Received: 28 February 2021 | Revised: 27 April 2021 | Accepted: 12 May 2021

DOI: 10.1111/bph.15573

Themed Issue: Advances in Opioid Pharmacology at the Time of the Opioid Epidemic

THEMED ISSUE REVIEW

The anomalous pharmacology of fentanyl

Eamonn Kelly | Katy Sutcliffe | Damiana Cavallo | Nokomis Ramos-Gonzalez | Norah Alhosan | Graeme Henderson

School of Physiology, Pharmacology and Neuroscience, University of Bristol, Bristol, UK

Correspondence
Prof. Graeme Henderson and Prof. Eamonn Kelly, School of Physiology, Pharmacology and Neuroscience, University of Bristol, Bristol BS8 1TD, UK.
Email: graeme.henderson@bristol.ac.uk; e.kelly@bristol.ac.uk

Funding information
Medical Research Council, Grant/Award Number: MR/S010890/1; Biotechnology and Biological Sciences Research Council (BBSRC); South West Biosciences (SWBio), Grant/Award Number: BB/J014400/1

Fentanyl is a key therapeutic, used in anaesthesia and pain management. It is also increasingly used illicitly and is responsible for a large and growing number of opioid overdose deaths, especially in North America. A number of factors have been suggested to contribute to fentanyl's lethality, including rapid onset of action, *in vivo* potency, ligand bias, induction of muscle rigidity and reduced sensitivity to reversal by naloxone. Some of these factors can be considered to represent 'anomalous' pharmacological properties of fentanyl when compared with prototypical opioid agonists such as morphine. In this review, we examine the nature of fentanyl's 'anomalous' properties, to determine whether there is really a pharmacological basis to support the existence of such properties, and also discuss whether such properties are likely to contribute to overdose deaths involving fentanyls.

LINKED ARTICLES: This article is part of a themed issue on Advances in Opioid Pharmacology at the Time of the Opioid Epidemic. To view the other articles in this section visit http://onlinelibrary.wiley.com/doi/10.1111/bph.v180.7/issuetoc

KEYWORDS
fentanyl, opioid, receptor

1 | INTRODUCTION

Fentanyl, a **μ receptor** agonist, was introduced into clinical medicine in 1963 as a potent, relatively short-acting intravenous analgesic agent (Stanley, 2014). Subsequently, several congeners including **alfentanil**, **sufentanil** and **remifentanil** were developed for medical use and **carfentanil** was introduced into veterinary practice. In human medicine, these fentanyls are used for the treatment of intractable and breakthough cancer pain, and to produce balanced intravenous anaesthesia. Over the last 10 years, fentanyl and structurally related medicinal and illicit compounds (generically referred to as 'fentanyls') have become major illicit drugs, especially in North America. There has been a dramatic rise in acute opioid overdose deaths involving fentanyls in the United States since 2013 (Jannetto et al., 2019). Of the 50,000 deaths in 2019 involving opioids, just over 36,000 involved fentanyls, exceeding those involving **heroin** or prescription opioids such as **oxycodone** (National Institute on Drug Abuse, 2021).

Despite the vast scientific literature describing the varied pharmacological properties of the fentanyls that have been produced over the past 60 years, some important misconceptions about how these drugs act are promulgated in both the scientific literature and media reports. In the present review, we will concentrate primarily on fentanyl itself and consider a number of anomalous aspects to the pharmacology of fentanyl, considering how these may have contributed to the misconceptions alluded to above. The term 'anomalies' here refers to fentanyl behaving pharmacologically in ways that appear to be different from those observed with other widely used μ receptor agonists such as **morphine** and **oxycodone** (Gill et al., 2019).

Anomalous pharmacological properties of fentanyl are as follows:

- *in vitro* and *in vivo* potency does not correlate with measurements of affinity or efficacy.

Abbreviations: cryo-EM, cryogenic electron microscopy; DAMGO, [D-Ala^2,*N*-MePhe^4,Gly-ol^5]-enkephalin; GIRK, G protein-coupled inwardly rectifying potassium channel; GRK, GPCR kinase; MD, Molecular Dynamics; β-CNA, β-chlornaltrexamine; β-FNA, β-funaltrexamine.

- the potential for the fentanyl molecule to orientate in various ways within the orthosteric binding pocket of the μ receptor.
- access to the orthosteric binding pocket via a lipophilic pathway.
- potential for arrestin-biased signalling.
- lower cross-tolerance to heroin *in vivo*.
- induction of respiratory muscle rigidity.
- reduced sensitivity to reversal by **naloxone** compared with other opioid agonists.

2 | COMPARISON OF THE AFFINITY AND EFFICACY OF FENTANYL AND MORPHINE AT THE μ RECEPTOR

A range of *in vivo* studies indicate that fentanyl is at least 50-fold and in some cases over 100-fold more potent than morphine for inducing μ receptor-related behavioural effects (Tables 1A and 1B) (Hill et al., 2020; Schwienteck et al., 2019; Suzuki & El-Haddad, 2017). This has led to the widespread assertion that fentanyl has a much higher affinity and/or efficacy than morphine at the μ receptor. However, in *in vitro* radioligand binding studies using recombinant or endogenous μ receptors, fentanyl has been shown to have similar affinity for the receptor as morphine (Table 1C). This is true whether assays were performed in the absence or presence of a physiologically relevant concentration of sodium ions. The presence of sodium ions reduces the affinity of high efficacy agonists to a greater extent than the affinity of lower efficacy (partial) agonists (Pert et al., 1973; Simon et al., 1975) and although this could have confounded the comparison of fentanyl and morphine, the fact that there was no difference in receptor affinity in the absence or presence of sodium negates this potential confounder. Thus, at the molecular level, the affinity of fentanyl for the μ receptor is not markedly different from that of morphine.

Furthermore, in the long-established [^{35}S]GTPγS binding assay to measure potency and efficacy for GPCR activation, fentanyl is reported to be only slightly more potent than morphine (2.4-fold, taken as an average of the 16 separate studies listed in Table 1D). It is also possible, when the E_{max} values are lower than that of the full agonist **[D-Ala^2,N-MePhe^4,Gly-ol^5]-enkephalin (DAMGO)**, to use the comparative E_{max} values of lower efficacy agonists as a surrogate measure of their relative agonist efficacies. In a number of such studies, fentanyl was observed to have efficacy that was slightly lower than, equal to, or slightly higher than that of morphine (Table 1D). In our own study of agonist-induced binding of [^{35}S]GTPγS to the μ receptor, we observed that fentanyl was slightly more potent than morphine. Using the operational model of Black and Leff (1983) to calculate operational efficacy, we determined that fentanyl had higher efficacy than morphine but less than that of DAMGO and methadone (McPherson et al., 2010).

These data on affinity of binding, agonist potency and efficacy do not fit with the concept that fentanyl has exceptionally high affinity at, or high efficacy for, the μ receptor. However, these experiments were all performed using membrane homogenates and when cell signalling assays were carried out using intact cells, a different picture emerged for fentanyl (Table 1E). In the case of whole-cell assays, the difference in potency between fentanyl and morphine is larger than for membrane or homogenate preparations. Overall, for cell-based assays, the difference in potency between fentanyl and morphine was marked (13.9-fold, taken as an average of the 20 studies listed in Table 1E; c.f. 2.4-fold difference for homogenate [^{35}S]GTPγS binding assay). This difference was present irrespective of the nature of the cell-based assay, be it G-protein activation, **G protein-coupled inwardly rectifying potassium channel (GIRK; K_{ir}3.x)** activation, inhibition of cAMP accumulation or GPCR kinase (GRK) and arrestin recruitment (Table 1E). The difference was independent of the amplification factor in the cellular assay, being evident in assays with low or non-existent amplification (arrestin recruitment) as well as in assays with high amplification (inhibition of cAMP accumulation).

This increased difference in potency between fentanyl and morphine in cell-based assays could potentially be explained by a relative increase in affinity and/or efficacy for fentanyl over morphine at the μ receptor in intact cells. Although measurements of fentanyl affinity for the μ receptor in intact cells as opposed to membranes are rarely performed, one study (Lambert et al., 1993) reported no difference in

TABLE 1A Comparison of fentanyl and morphine in *in vitro* and *in vivo* assay systems: Intact tissue assay

Assay	Species	Fentanyl EC_{50} (nM)	Morphine EC_{50} (nM)	Relative potency of fentanyl:morphine	Reference
Inhibition of nerve-evoked contractions	Guinea pig ileum	0.92	69	75-fold	Kosterlitz and Leslie (1978)

TABLE 1B Comparison of fentanyl and morphine in *in vitro* and *in vivo* assay systems: In vivo assays

Assay	Species	Fentanyl ED_{50} (mg·kg^{-1})	Morphine ED_{50} (mg·kg^{-1})	Relative potency of fentanyl:morphine	Reference
Antinociception (thermal tail flick)	Rat	0.049	8.07	165-fold	Schwienteck et al. (2019)
Antinociception (thermal tail flick)	Mouse	0.041	2.82	68-fold	Chan et al. (1995) and Sirohi et al. (2008)
Respiratory depression	Mouse	0.6	25	42-fold	Hill (2019)

TABLE 1C Comparison of fentanyl and morphine in *in vitro* and *in vivo* assay systems: Radioligand binding (membrane homogenates)

	Species of μ receptor (tissue)	Fentanyl (K_i, nM)	Morphine (K_i, nM)	Relative affinity of fentanyl:morphine	Reference
High Na^+ (100–137 mM)	Rat	158	250	1.6-fold	McPherson et al. (2010)
	Rat	157	132	0.8-fold	Emmerson et al. (1996)
	Guinea pig (brain)	162	177	1.1-fold	Kosterlitz and Leslie (1978)
	Human	2.8[a]	6.4[a]	2.2-fold	Schmid et al. (2017)
Zero Na^+	Human	1.6	4.0	2.6-fold	Hassanien et al. (2020)
	Human	0.5	0.8	1.6-fold	Heusler et al. (2015)
	Rat	0.135	0.252	1.9-fold	Eshleman et al. (2020)
	Rat	0.35	0.58	1.7-fold	Torralva et al. (2020)
	Rat	0.16	0.16	1.0-fold	Emmerson et al. (1996)
	Guinea pig (brain)	4.2	2.7	0.6-fold	Kosterlitz and Leslie (1978)

[a]In this study by Schmid et al., the authors state that the assay was performed in the presence of Na (100 mM), but the high affinity for both ligands (low nM values) would indicate the absence of Na. Either way it does not matter as the ratio is close to 1.

