development of OIH (see above for the thermal example) (Jensen & Finnerup, 2014; Yalcin, Charlet, Freund-Mercier, Barrot, & Poisbeau, 2009). It is also of particular importance that multiple pain modalities are tested in the context of OIH as the development of OIH in patients is usually related to both chronic use of opioids as well as an underlying pain states (Burma, Leduc-Pessah, & Trang, 2017; Marrone et al., 2017). Other modalities include mechanical stimulation of pain responses (e.g. Von Frey filaments) (Koblish et al., 2017), that can be performed in conjunction with

pro-inflammatory treatments (e.g. carrageenan or capsaicin injections) (Luo et al., 2008; Yalcin et al., 2009) as well as neuropathic models of pain (e.g. nerve ligation assays) (Chen et al., 2020).

6 In vitro measurements of opioid pharmacology and their relationship with opioid-induced effects in vivo

The antinociceptive, respiratory and gastrointestinal effects of opioid therapeutics are mainly caused by the activation of the MOR (Bachmutsky et al., 2021; Matthes et al., 1996; Roy et al., 1998; Varga et al., 2020). The MOR signals predominantly through the activation of $G\alpha_{i/o}$ and $\beta\gamma$ proteins. MOR activation alters neurotransmitter release through presynaptic inhibition of voltage-gated calcium channels (VGCC) and inhibits neuronal activity through hyperpolarisation caused by post-synaptic activation of G protein-coupled inwardly rectifying potassium (GIRK) channels. Moreover, G protein activation by MOR also results in inhibition of adenylate cyclase, resulting in decreased levels of the second messenger cAMP. As with most GPCRs, the G protein signalling of MORs is regulated by phosphorylation of intracellular domains, arrestin binding and internalisation. Numerous in vitro assays are available for the detection of MOR signalling and regulation in cell lines. These assays include sensors to monitor receptor, G protein and GIRK channel activation, measurements of cAMP levels, recruitment of GRK and arrestins and receptor internalisation (Table 3). However, with the exception of electrophysiological approaches (Birdsong & Williams, 2020), application of these approaches for the detection of MOR signalling in neuronal cultures remains slightly more challenging.

Despite the initial reports, recent evidence suggests that a clear separation of the signalling responsible for antinociceptive versus deleterious side effects of opioids is unlikely (Bachmutsky, Wei, Durand, & Yackle, 2021; Kliewer et al., 2019; Kliewer et al., 2020). Antinociception, respiratory depression and constipation are all mediated by G protein activation by MOR and retained in arrestin KO mice (Bachmutsky et al., 2021; Benyamin et al., 2008; Conibear & Kelly, 2019; Gillis et al., 2020b; Kliewer et al., 2019; Matthes et al., 1996; Montandon et al., 2016; Valentino & Volkow, 2018). However, this should not preclude the development of improved opioids that minimise adverse effects and provide wider therapeutic windows through other mechanisms or pharmacological properties different than biased agonism. For example, the role of ligand binding kinetics, as well as kinetic of effect has been proposed to underlie potential signalling differences among GPCR ligands (Klein Herenbrink et al., 2016; van der Velden, Heitman, & Rosenkilde, 2020) although this does not seem to be the case for some novel opioids (Pedersen et al., 2020).

It has also been proposed that low intrinsic efficacy may provide an alternative explanation to the improved therapeutic profiles (Azevedo Neto et al., 2020; Benredjem et al., 2019; Gillis, Gondin, et al., 2020; Kelly, 2013). This is supported by a recent study of clinically used opioids which found that intrinsic efficacy, rather than any G protein/β-arrestin bias, predicted the rate of reported adverse events (Benredjem et al., 2019), and by the actions of the opioid buprenorphine, an extremely low efficacy MOR agonist with a ceiling effect in respiratory depression and reduced overdose risk (Dahan et al., 2006; Walsh, Preston,