TABLE 1D Comparison of fentanyl and morphine in *in vitro* and *in vivo* assay systems: Stimulation of [^{35}S]GTPγS binding (membrane homogenates)

Species of μ receptor	Fentanyl EC_{50} (nM)	Morphine EC_{50} (nM)	Relative potency of fentanyl:morphine	E_{max} (relative efficacy) of fentanyl:morphine (c.f. DAMGO 100)	Reference
Recombinant receptors					
Human	32	150	4.7-fold	89:98	Hassanien et al. (2020)
Human	43	64	1.5-fold	80:81	Schmid et al. (2017)
Human	2.6	3.6	1.4-fold	112:111	Heusler et al. (2015)
Human	27.8	125	4.5-fold	107:90	Obeng et al. (2021)
Human					Saidak et al. (2006)
Ga_{i1}	119	213	1.8-fold	69:66	
Ga_{oA}	67	89	1.3-fold	72:88	
Rat	21.4	26.1	1.2-fold	89:82	Eshleman et al. (2020)
Rat	18	38	2.1-fold	92:86	Torralva et al. (2020)
Rat	56.8	97.5	1.7-fold	110:94	McPherson et al. (2010)
Rat	58	73	1.3-fold	86:74	Clark et al. (2006)
Rat	–	28.3	–	97:83	Emmerson et al. (1996)
Mouse	59.7	36.3	0.6-fold	–	Zaki et al. (2000)
Mouse	23	120	5.2-fold	110:106	Selley et al. (1997)
Native tissue (species)					
SK-N-SH cells (human)	37.5	138	3.7-fold	66:73	Selley et al. (1997)
SH-SY-5Y cells (human)	15.2	26.7	1.8-fold	91:75	Traynor and Nahorski (1995)
Spinal cord (mouse)	135	407	3.0-fold	83:78	Madia et al. (2012)
Thalamus (rat)	117	434	3.7-fold	58:56	Selley et al. (1997)

binding affinity of fentanyl and morphine at the μ receptor. However, it is possible that the suspension of SH-SY5Y cells used in that study disrupted any possible membrane-induced concentration gradient (see below). On the other hand, fentanyl does exhibit somewhat higher efficacy than morphine in cell-based assays. Precise estimations of agonist relative efficacy in cell-based assays comparing maximum responses are confounded because fentanyl behaves as a full agonist in most of these assays. However, in one study (Gillis, Gondin, et al., 2020) where functional receptor number was reduced by pretreatment of cells with the irreversible antagonist β-chlornaltrexamine (β-CNA), the E_{max} values for fentanyl- and morphine-induced activation of GIRK currents were 74% and 56%, respectively, with DAMGO taken as 100%. In another study of arrestin recruitment (Schmid et al., 2017), the relative E_{max} values for

TABLE 1E Comparison of fentanyl and morphine in *in vitro* and in *vivo* assay systems: Cell-based assays

Assay	Species of μ receptor	Fentanyl EC_{50} (nM)	Morphine EC_{50} (nM)	Relative potency of fentanyl:morphine	Reference
Inhibition of cAMP accumulation	Human	0.26	5.04	19.4-fold	Crowley et al. (2020)
	Human	0.13	3	23.1-fold	Zebala et al. (2020)
	Human	10.2	209	20.5-fold	Manabe et al., 2019
	Human	0.54	26	48.1-fold	Schmid et al. (2017)
	Mouse	0.63	7.9	12.5-fold	Gillis, Gondin, et al. (2020)
	Mouse	2.06	4.9	2.4-fold	Zaki et al. (2000)
Cell impedance	Human	15.1	251	16.6-fold	Manabe et al. (2019)
G-protein activation					
NB33	Mouse	43.6	213.8	4.9-fold	Gillis, Gondin, et al. (2020)
mG_{si}	Mouse	22.9	114.8	5.0-fold	Gillis, Gondin, et al. (2020)
$G\alpha_{i2}$	Mouse	2.1	19.1	9.1-fold	Gillis, Gondin, et al. (2020)
GIRK activation	Human	1.8	23.3	12.9-fold	Dasgupta et al. (2021)
	Mouse	0.5	10.5	21.0-fold	Gillis, Gondin, et al. (2020)
	Mouse	0.5	19.9	39.8-fold	Knapman et al. (2012)
GRK2 translocation	Mouse	46.8	166	3.5-fold	Gillis, Gondin, et al. (2020)
Arrestin translocation	Human	38	380	10-fold	Crowley et al. (2020)
	Human	35	352	10.1-fold	Zebala et al. (2020)
	Human	53	372	7.1-fold	Schmid et al. (2017)
	Rat	210	322	1.5-fold	McPherson et al. (2010)
	Mouse	79.4	331	4.2-fold	Gillis, Gondin, et al. (2020)
+GRK overexpression	Mouse	8.3	48.9	5.9-fold	Gillis, Gondin, et al. (2020)

fentanyl and morphine were 60% and 24%, respectively (again DAMGO taken as 100%). Whereas, in our own study of arrestin recruitment (McPherson et al., 2010), the efficacy of fentanyl relative to morphine, as calculated by operational analysis, was 2.9-fold greater. Thus, in cell-based assays, fentanyl may exhibit slightly greater efficacy over morphine, but this seems unlikely to be sufficient to explain the increased potency of fentanyl relative to morphine in this experimental setting.

A possible reason for the increased separation of fentanyl and morphine potency in cell-based assays is that the concentration of the highly lipophilic fentanyl in the immediate vicinity of the μ receptor may be substantially greater than that in the medium bathing the cells and that such a ligand-concentrating effect is less evident with the much less lipophilic morphine. The idea that lipophilic ligands become concentrated in the aqueous layer just above the intact cell membrane has been demonstrated for another GPCR, the **β_2 adrenoceptor** (Gherbi et al., 2018), this effect was due to the presence of the cell membrane (Figure 1). The higher lipid solubility of fentanyl compared with morphine will mean that more fentanyl may enter the cell membrane, thus leading to higher concentrations of fentanyl than morphine around the μ receptor, even though the concentration of these drugs in the general bathing medium was the same. If such a ligand-concentrating effect was weaker or absent with membrane fragments compared with intact cells, then this could lead to an apparent increase in fentanyl's potency in cell-based assays. The idea that the higher lipid solubility of fentanyl compared with morphine could contribute to apparent anomalous effects of fentanyl is further discussed in Section 4.

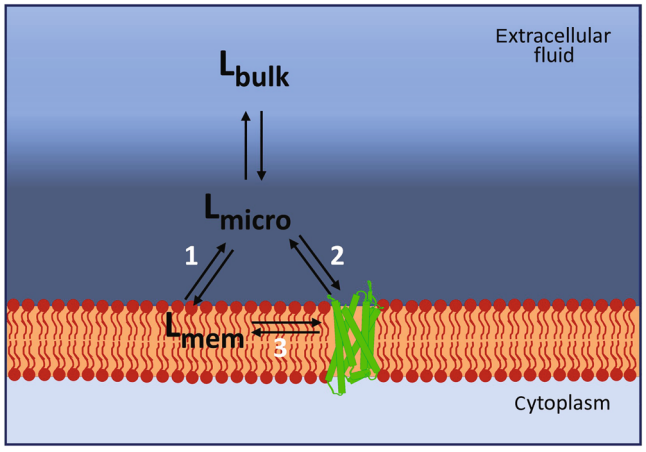

FIGURE 1 Schematic illustrating increased local ligand concentration in the vicinity of the cell membrane and pathways for ligand binding to the receptor: Pathway 1—ligand entering the lipid membrane; Pathway 2—ligand binding to the receptor via the aqueous route; and Pathway 3—ligand diffusing into the orthosteric binding pocket of the receptor from the lipid. Adapted from Gherbi et al. (2018)

It is also possible to estimate the efficacy of fentanyl relative to that of morphine *in vivo*. In one study, morphine or fentanyl was microinjected into the periaqueductal grey of rats after treatment with different doses of the irreversible μ receptor antagonist **β-funaltrexamine (β-FNA)** (Bobeck et al., 2012). It was found that β-FNA treatment produced a similar degree of rightward shift of the concentration–response curves for morphine- and fentanyl-induced analgesia, suggesting that the two agonists have similar efficacy at endogenous μ receptors in this experimental system. Similar effects were observed with β-FNA treatment in pigeon drug discrimination experiments (Barrett et al., 2003). Calculation of agonist operational efficacies in these experiments indicated that fentanyl and morphine had similar efficacy, whereas **methadone**, sufentanil and **etorphine** had significantly higher efficacies. On the other hand, another study of drug discrimination in pigeons reported fentanyl to have slightly higher efficacy than morphine (Morgan & Picker, 1998). Estimates of agonist efficacy for analgesia in thermal nociception in monkeys (Cornelissen et al., 2018) and analgesia for tail flick in rats (Adams et al., 1990) also indicated fentanyl to have slightly higher efficacy than morphine. Finally, in a series of studies (Madia et al., 2012; Pawar et al., 2007; Sirohi et al., 2008), operational analysis was employed to determine the *in vivo* efficacy of various opioid agonists to produce antinociception in mice. They calculated the efficacies (as τ values) of fentanyl and morphine to be 58 and 39, respectively. This reflects the ratio of efficacy values for fentanyl and morphine in [^{35}S]GTPγS experiments using membrane homogenates (Table 1D). Taken together, these studies indicate that as observed with *in vitro* assays, the *in vivo* efficacy of fentanyl relative to morphine is not markedly higher.

Overall, these studies indicate that the affinity of fentanyl for the μ receptor is similar to that of morphine and that although fentanyl's efficacy at the μ receptor is in some cases somewhat higher than that of morphine, it is less than that of high efficacy agonists such as DAMGO and methadone (McPherson et al., 2010). Because methadone has higher efficacy at the μ receptor than fentanyl, yet is not a particularly potent opioid *in vivo* (Schwienteck et al., 2019), then it seems likely that factors other than efficacy contribute to the high potency of fentanyl in cell-based assays and *in vivo*. The discussion of fentanyl's agonist efficacy at the μ receptor should not be viewed as an esoteric exercise of interest only to some molecular pharmacologists. As we describe below, several of the anomalous features of fentanyl's actions *in vivo* are readily explained if fentanyl is considered to be a high efficacy agonist; but is the fact that it exhibits somewhat higher efficacy than morphine in intact cell and *in vivo* assays sufficient to define it as a high efficacy agonist?

3 | FENTANYL BINDING WITHIN THE ORTHOSTERIC BINDING POCKET OF THE μ RECEPTOR

Atomistic molecular dynamics (MD) simulations provide high-resolution detail on the ligand binding pose and receptor residue interactions within the orthosteric binding pocket of GPCRs (Latorraca et al., 2017). Such studies have confirmed the details of the binding pose of morphinan and peptide ligands in the μ receptor originally obtained from agonist and antagonist bound crystal structures (Huang et al., 2015; Manglik et al., 2012) and cryogenic electron microscopy (cryo-EM) studies (Koehl et al., 2018). So far, there have been no structural (crystal or cryo-EM) studies for fentanyls at the μ receptor, but a number of molecular modelling studies have been undertaken, ranging from docking studies (Subramanian et al., 2000) through to more recent atomistic molecular dynamics simulations based on the published crystal structures of the μ receptor (Lipiński et al., 2019). Surprisingly, such studies reveal that *in silico*, fentanyl may interact with the orthosteric binding pocket of the μ receptor in more than one way, that is, with the fentanyl ligand positioned in different orientations in the orthosteric binding pocket. On the other hand, there is currently no evidence that morphinan ligands such as morphine can take up more than one general binding pose in the μ receptor (Kapoor et al., 2017).

Fentanyls are unlike most other opioid ligands in that the protonated nitrogen, which forms a key interaction with Asp147$^{3.32}$ of the μ receptor, is located in the middle of the molecule (Figure 2a). It is probably this central nitrogen along with the elongated and flexible structure of fentanyl that, at least *in silico*, allows it to adopt different orientations in the μ receptor pocket, whilst maintaining the amine–

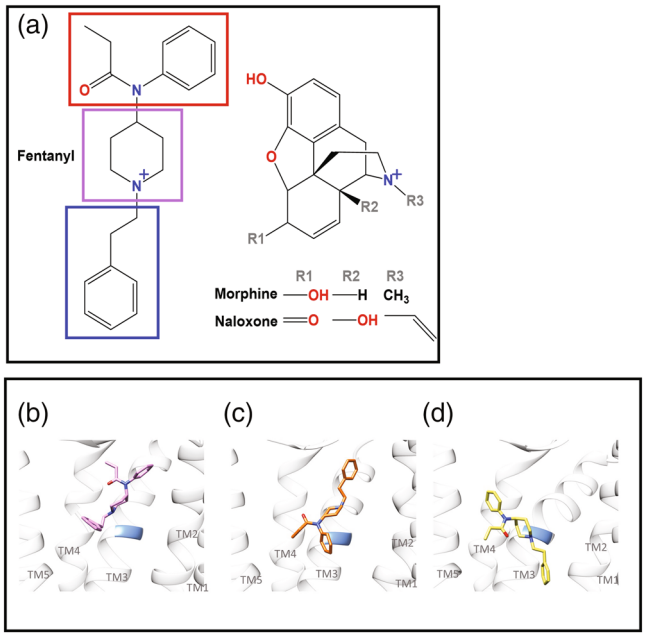

FIGURE 2 (a) Chemical structures of fentanyl, morphine and naloxone. Coloured boxes around fentanyl indicate the chemical moieties within the structure: red—*N*-phenylpropanamide; purple—piperidine ring; and blue—*N*-phenethyl. The three predicted binding poses of fentanyl in the μ receptor orthosteric binding pocket: (b) phenethyl group towards inside of cell, (c) phenethyl group towards outside of cell and (d) phenethyl towards inside of cell but in deeper pose. In each case, the position of Asp147$^{3.32}$ in transmembrane 3 is shown in blue

Asp147$^{3.32}$ interaction. One reported fentanyl orientation is with the phenethyl group positioned towards the intracellular side in the μ receptor pore and the *N*-phenylpropanamide group extended towards the extracellular side of the receptor (Figure 2b; Dosen-Micovic et al., 2006; Ellis et al., 2018; Eshleman et al., 2020; Lipiński et al., 2019; Subramanian et al., 2000). Another orientation is the opposite (180° rotation), with the phenylethyl group positioned towards the extracellular face of the pocket (Figure 2c; de Waal et al., 2020; Huang et al., 2000; Ricarte et al., 2021). Indeed, some studies have observed both stable poses for fentanyl (Jarończyk et al., 2017; Podlewska et al., 2020). However, either structural studies such as cryo-EM or *in vitro* receptor mutation studies will be required to determine which of these poses is the one that occurs *in vivo* or indeed whether both might occur. Although the idea that a ligand can switch orientations and adopt two poses in the binding pocket may intuitively seem unlikely given the normally high specificity of ligand–GPCR interactions, the ability of a ligand to switch between different binding orientations in the orthosteric site has previously been suggested from *in silico* analysis of adenosine binding to the **adenosine A_{2A} receptor** (Sabbadin et al., 2015).