Stitzer, Cone, & Bigelow, 1994). In this context, the response of partial agonists, compounds with low intrinsic efficacy, is very sensitive to the presence of receptor reserve (also known as spare receptors). As such, partial agonists will display robust responses in systems with high receptor reserve, whilst in systems with lower receptor reserve they will produce a partial maximum response even at full receptor occupancy. As receptor reserve is a property of the tissue and of the agonist (Buchwald, 2019; S. J. Hill, 2006), it therefore follows that the same drug may elicit full or partial responses depending on the tissue receptor reserve. Although difficult to prove experimentally, these differences may explain the effects of low efficacy opioids (A. E. Conibear & Kelly, 2019; Gillis, Sreenivasan, & Christie, 2020; Pineyro & Nagi, 2021; Uprety et al., 2021). The presence of receptor reserve in the responses of opioid agonists is a pharmacological concept that has been thoroughly explored in cultured cell lines (Carliss et al., 2009; Kelly, 2013) and utilised in electrophysiology (Lowe & Bailey, 2015). This is commonly assessed using irreversible antagonists (such as β-funaltrexamine (Takemori, Larson, & Portoghese, 1981), β-chlornaltrexamine (Portoghese, Larson, Jiang, Takemori, & Caruso, 1978) or methacinnamox (Broadbear et al., 2000) or genetic strategies to decrease receptor levels (Mizoguchi et al., 1999; Singleton et al., 2021; Sora et al., 2001).

As mentioned above, measurements of antinociceptive efficacy are limited by the imposed cut-offs. However, given that low efficacy agonists such as buprenorphine or oliceridine provide effective antinociception, it may be suggested that some receptor reserve is present in this system. Similarly, as tail flick and hot plate assays do not measure identical responses, differences in receptor reserve may also be relevant to consider. The relationship between tolerance and receptor reserve as received particular attention (Chavkin & Goldstein, 1984; Mjanger & Yaksh, 1991; Williams et al., 2013). A recent study, investigated the effect of irreversible antagonism in vitro and of reduced receptor expression in $MOR^{+/-}$ mice to interrogate the antinociceptive tolerance of oliceridine. Although a clear effect was observed in terms of ligand efficacy, oliceridine was still able to induce tolerance in mice expressing 50% less MOR than wild type (Singleton et al., 2021).

Despite the potential to explain the different actions of opioids in different tissues, the impact of receptor reserve in the other physiological responses of opioids such as respiratory depression and constipation have not as yet been studied and compared with the effect of receptor reserve on antinociception. Future studies addressing the relevance of receptor reserve for the effects of opioids in vivo are likely to provide a much-needed framework of efficacy to drug discovery programs that may suggest some new strategies for the development of improved analgesics.

7 Concluding remarks

- The inherent limitations in the experimental design of antinociception assays can influence determinations of efficacy of opioid drugs. When characterising novel opioid agonists, consideration should be taken into how these limitations affect the data.

- Differential strain sensitivity to the effects of opioids (including the potential for differences in drug pharmacokinetics) needs to be thoroughly studied. This is not only important when comparing actions of opioids across laboratories, but also when considering the use of genetically modified mice.
- Adverse side effects limit the therapeutic potential of novel opioid analgesics. It is important that all opioidergic behaviours induced by novel agonists are assessed at doses above those providing effective antinociception in order to understand the liabilities and risks associated with deliberate or accidental abuse.
- Whilst early in vitro assessment of novel opioids provides valuable insight into their actions, these assays are limited in their ability to predict side effect liabilities. Early ex vivo characterisation of novel opioids in native tissue may provide better indications for side effect liability. Recent measurements of respiratory depression in zebra fish provides an example of an innovative higher throughput screen for novel opioids (Zaig, da Silveira Scarpellini, & Montandon, 2021).
- Further assessments of the impact of receptor reserve and relative receptor expression levels across different tissues and animal strains are necessary to understand the relationship between efficacy and opioid-induced responses in vivo and provide a framework for the development of new opioid-based analgesics.

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Abbreviations

GPCR	G protein-coupled receptor
MOR	mu-opioid receptor
OIH	opioid-induced hyperalgesia
OIC	opioid-induced constipation
WBP	whole body plethysmography