Recently, it has been observed that fentanyl can, in addition to the poses mentioned above, adopt a deeper binding position in the active-state μ receptor (Vo et al., 2021; Figure 2d). In this pose, the phenethyl group extends down to the allosteric sodium site, disruption of which may be a mechanism for μ receptor activation (Sutcliffe et al., 2017). Whether or not these upper and lower poses of fentanyl in the binding site reflect different stages of binding of this ligand leading to receptor activation remains to be determined. As yet a similar pose for fentanyl with the *N*-phenylpropanamide group deep in the binding pocket has not been reported, neither has a deep binding pose for other opioid ligands such as morphine been reported.

In summary, the mode of binding of fentanyl (and probably all fentanyl-related molecules) in the μ receptor orthosteric binding pocket may be more complex than for other opioid ligands, which in part may result from the long, flexible nature of the fentanyl molecule. Currently, three fentanyl binding poses are evident from *in silico* studies, which if correct would indeed make fentanyl anomalous compared with other opioid agonists. Structural studies such as cryo-EM or *in vitro* mutation studies will however be needed to clarify which of these ligand orientations are relevant to fentanyl interaction with the μ receptor *in vivo*.

4 | FENTANYL LIPID PATHWAY

Fentanyl has high lipid solubility compared with many other opioid agonists (XlogP of 3.94 for fentanyl and 0.49 for morphine; https://www.guidetopharmacology.org/). This explains the ability of fentanyl to rapidly enter the CNS with consequent fast onset of centrally mediated effects relative to morphine and heroin (Hill et al., 2020). In addition, as discussed below, high lipid solubility may also be important with regard to the molecular mode of action of fentanyl.

In the field of molecular modelling, coarse-grained molecular dynamics simulations can be used to overcome the sampling issues of all-atom molecular dynamics and can enable the rare event of ligand binding to be visualized *in silico*. We have used coarse-grained molecular dynamics simulations of membrane-embedded μ receptor to investigate the interaction of fentanyl and morphine with the receptor (Sutcliffe et al., 2021). These simulations showed first that fentanyl, even in its protonated form, can penetrate the cell membrane to a significant depth, whereas morphine does not (Figure 3). This probably reflects the relative lipid solubilities of the two ligands. The movement of fentanyl in and out of the membrane in the vicinity of the μ receptor may increase the probability of fentanyl binding to the receptor simply due to the availability of fentanyl immediately above the receptor (Pathway 2 in Figure 1). Second, and most interestingly, following entry into the lipid membrane surrounding the μ receptor, we observed that fentanyl could penetrate the side of the μ receptor through a pore between transmembranes 6 and 7 and then enter the orthosteric pocket of the receptor (Figure 3a and Pathways 1 and 3 in Figure 1). In comparison, we only observed morphine to enter the orthosteric binding pocket of the μ receptor via the well-documented aqueous route from above the receptor (Figure 3b; Dror et al., 2011;

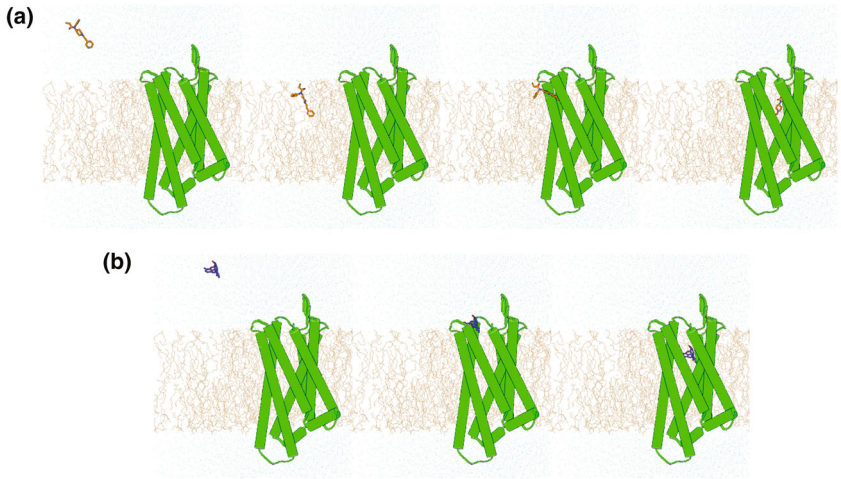

FIGURE 3 The lipid binding pathway for fentanyl identified by coarse-grained molecular dynamics simulations. (a) A molecule of fentanyl approaches and then enters the lipid membrane, before entering the μ receptor through a pore between transmembrane domains 6 and 7 of the receptor and eventually entering the orthosteric binding pocket. (b) A molecule of morphine approaches and then enters the μ receptor from above the receptor (the aqueous route)

Schneider et al., 2015, 2016). We further employed umbrella sampling and free energy calculations to demonstrate that fentanyl would be able to access the binding pocket by both aqueous and lipid routes (Sutcliffe et al., 2021). It may be that, in addition to high lipid solubility, the flexible nature of fentanyl's structure with six rotatable bonds is an essential property that enables fentanyl to penetrate the pore for entry into the receptor via this lipid route. However, as with the binding orientation of fentanyl in the orthosteric binding pocket discussed above, mutation experiments will be required to verify that this entry route to the μ receptor via a lipid pathway does actually occur.

The entry of fentanyl into the μ receptor from the lipid pathway has not been shown for any other opioid receptor ligands, but the lower resolution of the coarse-grained approach makes it highly likely that other fentanyls such as carfentanil will behave in the same way, whereas morphinan ligands, such as oxycodone and **naloxone**, with lower lipid solubility will not. Indeed, a major reason why the lipid route is probably not accessible to morphinan ligands is that, even if they could pass through a pore between transmembrane domains, they are not sufficiently lipid soluble to ever reach a high enough concentration around the pore to enter via this route. The entry of lipophilic ligands from the membrane through the transmembrane domains into the orthosteric pocket has previously been suggested for other GPCRs, for example, the endogenous cannabinoid **2-AG** at the **CB_2 receptor** (Hurst et al., 2010), the endogenous ligand at the **sphingosine-1-phosphate receptor ($S1P_1$**; Hanson et al., 2012) and the antagonist **vorapaxar** at **PAR1** (Bokoch et al., 2018). The significance of the present observation with fentanyl at the μ receptor is that it may explain a number of other anomalous findings that are discussed in this review. Thus, the enhanced potency of fentanyl relative to morphine in intact cells could occur because not only does the concentration of fentanyl increase around the μ receptor as the drug concentrates in the lipid membrane, but this high concentration of fentanyl in the membrane also makes the drug much more likely to enter the receptor via the transmembrane domains (Pathway 3 in Figure 1).

Whatever the case, this unique mode of entry of fentanyl into the μ receptor, if confirmed as a route of ligand entry to the μ receptor *in vitro* or *in vivo*, does mark fentanyl as having a novel receptor pharmacology, which may well contribute to other anomalous properties of the drug discussed in this review.

5 | FENTANYL, ARRESTIN SIGNALLING BIAS AND RESPIRATORY DEPRESSION

For GPCRs, ligand bias can be regarded as the propensity of an agonist, relative to a reference agonist, to selectively activate one downstream signalling pathway over another (Conibear & Kelly, 2019; Kelly, 2013). In most cases, the signalling pathways studied with regard to bias are those mediated by G proteins and arrestins. With regard to fentanyl-induced cell signalling and potential bias, our initial studies (McPherson et al., 2010; Rivero et al., 2012) did not find fentanyl displaying bias between G protein and arrestin pathways. More recently, it was reported that fentanyl is arrestin biased (Schmid et al., 2017), but in that study fentanyl was reported to be arrestin biased when G-protein activity was assessed by [^{35}S]GTPγS binding, yet was oppositely suggested to be G protein biased when G-protein activity was assessed by inhibition of cAMP accumulation. Other recent studies have concluded either that fentanyl displays the same moderate G-protein bias as morphine relative to DAMGO (Crowley et al., 2020) or that relative to morphine, fentanyl appeared to be either G protein or arrestin biased depending upon the type of bias calculation employed (Burgueño et al., 2017). In a comprehensive study covering five different assays of G-protein activation and compared with arrestin recruitment, fentanyl was found not to be arrestin biased; rather, it was unbiased in four G-protein activation assays and showed moderate G-protein bias in the fifth assay of GIRK activation as a measure of G-protein activity (Gillis, Gondin, et al., 2020). Overall, there is little evidence to support the idea of fentanyl being arrestin biased or indeed consistently biased in any way. This is important as the assumption that fentanyl is arrestin biased is repeated in other studies as a basis to understand fentanyl's pharmacological effects (e.g. de Waal et al., 2020; Mori et al., 2017). Instead, fentanyl is best regarded as unbiased and so in this aspect is not anomalous relative to standard agonists such as DAMGO or morphine.

Respiratory depression is the major cause of death in opioid overdose and results from activation of the μ receptor rather than other opioid receptor subtypes (Matthes et al., 1998). We know this to be true for fentanyl because depression of respiration is not observed in μ receptor knockout mice (Hill et al., 2020; Schmid et al., 2017). The opioid field and the development of new opioid analgesic drugs have, in recent years, been heavily influenced by the notion that opioid agonists acting at the μ receptor induce analgesia through G-protein signalling and induce respiratory depression through arrestin signalling. This hypothesis arose from the observation that in β-arrestin2 knockout mice, morphine analgesia was enhanced, whereas respiratory depression was greatly attenuated (Raehal et al., 2005). The same group subsequently reported that fentanyl, which they suggested showed β-arrestin2 bias over G-protein signalling (but see discussion above), was more likely to induce respiratory suppression at lower doses compared with morphine (Schmid et al., 2017). However, the idea that respiratory depression by opioids such as fentanyl is mediated by arrestin signalling has recently been refuted. First, three independent research groups collaborated to show that respiratory depression induced by morphine and fentanyl was not attenuated in β-arrestin2 knockout mice (Kliewer et al., 2020), a direct contradiction of the initial report cited above. Second, using mice that expressed a mutated form of the μ receptor, in which the COOH-terminal serine and threonine phosphorylation sites had been mutated to alanine (11S/T-A mice), thereby preventing phosphorylation by GRKs and arrestin binding, it was observed that both morphine and fentanyl still depressed respiration (Kliewer et al., 2019). There is therefore now considerable doubt about the validity of the hypothesis that respiratory depression by fentanyl and other μ receptor agonists results from arrestin signalling (Gillis, Gondin, et al., 2020; see also Gillis, Kliewer,

et al., 2020) with the evidence instead indicating that G-protein signalling largely mediates this effect (Montandon et al., 2016).

In summary, fentanyl should not be regarded as anomalous with regard to signalling bias as the evidence shows that it is not consistently biased in any direction. This together with the recent finding that β-arrestin2 does not mediate fentanyl-induced respiratory depression therefore provides no basis on which to propose that fentanyl is unusually effective at causing respiratory depression because it selectively engages arrestin signalling.

6 | TOLERANCE TO FENTANYL AND CROSS-TOLERANCE WITH OTHER OPIOIDS

On repeated or prolonged exposure to opioid agonists, tolerance may develop whereby the response produced by the same dose declines, to maintain the same level of response the dose must be increased (Williams et al., 2013). The extent to which tolerance develops varies with the pharmacological properties of the opioid drug and between different behavioural effects. In animal models, tolerance develops rapidly to the acute antinociceptive effect of morphine. In contrast, although tolerance to the respiratory depressant effects of morphine and methadone does develop, it is slower to develop than the tolerance to their antinociceptive effects (Hill et al., 2016). Indeed, using a single daily dosing regimen for morphine, Paronis and Woods (1997) failed to observe tolerance to respiratory depression in rhesus monkeys. White and Irvine (1999) have suggested that in man, tolerance develops more rapidly and to a greater extent to the desired (rewarding) effects of heroin than to respiratory depression.

An important question relating to fentanyl overdose is whether prolonged heroin use and the resulting induction of tolerance to opioid respiratory depression provide protection to fentanyl, that is, does heroin use induce high levels of cross-tolerance to fentanyl. People using illicit fentanyls may do so unknowingly because the heroin has been cut with fentanyls or they may switch from heroin or prescription opioids to fentanyls due to changes in the availability of different illicit opioids. In both instances, the degree of protection afforded by their previous opioid use will be important. Again, if tolerance results from a reduction in the number of functional μ receptors, then following tolerance induced by prolonged heroin use there may be less cross-tolerance to fentanyl because, if it is indeed a higher efficacy agonist (but see discussion above), fentanyl would have a greater receptor reserve and thus need to occupy fewer receptors to produce the same level of response. Therefore the loss of some functional receptors would impact fentanyl less. Indeed, in our study of morphine–fentanyl cross-tolerance to respiratory depression, we observed that prolonged morphine treatment produced a lower level of cross-tolerance to fentanyl than to morphine itself (Hill et al., 2020).

Numerous drug discrimination studies in non-human primates, rodents and pigeons have demonstrated the ability of fentanyl to substitute fully for morphine and other μ receptor agonists (Morgan & Picker, 1996; Obeng et al., 2021; Paronis & Holtzman, 1994; Platt et al., 2001; Walentiny et al., 2019; Walker et al., 1997). Similarly, morphine can substitute for fentanyl in rats trained to discriminate fentanyl (Emmett-Oglesby et al., 1988; Schwienteck et al., 2019). In such drug discrimination studies, the potency of fentanyl has been reported to be 40- to 200-fold higher than that of morphine, which is similar to their relative potency to produce antinociception (Suzuki & El-Haddad, 2017). Although in such tests repeated dosing with morphine has been reported to produce cross-tolerance to fentanyl (Emmett-Oglesby et al., 1988; Walker et al., 1997), the degree of cross-tolerance to fentanyl may be less than the tolerance to morphine itself (Paronis & Holtzman, 1994). Indeed, in one study, Hughes et al. (1996) failed to observe cross-tolerance between morphine and fentanyl.

Conversely, if users regularly take fentanyl, would they develop significant levels of tolerance? Tolerance induction is a function of dose, agonist efficacy and biological half-life, that is, it is a function of both the extent and duration of receptor activation. To study the influence of efficacy alone, Madia et al. (2009) and Sirohi et al. (2008) used infusions of opioid agonists and reported that fentanyl infusion induced less tolerance than infusions of equiactive analgesic doses of morphine or oxycodone. A number of other studies have also reported that following continuous infusion, higher efficacy opioids produce less analgesia tolerance at equieffective doses than lower efficacy agonists (Duttaroy & Yoburn, 1995; Kumar et al., 2008; Paronis & Holtzman, 1992; Pawar et al., 2007; Sirohi et al., 2008; Stevens & Yaksh, 1989). However, it is possible that repeated drug administrations may induce different levels of tolerance due to the peaks and troughs in drug levels that will occur.

Any discussion of the level of tolerance induced by fentanyl would certainly be easier if we fully understood its agonist intrinsic efficacy (see discussion above). In studies of antinociception, it was observed that the efficacy of fentanyl was greater than that of morphine (Madia et al., 2009; Pawar et al., 2007; Sirohi et al., 2008), but it is unclear whether the difference is sufficient to describe fentanyl as having 'high' efficacy and morphine as having 'low' efficacy. Differential development of tolerance between true high and low efficacy agonists would be predicted if tolerance is a direct consequence of receptor occupancy and subsequent loss of receptor function (desensitization) as with equiactive doses of the agonists. Lower receptor occupancy would be required for a high efficacy agonist to produce the same response as a low efficacy agonist and thus resulting in less receptor desensitization. Furthermore, given that *in vivo* the duration of action of fentanyl is shorter than that of morphine, as fentanyl is sequestered into fat, then if a repeated dosing protocol was used to mimic illicit opioid use it might be predicted that not only would fewer receptors be occupied by fentanyl but the duration of receptor occupancy would also be less, further reducing the degree of tolerance development induced by fentanyl. In behavioural tests, it has been reported that even continuous fentanyl treatment did not produce tolerance to its discriminative stimulus effects nor cross-tolerance to morphine (Paronis & Holtzman, 1994). However, others have reported that with higher doses and frequent or continuous administration of fentanyl, tolerance to its discriminative stimulus effects and cross-

tolerance to those of morphine can be observed (Emmett-Oglesby et al., 1988, 1989; Walker et al., 1997).

It is possible therefore that with illicit drug use, prior intermittent use of other opioid agonists such as heroin may not convey significant tolerance to fentanyl and also that intermittent fentanyl use by itself may not induce significant tolerance. Although multiple molecular mechanisms have been proposed to underlie μ receptor desensitization and the development of tolerance to different opioid agonists (for comprehensive review, see Williams et al., 2013), it might be expected that symmetric cross-tolerance would be evident even if the receptor is desensitized by different molecular mechanisms by different agonists. Further studies on tolerance to the respiratory depressant effects of fentanyls and cross-tolerance with other opioid agonists are definitely required as differential tolerance represents a crucial risk factor in fentanyl overdose deaths.

7 | MUSCLE RIGIDITY

Another anomaly of the fentanyls is their ability in humans to produce skeletal muscle rigidity, whereas other opioids such as heroin seem to have little propensity to produce such an effect even in overdose. Skeletal muscle rigidity is a well-established clinical complication of administering high intravenous doses of medicinal fentanyls (Comstock et al., 1981; Grell et al., 1970; Jaffe & Ramsey, 1983). The onset and degree of rigidity is directly correlated with the dose and speed of injection (Grell et al., 1970). Although rigidity induced by fentanyls can be observed in skeletal muscles throughout the body, it is the glottic and supraglottic airway obstruction (Abrams et al., 1996; Bennett et al., 1997), along with sustained contracture of the intercostal muscles and diaphragm (Benthuysen et al., 1986), that produces what is described colloquially as 'wooden chest syndrome' that reduces the ability to breathe. Respiratory muscle rigidity induced by fentanyls is reported to be reversed by the opioid antagonist, naloxone (Ackerman et al., 1990; Çoruh et al., 2013; Dewhirst et al., 2012; Vaughn & Bennett, 1981), but in general anaesthetic procedures, it is normally counteracted by the administration of a neuromuscular blocking agent and artificial ventilation in order to retain fentanyl-induced analgesia (Comstock et al., 1981; Jaffe & Ramsey, 1983). In overdose deaths involving illicit fentanyls, upper airway obstruction and respiratory muscle rigidity are likely to be significant factors (Burns et al., 2016).

In experimental animal studies, opioid-induced muscle rigidity has been observed in both respiratory and non-respiratory muscle groups and results from μ receptor activation in the brain, resulting in enhanced asynchronous motor output that can be recorded either as an increase in peripheral motor nerve activity (Willette et al., 1982) or as an increase in electromyographic (EMG) activity (Blasco et al., 1986; Campbell et al., 1995; Lui et al., 1989; Yang et al., 1992). Muscle rigidity is not only produced by fentanyls, **etonitazene** has also been reported to increase EMG activity (Rackam, 1980) as have the opioid peptide agonists DAMGO and **β-endorphin**, but as these peptides have low lipid solubility and do not penetrate the blood–brain barrier, their effect on muscle stiffness is only observed when they are injected directly into the brain (Slater & Starkie, 1987; Vankova et al., 1996; Widdowson et al., 1986). It has been suggested that because the fentanyls have high lipid solubility, they are more likely to produce rigidity than other as less lipid soluble opioids, such as morphine and heroin, as they will permeate the brain rapidly, resulting in relatively high peak brain concentrations following peripheral administration (Bowdle, 1998). We have used an *in situ* perfused heart–brainstem preparation (Levitt et al., 2015; Paton, 1996) to measure rat respiratory muscle EMG activity (Pearson et al., 2005) at steady-state drug concentrations and observed that the ability of opioids (fentanyls and non fentanyls) to produce muscle stiffness is a function of agonist efficacy (Cavallo et al., 2021). Thus, it is likely that all opioid agonists that have sufficient lipid solubility to penetrate the brain will have a reasonably high efficacy to induce rigidity.

In rodents, muscle rigidity induced by fentanyls can be blocked by pretreatment with the α_1 adrenoceptor antagonist **prazosin** (Tsou et al., 1989). It has been suggested that fentanyls could bind to and activate **α_1 adrenoceptors** (Torralva & Janowsky, 2019). However, fentanyl and carfentanil have micromolar affinity for binding to α_1 adrenoceptors and in functional assays, they act as α_1 adrenoceptor antagonists not as agonists (Torralva et al., 2020). Furthermore, clinical case reports indicate that chest wall rigidity following fentanyl administration can be reversed by naloxone (Ackerman et al., 1990; Çoruh et al., 2013; Dewhirst et al., 2012; Vaughn & Bennett, 1981) and in rodent studies, fentanyl-induced muscle stiffness was reversed by microinjection of the quaternary opioid antagonist methylnaloxonium into discrete brain regions (Blasco et al., 1986; Weinger et al., 1991). These observations are more compatible with fentanyl activating the μ receptor rather than binding to α_1 adrenoceptors.

Another possibility is that fentanyls activate descending noradrenergic projections from the locus coeruleus to release **noradrenaline** onto α_1 adrenoceptors in the ventral horn of the spinal cord (Fu et al., 1997; Lui et al., 1993). However, the predominant effect of μ receptor agonists on locus coeruleus neurones is inhibitory through activation of G protein-activated inwardly rectifying potassium channels (Pepper & Henderson, 1980; Travagli et al., 1995; Williams et al., 1982). There have been two reports of fentanyl exciting locus coeruleus neurones (Pan et al., 2004; Rasmussen & Jacobs, 1985), but the effects observed were modest, showing at most a doubling of firing frequency, and are likely to be below the level required to produce profound muscle rigidity. There is a need for further studies to validate the role of locus coeruleus excitation in the respiratory muscle rigidity and airway obstruction induced by fentanyls and other opioid agonists in rodents and of the applicability of this phenomenon to the situation in humans. It raises the possibility that the combination of an α_1 adrenoceptor antagonist plus an opioid antagonist might be a better antidote in fentanyl overdose that an opioid antagonist such as naloxone alone (Torralva & Janowsky, 2019).

8 | NALOXONE REVERSAL

In recent years, there have been numerous reports suggesting that more naloxone, in the form of multiple or higher doses, is required to reverse overdoses involving fentanyls compared with overdoses involving other opioids such as heroin (Mahonski et al., 2020; Mayer et al., 2018; Moe et al., 2020; Moss & Carlo, 2019; Somerville et al., 2017; Sutter et al., 2017). In the treatment of opioid overdose, the antagonist, usually naloxone, is administered to counteract the effect of the drug causing the overdose; that is, it is administered after the response to the agonist has developed. This is not the same as in pharmacological studies of antagonism (e.g. in studies determining antagonist pA_2 values) where the antagonist is administered first and allowed to come to equilibrium with the receptors before the agonist is administered. Below, we focus on studies in which the antagonist is administered after the opioid agonist.