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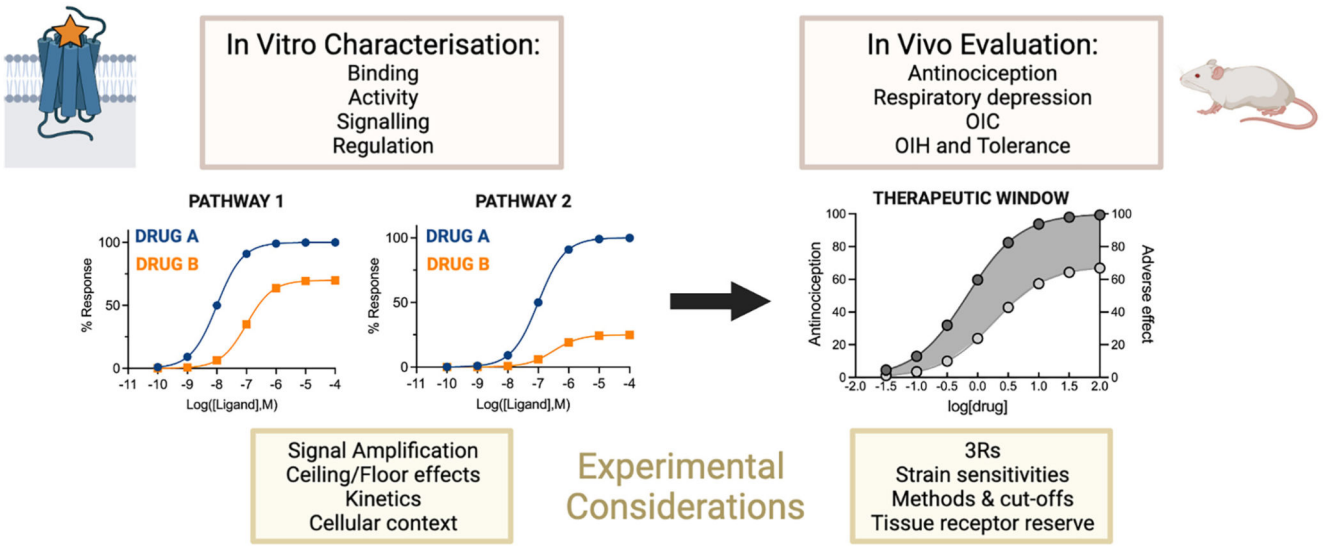

Fig. 1.

Experimental considerations for the assessment of opioid pharmacology in vitro and in vivo. In vitro and in vivo characterisations of opioid action are both essential in drug discovery. However, important factors need to be considered to provide a consistent and systematic framework of opioid action. In vitro, compounds are tested for their binding to MOR, and for their ability to activate downstream signalling. These assays need to take into account distinct variables than can affect the measured outcome. In vivo, candidates are usually first assessed for their antinociceptive efficacy, and then for their ability to elicit adverse effects. OIC: Opioid-induced constipation, OIH: Opioid-induced hyperalgesia.

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Table 1
Opioid-induced antinociception assays used in MOR drug discovery.

Compound	Antinociception assay – species	Strain	Temp & cut-off	Reference
TRV130	Hot plate – mouse	C57/BL6J	56 °C, 30 s	(DeWireet al., 2013)
		C57/BL6	54 °C, 20 s	(Gillis, Gondin, et al., 2020)
		ICR	55 °C, 30 s	(Mori et al., 2021)
	Tail flick – mouse	Swiss Webster	52 °C,10 s	(Altarifi et al., 2017)
		C57/BL6	Variable, 10 s	(Liang, Li, Nwaneshiudu, Irvine, & Clark, 2019)
	Hot plate – rat	Sprague-Dawley	52 °C, 30 s	(DeWireet al., 2013)
	Tail flick – rat	Sprague-Dawley	Variable, 15 s	(DeWireet al., 2013)
		Sprague-Dawley	50 °C, 20 s	(Schwienteck et al., 2019)
TRV0109101	Hot plate – mouse	C57/BL6	56 °C, 30 s	(Koblish et al., 2017)
	Hot plate – rat	Sprague-Dawley	52 °C, 30 s	
	Tail flick – rat	Sprague-Dawley	Variable, 15 s	
	Von Frey filaments – mouse	C57/BL6	0.4g	
PZM21	Hot plate – mouse	C57/BL6J	55 °C, 30 s	(Mangliket al., 2016)
		C57/BL6J	52.5 °C, 30 s	(iKudla et al., 2019)
		C57/BL6	54 °C, 20 s	(Gillis, Gondin, et al., 2020)
		CD-1	52.5 °C, 30 s	(Hill et al., 2018)
	Tail flick – mouse	C57/BL6J	56 °C,10 s	(Mangliket al., 2016)
		C57/BL6J	Variable, 9 s	(iKudla et al., 2019)
	Affective vs reflexive – hot plate – mouse	C57/BL6J	52.5 °C, 30 s	(Mangliket al., 2016)
	Formalin chemical assay – mouse	C57/BL6J	1% formalin	(Mangliket al., 2016)
SR-17018	Hot plate – mouse	C57/BL6J	52 °C, 20 s	(Grim et al., 2020; Schmid et al., 2017)
		C57/BL6	54 °C, 20 s	(Gillis, Gondin, et al., 2020)
	Tail flick – mouse	C57/BL6J	49 °C, 30 s	(Grim et al., 2020; Schmid et al., 2017)
Mitragynine	Tail flick – mouse	CD-1	Variable, 3× baseline	(Varadi et al., 2016)
		C57/BL6	Variable, 3× baseline	
		C57/BL6J	48 °C, 30 s	(Wilson et al., 2021)
		129S1	Variable, 10 s	(Kruegel et al., 2019)
7-OH Mitragynine	Tail flick – mouse	CD-1	Variable, 3× baseline	(Varadi et al., 2016)
		129S1	Variable, 10 s	(Kruegel et al., 2019)
Mitragynine Pseudoindoxyl	Hot plate – mouse	CD-1	55 °C, 30 s	(Varadi et al., 2016)
	Tail flick – mouse	CD-1	Variable, 3× baseline	(Varadi et al., 2016), (Wilson et al., 2021)
		C57/BL6	Variable, 3× baseline	
		129Sv6	Variable, 3× baseline	
		C57/BL6J	48 °C, 30 s	