One reason why more naloxone may be required to reverse overdoses involving fentanyls might be that, given the high potency of many fentanyls *in vivo*, it would be relatively easy to 'over' overdose, that is, inject a much too high dose of a fentanyl (Rzasa Lynn & Galinkin, 2018). To investigate naloxone sensitivity, we chose to examine the ability of naloxone to reverse respiratory depression in response to equiactive, sublethal doses of morphine and fentanyl (Hill et al., 2020). By allowing the effect of both drugs to reach steady state before administering naloxone, we also avoided any possible pharmacokinetic complications arising from the more rapid onset of action of fentanyl. What we observed was that it required a 10-fold higher dose of naloxone to reverse fentanyl than morphine (Figure 4). This finding demonstrates that even relatively low doses of fentanyl exhibit reduced sensitivity to reversal by naloxone. This lower sensitivity to naloxone was not due to fentanyl acting on other, less naloxone-sensitive, opioid receptor types (i.e. the **δ receptor** or the **κ receptor**) to depress respiration as fentanyl did not depress respiration in μ receptor knockout mice. Interestingly, the lipophilic opioid antagonist, **diprenorphine** (Revivon®), was equieffective in reversing fentanyl and morphine depression of respiration (Hill et al., 2020). These observations suggest that there may be a non-equilibrium interaction between naloxone and fentanyl at the μ receptor as, under equilibrium conditions, naloxone, a competitive antagonist, should reverse both agonists equally irrespective of their affinity and efficacy at the receptor. Indeed, a similar ability of competitive μ receptor antagonists to antagonize morphine and fentanyl has been observed in studies of various opioid responses where the antagonist is administered first and pA_2 values determined (Negus, Burke, et al., 1993; Pitts et al., 1996; Walker et al., 1994). The irreversible μ receptor antagonist, methocinnamox, has been observed to prevent respiratory depression and antinociception induced by fentanyl in rats (Gerak et al., 2019). It remains to be determined whether, when administered after the opioid agonist, methocinnamox shows differential antagonism between heroin/morphine and fentanyl.

It has also been suggested that muscle rigidity is not reversed by naloxone (Davis & Behm, 2020), but of the references cited in that review to support that view, none studied the effects of naloxone; rather, they examined the possible involvement of noradrenergic mechanisms, which may in fact be downstream of μ receptor activation by fentanyls (see above). There is in fact good evidence for opioid antagonists, when administered in adequate

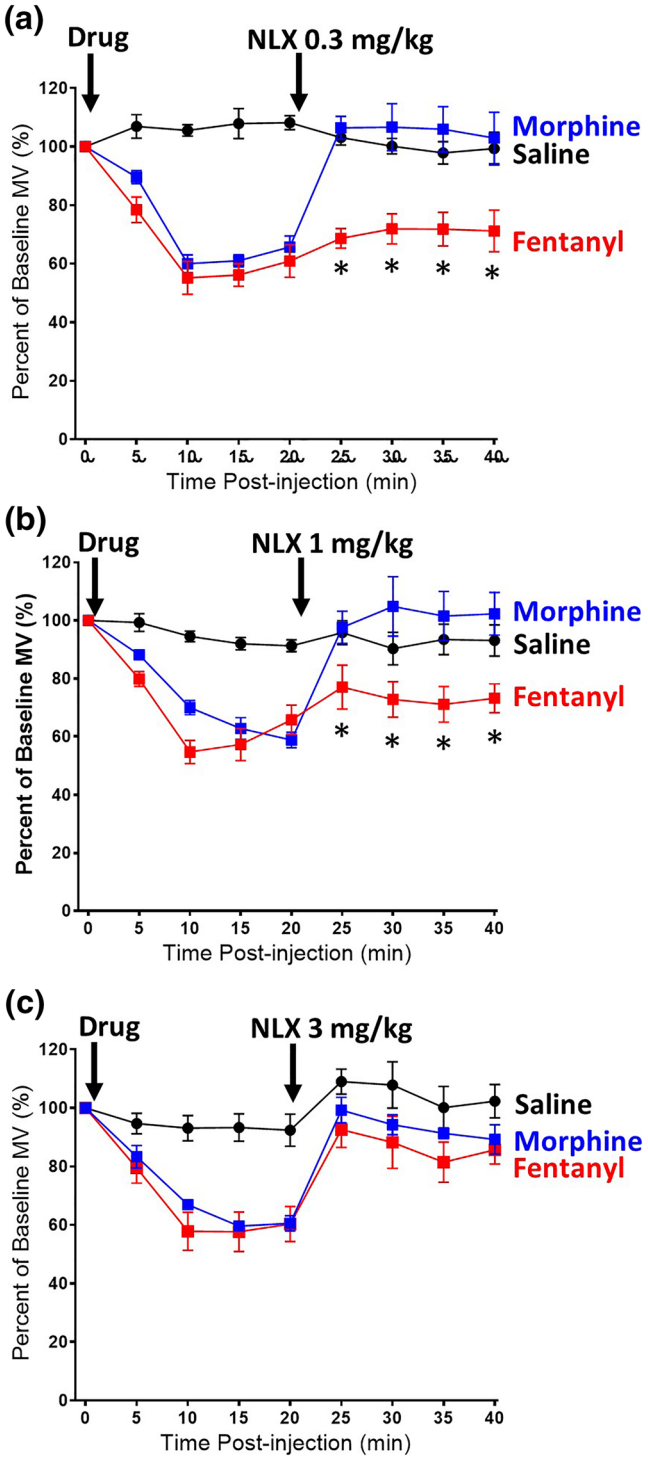

FIGURE 4 A higher concentration of naloxone is required to reverse respiratory depression by fentanyl than by morphine. Data are from Hill et al. (2020) in which respiration was monitored in freely moving mice by plethysmography and drugs injected intraperitoneally

doses, being able to reverse muscle rigidity induced by fentanyls in animal studies (Blasco et al., 1986; Negus, Pasternak, et al., 1993; Weinger et al., 1991, 1995; Weinger & Taurek, 1990; Wilcox & Levitt, 1978) and wooden chest syndrome in humans (Ackerman et al., 1990; Çoruh et al., 2013; Dewhirst et al., 2012; Vaughn & Bennett, 1981). Indeed, Negus, Pasternak, et al. (1993) reported that fentanyl-induced antinociception and muscle rigidity were equally reversed by naloxone. The effect of naloxone on upper airway obstruction by fentanyls does however still need to be determined.

9 | CONCLUSIONS

Does fentanyl exhibit anomalous pharmacological properties? Do such properties contribute to overdose deaths due to fentanyl? The aim of this review was to examine the pharmacological evidence concerning these questions and we suggest that on balance, the answer to both questions is 'yes'. An important property of fentanyl that is likely to contribute to what might be regarded as anomalous behaviour is its high lipid solubility. This physicochemical property, apart from causing the very rapid movement of fentanyl into the brain from the periphery, may underlie some of the anomalous properties discussed here. Further experimentation is however now required to verify this. In the longer term, it is hoped that such studies will facilitate new approaches that will significantly reduce the risk of death from the illicit use of fentanyl.

9.1 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019).

ACKNOWLEDGEMENTS

This study was supported by UKRI Medical Research Council (MRC) grant (MR/S010890/1) as well as UKRI Biotechnology and Biological Sciences Research Council (BBSRC) studentships awarded to K.S. and N.R.-G. under the South West Biosciences (SWBio) DTP scheme (Grant BB/J014400/1).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

ORCID

Eamonn Kelly https://orcid.org/0000-0002-9183-3993
Katy Sutcliffe https://orcid.org/0000-0002-8971-440X
Graeme Henderson https://orcid.org/0000-0001-6050-0048

REFERENCES

Abrams, J. T., Horrow, J. C., Bennett, J. A., Van Riper, D. F., & Storella, R. J. (1996). Upper airway closure: A primary source of difficult ventilation with sufentanil induction of anesthesia. *Anesthesia & Analgesia*, *83*(3), 629–632. https://doi.org/10.1097/00000539-199609000-00034

Ackerman, W. E., Phero, J. C., & Theodore, G. T. (1990). Ineffective ventilation during conscious sedation due to chest wall rigidity after intravenous midazolam and fentanyl. *Anesthesia Progress*, *37*(1), 46–48. PMID: 2077987

Adams, J. U., Paronis, C. A., & Holtzman, S. G. (1990). Assessment of relative intrinsic activity of mu-opioid analgesics in vivo by using beta-funaltrexamine. *Journal of Pharmacology and Experimental Therapeutics*, *255*(3), 1027–1032. PMID: 2175793

Alexander, S. P. H., Christopoulos, A., Davenport, A. P., Kelly, E., Mathie, A., Peters, J. A., Veale, E. L., Armstrong, J. F., Faccenda, E., Harding, S. D., Pawson, A. J., Sharman, J. L., Southan, C., Davies, J. A., & CGTP Collaborators. (2019). The Concise Guide to PHARMACOLOGY 2019/20: G protein-coupled receptors. *British Journal of Pharmacology*, *176*(S1), S21–S141. https://doi.org/10.1111/bph.14748

Barrett, A. C., Smith, E. S., & Picker, M. J. (2003). Use of irreversible antagonists to determine the relative efficacy of μ-opioids in a pigeon drug discrimination procedure: Comparison of β-funaltrexamine and clocinnamox. *Journal of Pharmacology and Experimental Therapeutics*, *305*(3), 1061–1070. https://doi.org/10.1124/jpet.102.047068

Bennett, J. A., Abrams, J. T., Van Riper, D. F., & Horrow, J. C. (1997). Difficult or impossible ventilation after sufentanil-induced anesthesia is caused primarily by vocal cord closure. *Anesthesiology*, *87*(5), 1070–1074. https://doi.org/10.1097/00000542-199711000-00010

Benthuysen, J. L., Smith, N. T., Sanford, T. J., Head, N., & Dec-Silver, H. (1986). Physiology of alfentanil-induced rigidity. *Anesthesiology*, *64*(4), 440–446. https://doi.org/10.1097/00000542-198604000-00005

Black, J. W., & Leff, P. (1983). Operational models of pharmacological agonism. *Proceedings of the Royal Society of London B: Biological Sciences*, *220*(1219), 141–162. https://doi.org/10.1098/rspb.1983.0093

Blasco, T. A., Lee, D., Amalric, M., Swerdlow, N. R., Smith, N. T., & Koob, G. F. (1986). The role of the nucleus raphe pontis and the caudate nucleus in alfentanil rigidity in the rat. *Brain Research*, *386*(1–2), 280–286. https://doi.org/10.1016/0006-8993(86)90164-2

Bobeck, E. N., Haseman, R. A., Hong, D., Ingram, S. L., & Morgan, M. M. (2012). Differential development of antinociceptive tolerance to morphine and fentanyl is not linked to efficacy in the ventrolateral periaqueductal gray of the rat. *The Journal of Pain*, *13*(8), 799–807. https://doi.org/10.1016/j.jpain.2012.05.005

Bokoch, M. P., Jo, H., Valcourt, J. R., Srinivasan, Y., Pan, A. C., Capponi, S., Grabe, M., Dror, R. O., Shaw, D. E., DeGrado, W. F., & Coughlin, S. R. (2018). Entry from the lipid bilayer: A possible pathway for inhibition of a peptide G protein-coupled receptor by a lipophilic small molecule. *Biochemistry*, *57*(39), 5748–5758. https://doi.org/10.1021/acs.biochem.8b00577

Bowdle, T. A. (1998). Adverse effects of opioid agonists and agonist-antagonists in anaesthesia. *Drug Safety*, *19*(3), 173–189. https://doi.org/10.2165/00002018-199819030-00002

Burgueño, J., Pujol, M., Monroy, X., Roche, D., Varela, M. J., Merlos, M., & Giraldo, J. (2017). A complementary scale of biased agonism for

agonists with differing maximal responses. *Scientific Reports*, *7*(1), 15389. https://doi.org/10.1038/s41598-017-15258-z

Burns, G., DeRienz, R. T., Baker, D. D., Casavant, M., & Spiller, H. A. (2016). Could chest wall rigidity be a factor in rapid death from illicit fentanyl abuse? *Clinical Toxicology (Philadelphia, Pa.)*, *54*(5), 420–423. https://doi.org/10.3109/15563650.2016.1157722

Campbell, C., Weinger, M. B., & Quinn, M. (1995). Alterations in diaphragm EMG activity during opiate-induced respiratory depression. *Respiratory Physiology*, *100*(2), 107–117. https://doi.org/10.1016/0034-5687(94)00119-k

Cavallo, D., Kelly, E., Henderson, G., & Abdala Sheikh, A. P. (2021). The ability of fentanyls and other opioids to increase EMG amplitude in respiratory muscles correlates with their agonist efficacy. *The FASEB Journal*, *35*(S1). https://doi.org/10.1096/fasebj.2021.35.S1.03977

Chan, K., Brodsky, M., Davis, T., Franklin, S., Inturrisi, C. E., & Yoburn, B. C. (1995). The effect of the irreversible μ-opioid receptor antagonist clocinnamox on morphine potency, receptor binding and receptor mRNA. *European Journal of Pharmacology*, *287*(2), 135–143. https://doi.org/10.1016/0014-2999(95)00488-2

Clark, M. J., Furman, C. A., Gilson, T. D., & Traynor, J. R. (2006). Comparison of the relative efficacy and potency of μ-opioid agonists to activate $G\alpha_{i/o}$ proteins containing a pertussis toxin-insensitive mutation. *Journal of Pharmacology and Experimental Therapeutics*, *317*(2), 858–864. https://doi.org/10.1124/jpet.105.096818

Comstock, M. K., Carter, J. G., Moyers, J. R., & Stevens, W. C. (1981). Rigidity and hypercarbia associated with high dose fentanyl induction of anesthesia. *Anesthesia & Analgesia*, *60*(5), 362–363. PMID: 7194606

Conibear, A. E., & Kelly, E. (2019). A biased view of μ-opioid receptors? *Molecular Pharmacology*, *96*(5), 542–549. https://doi.org/10.1124/mol.119.115956

Cornelissen, J. C., Obeng, S., Rice, K. C., Zhang, Y., Negus, S. S., & Banks, M. L. (2018). Application of receptor theory to the design and use of fixed-proportion μ-opioid agonist and antagonist mixtures in rhesus monkeys. *Journal of Pharmacology and Experimental Therapeutics*, *365*(1), 37–47. https://doi.org/10.1124/jpet.117.246439

Çoruh, B., Tonelli, M. R., & Park, D. R. (2013). Fentanyl-induced chest wall rigidity. *Chest*, *143*(4), 1145–1146. https://doi.org/10.1378/chest.12-2131

Crowley, R. S., Riley, A. P., Alder, A. F., Anderson, R. J. 3rd, Luo, D., Kaska, S., Maynez, P., Kivell, B. M., & Prisinzano, T. E. (2020). Synthetic studies of neoclerodane diterpenes from Salvia divinorum: Design, synthesis, and evaluation of analogues with improved potency and G-protein activation bias at the μ-opioid receptor. *ACS Chemical Neuroscience*, *11*(12), 1781–1790. https://doi.org/10.1021/acschemneuro.0c00191

Dasgupta, P., Günther, T., Reinscheid, R. K., Zaveri, N. T., & Schulz, S. (2021). Rapid assessment of G protein signaling of four opioid receptors using a real-time fluorescence-based membrane potential assay. *European Journal of Pharmacology*, *890*, 173640. https://doi.org/10.1016/j.ejphar.2020.173640

Davis, M. P., & Behm, B. (2020). Reasons to avoid fentanyl. *Annals of Palliative Medicine*, *9*(2), 611–624. https://doi.org/10.21037/apm.2020.01.12

de Waal, P. W., Shi, J., You, E., Wang, X., Melcher, K., Jiang, Y., Xu, E. X., & Dickson, B. M. (2020). Molecular mechanisms of fentanyl mediated β-arrestin biased signaling. *PLoS Computational Biology*, *16*(4), e1007394. https://doi.org/10.1371/journal.pcbi.1007394

Dewhirst, E., Naguib, A., & Tobias, J. D. (2012). Chest wall rigidity in two infants after low-dose fentanyl administration. *Pediatric Emergency Care*, *28*(5), 465–468. https://doi.org/10.1097/PEC.0b013e3182535a2a

Dosen-Micovic, L., Ivanovic, M., & Vuk Micovic, V. (2006). Steric interactions and the activity of fentanyl analogs at the μ-opioid receptor. *Bioorganic and Medicinal Chemistry*, *14*(9), 2887–2895. https://doi.org/10.1016/j.bmc.2005.12.010

Dror, R. O., Pan, A. C., Arlow, D. H., Borhani, D. W., Maragakis, P., Shan, Y., Xu, H., & Shaw, D. E. (2011). Pathway and mechanism of drug binding to G-protein-coupled receptors. *Proceedings of the National Academy of Science U S A*, *108*(32), 13118–13123. https://doi.org/10.1073/pnas.1104614108

Duttaroy, A., & Yoburn, B. C. (1995). The effect of intrinsic efficacy on opioid tolerance. *Anesthesiology*, *82*(5), 1226–1236. https://doi.org/10.1097/00000542-199505000-00018

Ellis, C. R., Kruhlak, N. L., Kim, M. T., Hawkins, E. G., & Stavitskaya, L. (2018). Predicting opioid receptor binding affinity of pharmacologically unclassified designer substances using molecular docking. *PLoS ONE*, *13*(5), e0197734. https://doi.org/doi/10.1371/journal.pone.0197734

Emmerson, P. J., Clark, M. J., Mansour, A., Akil, H., Woods, J. H., & Medzihradsky, F. J. (1996). Characterization of opioid agonist efficacy in a C6 glioma cell line expressing the μ opioid receptor. *Journal of Pharmacology and Experimental Therapeutics*, *278*(3), 1121–1127. PMID: 8819494

Emmett-Oglesby, M. W., Shippenberg, T. S., & Herz, A. (1988). Tolerance and cross-tolerance to the discriminative stimulus properties of fentanyl and morphine. *The Journal of Pharmacology and Experimental Therapeutics*, *245*(1), 17–23. PMID: 3361440

Emmett-Oglesby, M. W., Shippenberg, T. S., & Herz, A. (1989). Fentanyl and morphine discrimination in rats continuously infused with fentanyl. *Behavioural Pharmacology*, *1*(1), 3–11. PMID: 11175382

Eshleman, A. J., Nagarajan, S., Wolfrum, K. M., Reed, J. F., Nilsen, A., Torralva, R., & Janowsky, A. (2020). Affinity, potency, efficacy, selectivity, and molecular modeling of substituted fentanyls at opioid receptors. *Biochemical Pharmacology*, *182*, 114293. https://doi.org/10.1016/j.bcp.2020.114293

Fu, M. J., Tsen, L. Y., Lee, T. Y., Lui, P. W., & Chan, S. H. (1997). Involvement of cerulospinal glutamatergic neurotransmission in fentanyl-induced muscular rigidity in the rat. *Anesthesiology*, *87*(6), 1450–1459. https://doi.org/10.1097/00000542-199712000-00024

Gerak, L. R., Minervini, V., Latham, E., Ghodrati, S., Lillis, K. V., Wooden, J., Disney, A., Husbands, S. M., & France, C. P. (2019). Methocinnamox produces long-lasting antagonism of the behavioral effects of μ-opioid receptor agonists but not prolonged precipitated withdrawal in rats. *Journal of Pharmacology and Experimental Therapeutics*, *371*, 507–516. https://doi.org/10.1124/jpet.119.260331

Gherbi, K., Briddon, S. J., & Charlton, S. J. (2018). Micro-pharmacokinetics: Quantifying local drug concentration at live cell membranes. *Scientific Reports*, *8*(1), 3479. https://doi.org/10.1038/s41598-018-21100-x

Gill, H., Kelly, E., & Henderson, G. (2019). How the complex pharmacology of the fentanyls contributes to their lethality. *Addiction*, *114*(9), 1524–1525. https://doi.org/10.1111/add.14614

Gillis, A., Gondin, A. B., Kliewer, A., Sanchez, J., Lim, H. D., Alamein, C., Manandhar, P., Santiago, M., Fritzwanker, S., Schmiedel, F., Katte, T. A., Reekie, T., Grimsey, N. L., Kassiou, M., Kellam, B., Krasel, C., Halls, M. L., Connor, M., Lane, J. R., … Canals, M. (2020). Low intrinsic efficacy for G protein activation can explain the improved side effect profiles of new opioid agonists. *Science Signaling*, *13*(625), eaaz3140. https://doi.org/10.1126/scisignal.aaz3140

Gillis, A., Kliewer, A., Kelly, E., Henderson, G., Christie, M. J., Schulz, S., & Canals, M. (2020). Critical assessment of G protein-biased agonism at the μ-opioid receptor. *Trends in Pharmacological Sciences*, *41*(12), 947–959. https://doi.org/10.1016/j.tips.2020.09.009

Grell, F. L., Koons, R. A., & Denson, J. S. (1970). Fentanyl in anesthesia: A report of 500 cases. *Anesthesia and Analgesia*, *49*(4), 523–532. PMID: 5534663

Hanson, M. A., Roth, C. B., Jo, E., Griffith, M. T., Scott, F. L., Reinhart, G., Desale, H., Clemons, B., Cahalan, S. M., Schuerer, S. C., Sanna, M. G., Han, G. W., Kuhn, P., Rosen, H., & Stevens, R. C. (2012). Crystal structure of a lipid G protein-coupled receptor. *Science*, *335*(6070), 851–855. https://doi.org/10.1126/science.1215904

Hassanien, S. H., Bassman, J. R., Perrien Naccarato, C. M., Twarozynski, J. J., Traynor, J. R., Iula, D. M., & Anand, J. P. (2020). In vitro pharmacology of fentanyl analogs at the human μ opioid receptor and their spectroscopic analysis. *Drug Testing and Analysis*, *12*(8), 1212–1221. https://doi.org/10.1002/dta.2822

Heusler, P., Tardif, S., & Cussac, D. (2015). Agonist stimulation at human μ opioid receptors in a [^{35}S]GTPγS incorporation assay: Observation of "bell-shaped" concentration–response relationships under conditions of strong receptor G protein coupling. *Journal of Receptor and Signal Transduction Research*, *36*(2), 158–166. https://doi.org/10.3109/10799893.2015.1069845

Hill, R. (2019). Polydrug opioid abuse and mechanisms of tolerance to opioid respiratory depression. PhD thesis, University of Bristol, UK. Retrieved from https://research-information.bris.ac.uk/en/studentTheses/polydrug-opioid-abuse-mechanisms-of-tolerance-to-opioid-respiration

Hill, R., Lyndon, A., Withey, S., Roberts, J., Kershaw, Y., MacLachlan, J., Lingford-Hughes, A., Kelly, E., Bailey, C., Hickman, M., & Henderson, G. (2016). Ethanol reversal of tolerance to the respiratory depressant effects of morphine. *Neuropsychopharmacology*, *41*, 762–773. PMID: 26171718

Hill, R., Santhakumar, R., Dewey, W., Kelly, E., & Henderson, G. (2020). Fentanyl depression of respiration: Comparison with heroin and morphine. *British Journal of Pharmacology*, *177*(2), 254–266. https://doi.org/10.1111/bph.14860

Huang, W., Manglik, A., Venkatakrishnan, A. J., Laeremans, T., Feinberg, E. N., Sanborn, A. L., Kato, H. E., Livingston, K. E., Thorsen, T. S., Kling, R. C., Granier, S., Gmeiner, P., Husbands, S. M., Traynor, J. R., Weis, W. I., Steyaert, J., Dror, R. O., & Kobilka, B. K. (2015). Structural insights into μ-opioid receptor activation. *Nature*, *524*(7565), 315–321. https://doi.org/10.1038/nature14886

Huang, X. Q., Jiang, H. L., Luo, X. M., Rong, S. B., Gu, J. D., Tan, X. J., Zhu, Y. C., Chen, K. X., Ji, R. Y., & Cao, Y. (2000). Molecular modeling on solvent effect and interaction mechanism of fentanyl analogs to μ-opioid receptor. *Acta Pharmacologica Sinica*, *21*(1), 46–54. PMID: 11263247

Hughes, C. E., Dykstra, L. A., & Picker, M. J. (1996). Behavioral tolerance and cross-tolerance to the response rate-decreasing effects of mu opioids in rats. *Behavioural Pharmacology*, *7*(3), 228–236. PMID: 11224415