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Compound	Antinociception assay – species	Strain	Temp & cut-off	Reference
Tianeptine	Hot plate – mouse	C57/BL6	50 or 55 °C, 30 s	(Samuels et al., 2017)
MP-1208 & MP1207	Tail flick – mouse	C57BL/6	55 °C,15 s	(Uprety et al., 2021)
CYM51010	Tail flick – mouse	C57/BL6	Heat intensity	(Gomes et al., 2013)
			10 (IITC LifeScience), 20 s	(Faouzi et al., 2020)
		C57/BL6	Variable, 10 s	
		CD-1	Variable, 10 s	
		129	Variable, 10 s	
MP135	Tail flick – mouse	C57/BL6	Variable, 10 s	(Faouzi et al., 2020)
		CD-1	Variable, 10 s	
		129	Variable, 10 s	
	Hargreaves – rat	Long Evans	not given	
AT-201	Tail flick – mouse	ICR	Radial beam	(Khroyan et al., 2007; Toll et al., 2009)
			(Stoelting, Wood Day, IL), 15 s	
Endomorphin analogues	Tail flick – mouse	CD-1	Variable, 9 s	(Zadina et al., 2016)
	Tail flick – rat	Sprague-Dawley	Variable, 9 s	

Summary of the antinociception assay, species, strain and cut-offs used for the evaluation of novel opioids.

Table 2

Strain differences in opioid-induced behaviours.

Drug	Assay	Strains	Observations	References
Morphine	Hot water tail flick – 49 °C and 15 s cutoff Four day escalating dose ofmorphine (10-40 mg/kg) used to induce tolerance.	*Strain – baseline (s)* 129P3-2.4 ± 0.1 A – 2.4 ± 0.1 AKR – 2.9 ± 0.2 ABLB/c – 3.1 ± 0.2 C3H/He – 1.8 ± 0.1 C57BL/6-2.2 ± 0.1 CBA – 1.9 ±0.1 DBA/2-2.5 ± 0.1 LP – 2.8 ± 0.1 SJL – 2.2 ± 0.1 SWR – 1.8 ± 0.1	• Significant differences in baseline latencies. • Significant differences in the relative degree of tolerance (from 0.8 to 10.8 fold change in morphine ED_{50} values (day 1 vs day 4).	(Kestet al., 2002)
Morphine	Hot water tail flick – 55 °C and 10 s cutoff	*Strain – baseline (s)* SW – 2.7 ± 0.6 129/SvEv – 3.5 ± 0.6	• Significant differences in baseline latencies. • Differences in duration of antinociception (>6 h in 129/SvEv vs 2 h in SW). • 129/SvEv mice were approx. 10-fold more sensitive to morphine	(Crain and Shen, 2000)
Morphine	Hot water tail flick – 49 °C and 15 s cutoff	*Strain – baseline (s)* 129/J – 4.7 ± 0.2 129/SvJ – 4.5 ± 0.1 B6-2.6 ± 0.1	• Significant differences in baseline latencies. • Right shifted dose-response curve to cumulative morphine in B6 mice.	(Mogil & Wilson, 1997)
Morphine	LD_{50} determination by percent survival – measured by return to regular breathing	*Strain and sex – LD_{50} (mg/kg)* 129S1/SvlmJ *F* – 631.3 129S1/SvlmJ *M* -664.2 A/J *F* – 212.2 A/J *M* – 225.2 C57BL/6 J *F* – 311.6 C57BL/6 J *M* – 254.3 CAST/EiJ *F* – 882.2 CAST/EiJ *M* – 429.9 NOD/ShiLtJ *F* – 811.0 NOD/ShiLtJ *M* – 588.8 NZO/HILtJ *F* – 333.9 NZO/HILtJ *M* – 324.4 PWK/PhJ *F* – 261.0 PWK/PhJ *M* – 359.1 WSB/EiJ *F* – 526.4 WSB/EiJ *M* – 695.6	• Baseline respiration data not reported. • Significant differences in LD50 values between strains and sex.	(Bubier et al., 2020)
Morphine	Measurement of respiration (whole body plethysmography) and constipation (Charcoal transit)	C57BL/6 J A/J	• Baseline values not compared between strains. • Enhanced sensitivity to morphine respiratory depression and significantly greater constipation in C57BL/6 J mice vs A/J mice	(Young et al., 2018)

Summary of the assays, strains and results reported in the studies addressing strain differences to opioid sensitivity.