Hurst, D. P., Grossfield, A., Lynch, D. L., Feller, S., Romo, T. D., Gawrisch, K., Pitman, M. C., & Reggio, P. H. (2010). A lipid pathway for ligand binding is necessary for a cannabinoid G protein-coupled receptor. *Journal of Biological Chemistry*, *285*(23), 17954–17964. https://doi.org/10.1074/jbc.M109.041590

Jaffe, T. B., & Ramsey, F. M. (1983). Attenuation of fentanyl-induced truncal rigidity. *Anesthesiology*, *58*(6), 562–564. https://doi.org/10.1097/00000542-198306000-00015

Jannetto, P. J., Helander, A., Garg, U., Janis, G. C., Goldberger, B., & Ketha, H. (2019). The fentanyl epidemic and evolution of fentanyl analogs in the United States and the European Union. *Clinical Chemistry*, *65*(2), 242–253. https://doi.org/10.1373/clinchem.2017.281626

Jarończyk, M., Lipiński, P. F. J., Dobrowolski, J. C., & Sadlej, J. (2017). The FMO analysis of the molecular interaction of fentanyl derivatives with the μ-opioid receptor. *Chemical Papers*, *71*, 1429–1443. https://link.springer.com/article/10.1007/s11696-017-0136-5

Kapoor, A., Martinez-Rosell, G., Provasi, D., de Fabritiis, G., & Filizola, M. (2017). Dynamic and kinetic elements of μ-opioid receptor functional selectivity. *Scientific Reports*, *7*(1), 11255. https://doi.org/10.1038/s41598-017-11483-8

Kelly, E. (2013). Efficacy and ligand bias at the μ-opioid receptor. *British Journal of Pharmacology*, *169*(7), 1430–1446. https://doi.org/10.1111/bph.12222

Kliewer, A., Gillis, A., Hill, R., Schmidel, F., Bailey, C., Kelly, E., Henderson, G., Christie, M. J., & Schulz, S. (2020). Morphine-induced respiratory depression is independent of β-arrestin 2 signalling. *British Journal of Pharmacology*, *177*, 2923–2931. https://doi.org/10.1111/bph.15004

Kliewer, A., Schmiedel, F., Sianati, S., Bailey, A., Bateman, J. T., Levitt, E. S., Williams, J. T., Christie, M. J., & Schulz, S. (2019). Phosphorylation-deficient G-protein-biased μ-opioid receptors improve analgesia and diminish tolerance but worsen opioid side effects. *Nature Communications*, *10*, 367. https://doi.org/10.1038/s41467-018-08162-1

Knapman, A., Santiago, M., Du, Y. P., Bennallack, P. R., Christie, M. J., & Connor, M. (2012). A continuous, fluorescence-based assay of μ-opioid receptor activation in AtT-20 cells. *Journal of Biomolecular Screening*, *18*(3), 269–276. https://doi.org/10.1177/1087057112461376

Koehl, A., Hu, H., Maeda, S., Zhang, Y., Qu, Q., Paggi, J. M., Latorraca, N. R., Hilger, D., Dawson, R., Matile, H., Schertler, G. F. X., Granier, S., Weis, W. I., Dror, R. O., Manglik, A., Skiniotis, G., & Kobilka, B. K. (2018). Structure of the μ-opioid receptor–G_i protein complex. *Nature*, *558*(7711), 547–552. https://doi.org/10.1038/s41586-018-0219-7

Kosterlitz, H. W., & Leslie, F. M. (1978). Comparison of the receptor binding characteristics of opiate agonists interacting with μ- or κ-receptors. *British Journal of Pharmacology*, *64*(4), 607–614. https://doi.org/10.1111/j.1476-5381.1978.tb17323.x

Kumar, P., Sunkaraneni, S., Sirohi, S., Dighe, S. V., Walker, E. A., & Yoburn, B. C. (2008). Hydromorphone efficacy and treatment protocol impact on tolerance and μ-opioid receptor regulation. *European Journal of Pharmacology*, *597*(1–3), 39–45. https://doi.org/10.1016/j.ejphar.2008.08.025

Lambert, D. G., Atcheson, R., Hirst, R. A., & Rowbotham, D. J. (1993). Effects of morphine and its metabolites on opiate receptor binding, cAMP formation and [^{3}H]noradrenaline release from SH-SY5Y cells. *Biochemical Pharmacology*, *46*(7), 1145–1150. https://doi.org/10.1016/0006-2952(93)90462-6

Latorraca, N. R., Venkatakrishnan, A. J., & Dror, R. O. (2017). GPCR dynamics: Structures in motion. *Chemical Reviews*, *117*(1), 139–155. https://doi.org/10.1021/acs.chemrev.6b00177

Levitt, E. S., Abdala, A. P., Paton, J. F. R., Bissonnette, J. M., & Williams, J. T. (2015). μ opioid receptors activation hyperpolarizes respiratory-controlling Kölliker–Fuse neurons and suppresses post-inspiratory drive. *The Journal of Physiology*, *593*, 4453–4469. PMID: 26175072

Lipiński, P. F. J., Jarończyk, M., Dobrowolski, J. C., & Sadlej, J. (2019). Molecular dynamics of fentanyl bound to μ-opioid receptor. *Journal of Molecular Modeling*, *25*(5), 144. https://doi.org/10.1007/s00894-019-3999-2

Lui, P. W., Chang, G. J., Lee, T. Y., & Chan, S. H. (1993). Antagonization of fentanyl-induced muscular rigidity by denervation of the coerulospinal noradrenergic pathway in the rat. *Neuroscience Letters*, *157*(2), 145–148. https://doi.org/10.1016/0304-3940(93)90723-x

Lui, P. W., Lee, T. Y., & Chan, S. H. (1989). Involvement of locus coeruleus and noradrenergic neurotransmission in fentanyl-induced muscular rigidity in the rat. *Neuroscience Letters*, *96*(1), 114–119. https://doi.org/10.1016/0304-3940(89)90252-8

Madia, P. A., Dighe, S. V., Sirohi, S., Walker, E. A., & Yoburn, B. C. (2009). Dosing protocol and analgesic efficacy determine opioid tolerance in the mouse. *Psychopharmacology (Berlin)*, *207*(3), 413–422. https://doi.org/10.1007/s00213-009-1673-6

Madia, P. A., Navani, D. M., & Yoburn, B. C. (2012). [^{35}S]GTPγS binding and opioid tolerance and efficacy in mouse spinal cord. *Pharmacology, Biochemistry and Behaviour*, *101*(1), 155–165. https://doi.org/10.1016/j.pbb.2011.11.001

Mahonski, S. G., Leonard, J. B., Gatz, J. D., Seung, H., Haas, E. E., & Kim, H. K. (2020). Prepacked naloxone administration for suspected opioid overdose in the era of illicitly manufactured fentanyl: A retrospective study of regional poison center data. *Clinical Toxicology*

(Philadelphia, Pa.), 58(2), 117–123. https://doi.org/10.1080/15563650.2019.1615622

Manabe, S., Miyano, K., Fujii, Y., Ohshima, K., Yoshida, Y., Nonaka, M., Uzu, M., Matsuoka, Y., Sato, T., Uezono, Y., & Morimatsu, H. (2019). Possible biased analgesic of hydromorphone through the G protein-over β-arrestin-mediated pathway: cAMP, CellKey™, and receptor internalization analyses. *Journal of Pharmacological Sciences*, *140*(2), 171–177. https://doi.org/10.1016/j.jphs.2019.06

Manglik, A., Kruse, A. C., Kobilka, T. S., Thian, F. S., Mathiesen, J. M., Sunahara, R. K., Pardo, L., Weis, W. I., Kobilka, B. K., & Granier, S. (2012). Crystal structure of the μ-opioid receptor bound to a morphinan antagonist. *Nature*, *485*(7398), 321–326. https://doi.org/10.1038/nature10954

Matthes, H. W., Smadja, C., Valverde, O., Vonesch, J. L., Foutz, A. S., Boudinot, E., Denavit-Saubié, M., Severini, C., Negri, L., Roques, B. P., Maldonado, R., & Kieffer, B. L. (1998). Activity of the δ-opioid receptor is partially reduced, whereas activity of the κ-receptor is maintained in mice lacking the μ-receptor. *Journal of Neuroscience*, *18*(18), 7285–7295. https://doi.org/10.1523/JNEUROSCI.18-18-07285.1998

Mayer, S., Boyd, J., Collins, A., Kennedy, M. C., Fairbairn, N., & McNeil, R. (2018). Characterizing fentanyl-related overdoses and implications for overdose response: Findings from a rapid ethnographic study in Vancouver, Canada. *Drug and Alcohol Dependence*, *193*, 69–74. https://doi.org/10.1016/j.drugalcdep.2018.09.006

McPherson, J., Rivero, G., Baptist, M., Llorente, J., Al-Sabah, S., Krasel, C., Dewey, W. L., Bailey, C. P., Rosethorne, E. M., Charlton, S. J., Henderson, G., & Kelly, E. (2010). μ-Opioid receptors: Correlation of agonist efficacy for signalling with ability to activate internalization. *Molecular Pharmacology*, *78*, 756–766. https://doi.org/10.1124/mol.110.066613

Moe, J., Godwin, J., Purssell, R., O'Sullivan, F., Hau, J. P., Purssell, E., Curran, J., Doyle-Waters, M. M., Brasher, P. M. A., Buxton, J. A., & Hohl, C. M. (2020). Naloxone dosing in the era of ultra-potent opioid overdoses: A systematic review. *Canadian Journal of Emergency Medicine*, *2*(2), 178–186. https://doi.org/10.1017/cem.2019.471

Montandon, G., Liu, H., & Horner, R. L. (2016). Contribution of the respiratory network to rhythm and motor output revealed by modulation of GIRK channels, somatostatin and neurokinin-1 receptors. *Scientific Reports*, *6*, 32707. https://doi.org/10.1038/srep32707

Morgan, D., & Picker, M. J. (1996). Contribution of individual differences to discriminative stimulus, antinociceptive and rate-decreasing effects of opioids: Importance of the drug's relative intrinsic efficacy at the mu receptor. *Behavioural Pharmacology*, *7*(3), 261–284. PMID: 11224419

Morgan, D., & Picker, M. J. (1998). The μ opioid irreversible antagonist beta-funaltrexamine differentiates the discriminative stimulus effects of opioids with high and low efficacy at the μ opioid receptor. *Psychopharmacology (Berlin)*, *140*(1), 20–28. https://doi.org/10.1007/s002130050734

Mori, T., Kuzumaki, N., Arima, T., Narita, M., Tateishi, R., Kondo, T., Hamada, Y., Kuwata, H., Kawata, M., Yamazaki, M., Sugita, K., Matsuzawa, A., Baba, K., Yamauchi, T., Higashiyama, K., Nonaka, M., Miyano, K., Uezono, Y., & Narita, M. (2017). Usefulness for the combination of G-protein- and β-arrestin-biased ligands of μ-opioid receptors: Prevention of antinociceptive tolerance. *Molecular Pain*, *13*, 1744806917740030. https://doi.org/10.1177/1744806917740030

Moss, R. B., & Carlo, D. J. (2019). Higher doses of naloxone are needed in the synthetic opioid era. *Substance Abuse Treatment, Prevention, and Policy*, *14*(1), 6. https://doi.org/10.1186/s13011-019-0195-4

National Institute on Drug Abuse. (2021). Overdose death rates. Retrieved from https://www.drugabuse.gov/drug-topics/trends-statistics/overdose-death-rates

Negus, S. S., Burke, T. F., Medzihradsky, F., & Woods, J. H. (1993). Effects of opioid agonists selective for μ, κ and δ opioid receptors on schedule-controlled responding in rhesus monkeys: Antagonism by quadazocine. *The Journal of Pharmacology and Experimental Therapeutics*, *267*, 896–903. PMID: 8246165

Negus, S. S., Pasternak, G. W., Koob, G. F., & Weinger, M. B. (1993). Antagonist effects of β-funaltrexamine and naloxonazine on alfentanil-induced antinociception and muscle rigidity in the rat. *Journal of Pharmacology and Experimental Therapeutics*, *264*(2), 739–745. PMID: 8437122