Table 3
In vitro signalling assays used in MOR drug discovery.

Compound	Response	Cell type	Assays	References
TRV130 (Oliceridine)	β-Arrestin recruitment Nb33 recruitment mGsi recruitment G protein activation GIRK/VGC channel modulation Inhibition of Fsk-induced cAMP accumulation Internalisation MOPr phosphorylation	HEK293	DiscoverX PathHunter, BRET, FRET BRET BRET BRET, FRET Membrane potential assay, patch-clamp electrophysiology cAMP HiRange Kit, BRET, GloSensor DiscoverX PathHunterBRET, TR-FRET, imaging Western blot	(DeWire et al., 2013, Gillis, Gondin, et al., 2020, Pedersen et al., 2020, Mori et al., 2021, Ehrlich et al., 2019, Yudin et al., 2019)
TRV0109101	β-Arrestin recruitment Inhibition of Fsk-induced cAMP accumulation Internalisation MOPr phosphorylation	HEK293	DiscoverX PathHunter cAMP HiRange Kit DiscoverX PathHunter pSer^{375} Western blot	(Koblish et al., 2017)
PZM21	β-Arrestin recruitment Nb33 recruitment mGsi recruitment G protein activation GIRK/VGC channel modulation Calcium release Inhibition of Fsk-induced cAMP accumulation Internalisation MOPr phosphorylation	HEK293	DiscoverX PathHunter, BRET, FRET BRET BRET BRET, GTPγS binding, FRET Membrane potential assay, patch-clamp electrophysiology FLIPR (Fluo-4 dye) BRET, GloSensor DiscoverX PathHunterBRET Western blot	(Manglik et al., 2016) (Gillis, Gondin, et al., 2020) (Hill et al., 2018) (Yudin et al., 2019)
SR-17018	β-Arrestin recruitment Nb33 recruitment mGsi recruitment G protein activation GIRK channel activation Inhibition of Fsk-induced cAMP accumulation Internalisation MOPr phosphorylation	CHO-K1, U2OS, HEK293	DiscoverX PathHunter, BRET, FRET, imaging BRET BRET BRET, GTPγS binding Membrane potential assay BRET, cAMP HiRange Kit BRET, imaging Western blot	(Grim et al., 2020; Schmid et al., 2017) (Gillis, Gondin, et al., 2020)
Mitragynine	G protein activation β-Arrestin recruitment	CHO	GTPγS binding DiscoverX PathHunter	(Varadi et al., 2016) (Wilson et al., 2021)
7-OH Mitragynine	G protein activation β-Arrestin recruitment	CHO	GTPγS binding DiscoverX PathHunter	(Varadi et al., 2016)
Mitragynine Pseudoindoxyl	G protein activation β-Arrestin recruitment	CHO	GTPγS binding DiscoverX PathHunter	(Varadi et al., 2016)
Tianeptine	G protein activation Inhibition of Fsk-induced cAMP accumulation	HEK293T	BRET BRET	(Gassaway et al., 2014)
MP-1208&MP1207	β-Arrestin recruitment G protein activation Inhibition of Fsk-induced cAMP accumulation	HEK293, HTLA	Tango, BRET BRET, GTPγS binding GloSensor	(Uprety et al., 2021)
CYM51010	β-Arrestin recruitment G protein activation	U2OS	 DiscoverX PathHunter BRET, GTPγS binding	(Gomes et al., 2013) (Faouzi et al., 2020)
MP135	β-Arrestin recruitment G protein activation	U2OS	DiscoverX PathHunter BRET, GTPγS binding	(Faouzi et al., 2020)

Summary of the cell signalling assays commonly used for opioid drug discovery. Fsk; forskolin, ORs; opioid receptors, BRET: Bioluminescence Resonance Energy Transfer, FRET; Förster Resonance Energy Transfer, TR-FRET; Time-Resolve FRET; GIRK channel, G protein inwardly rectifying channel; VGCC, voltage-gated calcium channel.