Obeng, S., Wilkerson, J. L., León, F., Reeves, M. E., Restrepo, L. F., Gamez-Jimenez, L. R., Patel, A., Pennington, A. E., Taylor, V. A., Ho, N. P., Braun, T., Fortner, J. D., Crowley, M. L., Williamson, M. R., Pallares, V. L. C., Mottinelli, M., Lopera-Londoño, C., McCurdy, C. R., McMahon, L. R., & Hiranita, T. (2021). Pharmacological comparison of mitragynine and 7-hydroxymitragynine: In vitro affinity and efficacy for μ-opioid receptor and opioid-like behavioral effects in rats. *Journal of Pharmacology and Experimental Therapeutics*, *376*(3), 410–427. https://doi.org/10.1124/jpet.120.000189

Pan, Y. Z., Li, D. P., Chen, S. R., & Pan, H. L. (2004). Activation of μ-opioid receptors excites a population of locus coeruleus-spinal neurons through presynaptic disinhibition. *Brain Research*, *997*(1), 67–78. https://doi.org/10.1016/j.brainres.2003.10.050

Paronis, C. A., & Holtzman, S. G. (1994). Sensitization and tolerance to the discriminative stimulus effects of μ-opioid agonists. *Psychopharmacology*, *114*(4), 601–610. https://doi.org/10.1007/BF02244991

Paronis, C. A., & Holtzman, S. G. J. (1992). Development of tolerance to the analgesic activity of μ agonists after continuous infusion of morphine, meperidine or fentanyl in rats. *Journal of Pharmacology and Experimental Therapeutics*, *262*(1), 1–9. PMID: 1625189

Paronis, C. A., & Woods, J. H. (1997). Ventilation in morphine-maintained rhesus monkeys. II: Tolerance to the antinociceptive but not the ventilatory effects of morphine. *Journal of Pharmacology and Experimental Therapeutics*, *282*(1), 355–362. PMID: 9223574

Paton, J. F. (1996). A working heart-brainstem preparation of the mouse. *Journal of Neuroscience Methods*, *65*(1), 63–68. https://doi.org/10.1016/0165-0270(95)00147-6

Pawar, M., Kumar, P., Sunkaraneni, S., Sirohi, S., Walker, E. A., & Yoburn, B. C. (2007). Opioid agonist efficacy predicts the magnitude of tolerance and the regulation of μ-opioid receptors and dynamin-2. *European Journal of Pharmacology*, *563*(1–3), 92–101. https://doi.org/10.1016/j.ejphar.2007.01.059

Pearson, K. G., Acharya, H., & Fouad, K. (2005). A new electrode configuration for recording electromyographic activity in behaving mice. *Journal of Neuroscience Methods*, *148*, 36–42. PMID: 15908013

Pepper, C. M., & Henderson, G. (1980). Opiates and opioid peptides hyperpolarize locus coeruleus neurons in vitro. *Science*, *209*(4454), 394–395. https://doi.org/10.1126/science.7384811

Pert, C. B., Pasternak, G., & Snyder, S. H. (1973). Opiate agonists and antagonists discriminated by receptor binding in brain. *Science*, *182*(4119), 1359–1361. https://doi.org/10.1126/science.182.4119.1359

Pitts, R. C., West, J. P., Morgan, D., Dykstra, L. A., & Picker, M. J. (1996). Opioids and rate of positively reinforced behaviour: Differential antagonism by naloxone. *Behavioural Pharmacology*, *7*, 205–215. PMID: 11224413

Platt, D. M., Rowlett, J. K., & Spealman, R. D. (2001). Discriminative stimulus effects of intravenous heroin and its metabolites in rhesus monkeys: Opioid and dopaminergic mechanisms. *The Journal of Pharmacology and Experimental Therapeutics*, *299*(2), 760–767. PMID: 11602692

Podlewska, S., Bugno, R., Kudla, L., Bojarski, A. J., & Przewlocki, R. (2020). Molecular modeling of μ opioid receptor ligands with various functional properties: PZM21, SR-17018, morphine, and fentanyl-simulated interaction patterns confronted with experimental data. *Molecules*, *25*(20), 4636. https://doi.org/10.3390/molecules25204636

Rackam, A. (1980). Opiate-induced muscle rigidity in the rat: Effects of centrally acting agents. *Neuropharmacology*, *19*(9), 855–859. https://doi.org/10.1016/0028-3908(80)90083-0

Raehal, K. M., Walker, J. K., & Bohn, L. M. (2005). Morphine side effects in β-arrestin2 knockout mice. *The Journal of Pharmacology and Experimental Therapeutics*, *314*, 1195–1201. https://doi.org/10.1124/jpet.105.087254

Rasmussen, K., & Jacobs, B. L. (1985). Locus coeruleus unit activity in freely moving cats is increased following systemic morphine administration. *Brain Research*, *344*(2), 240–248. https://doi.org/10.1016/0006-8993(85)90801-7

Ricarte, A., Dalton, J. A. R., & Giraldo, J. (2021). Structural assessment of agonist efficacy in the μ-opioid receptor: Morphine and fentanyl elicit different activation patterns. *Journal of Chemical Information and Modeling*, *61*, 1251–1274. online ahead of print. https://doi.org/10.1021/acs.jcim.0c00890

Rivero, G., Llorente, J., McPherson, J., Cooke, A., Mundell, S. J., McArdle, C. A., Rosethorne, E. M., Charlton, S. J., Krasel, C., Bailey, C. P., Henderson, G., & Kelly, E. (2012). Endomorphin-2: A biased agonist at the μ-opioid receptor. *Molecular Pharmacology*, *82*(2), 178–188. https://doi.org/10.1124/mol.112.078659

Rzasa Lynn, R., & Galinkin, J. L. (2018). Naloxone dosage for opioid reversal: Current evidence and clinical implications. *Therapeutic Advances in Drug Safety*, *9*(1), 63–88. https://doi.org/10.1177/2042098617744161

Sabbadin, D., Ciancetta, A., Deganutti, G., Cuzzolin, A., & Moro, S. (2015). Exploring the recognition pathway at the human A_{2A} adenosine receptor of the endogenous agonist adenosine using supervised molecular dynamics simulations. *MedChemComm*, *6*, 1081–1085. https://doi.org/10.1039/C5MD00016E

Saidak, Z., Blake-Palmer, K., Hay, D. L., Northup, J. K., & Glass, M. (2006). Differential activation of G-proteins by μ-opioid receptor agonists. *British Journal of Pharmacology*, *147*(6), 671–680. https://doi.org/10.1038/sj.bjp.0706661

Schmid, C. L., Kennedy, N. M., Ross, N. C., Lovell, K. M., Yue, Z., Morgenweck, J., Cameron, M. D., Bannister, T. D., & Bohn, L. M. (2017). Bias factor and therapeutic window correlate to predict safer opioid analgesics. *Cell*, *171*, 1165–1175. https://doi.org/10.1016/j.cell.2017.10.035

Schneider, S., Provasi, D., & Filizola, M. (2015). The dynamic process of drug–GPCR binding at either orthosteric or allosteric sites evaluated by metadynamics. *Methods in Molecular Biology*, *1335*, 277–294. https://doi.org/10.1007/978-1-4939-2914-6_18

Schneider, S., Provasi, D., & Filizola, M. (2016). How oliceridine (TRV-130) binds and stabilizes a μ-opioid receptor conformational state that selectively triggers G protein signaling pathways. *Biochemistry*, *55*(46), 6456–6466. https://doi.org/10.1021/acs.biochem.6b00948

Schwienteck, K. L., Faunce, K. E., Rice, K. C., Obeng, S., Zhang, Y., Blough, B. E., Grim, T. W., Negus, S. S., & Banks, M. L. (2019). Effectiveness comparisons of G protein biased and unbiased μ opioid receptor ligands in warm water tail-withdrawal and drug discrimination in male and female rats. *Neuropharmacology*, *150*, 200–209. https://doi.org/10.1016/j.neuropharm.2019.01.020

Selley, D. E., Sim, L. J., Xiao, R., Liu, Q., & Childers, S. R. (1997). μ-Opioid receptor-stimulated guanosine-5′-*O*-(γ-thio)-triphosphate binding in rat thalamus and cultured cell lines: Signal transduction mechanisms underlying agonist efficacy. *Molecular Pharmacology*, *51*(1), 87–96. https://doi.org/10.1124/mol.51.1.87

Simon, E. J., Hiller, J. M., Growth, J., & Edelman, I. (1975). Further properties of stereospecific opiate binding sites in rat brain: On the nature of the sodium effect. *Journal of Pharmacology and Experimental Therapeutics*, *192*(3), 531–537. PMID: 235639

Sirohi, S., Dighe, S. V., Walker, E. A., & Yoburn, B. C. (2008). The analgesic efficacy of fentanyl: Relationship to tolerance and μ-opioid receptor regulation. *Pharmacology, Biochemistry and Behaviour*, *91*(1), 115–120. https://doi.org/10.1016/j.pbb.2008.06.019

Slater, P., & Starkie, D. A. (1987). Changes in limb tone produced by regional injections of opiates into rat brain. *Naunyn Schmiedebergs Archives of Pharmacology*, *335*(1), 54–58. https://doi.org/10.1007/BF00165036

Somerville, N. J., O'Donnell, J., Gladden, R. M., Zibbell, J. E., Green, T. C., Younkin, M., Ruiz, S., Babakhanlou-Chase, H., Chan, M., Callis, B. P., Kuramoto-Crawford, J., Nields, H. M., & Walley, A. Y. (2017). Characteristics of fentanyl overdose—Massachusetts, 2014–2016. *The Morbidity and Mortality Weekly Report*, *66*(14), 382–386. https://doi.org/10.15585/mmwr.mm6614a2

Stanley, T. H. (2014). The fentanyl story. *The Journal of Pain*, *15*(12), 1215–1226. https://doi.org/10.1016/j.jpain.2014.08.010

Stevens, C. W., & Yaksh, T. L. (1989). Potency of infused spinal antinociceptive agents is inversely related to magnitude of tolerance after continuous infusion. *Journal of Pharmacology and Experimental Therapeutics*, *250*(1), 1–8. PMID: 2526212

Subramanian, G., Paterlini, M. G., Portoghese, P. S., & Ferguson, D. M. (2000). Molecular docking reveals a novel binding site model for fentanyl at the μ-opioid receptor. *Journal of Medicinal Chemistry*, *43*(3), 381–391. https://doi.org/10.1021/jm9903702

Sutcliffe, K. J., Corey, R. A., Charlton, S. J., Sessions, R. B., Henderson, G., & Kelly, E. (2021). Fentanyl binds to the μ-opioid receptor via the lipid membrane and transmembrane helices. *bioRxiv*. https://doi.org/10.1101/2021.02.04.429703

Sutcliffe, K. J., Henderson, G., Kelly, E., & Sessions, R. B. (2017). Drug binding poses relate structure with efficacy in the μ opioid receptor. *Journal of Molecular Biology*, *429*(12), 1840–1851. https://doi.org/10.1016/j.jmb.2017.05.009

Sutter, M. E., Gerona, R. R., Davis, M. T., Roche, B. M., Colby, D. K., Chenoweth, J. A., Adams, A. J., Owen, K. P., Ford, J. B., Black, H. B., & Albertson, T. E. (2017). Fatal fentanyl: One pill can kill. *Academic Emergency Medicine*, *24*(1), 106–113. https://doi.org/10.1111/acem.13034

Suzuki, J., & El-Haddad, S. (2017). A review: Fentanyl and non-pharmaceutical fentanyls. *Drug and Alcohol Dependence*, *171*, 107–116. https://doi.org/10.1016/j.drugalcdep.2016.11.033

Torralva, R., Eshleman, A. J., Swanson, T. L., Schmachtenberg, J. L., Schutzer, W. E., Bloom, S. H., Wolfrum, K. M., Reed, J. F., & Janowsky, A. (2020). Fentanyl but not morphine interacts with non-opioid recombinant human neurotransmitter receptors and transporters. *Journal of Pharmacology and Experimental Therapeutics*, *374*(3), 376–391. https://doi.org/10.1124/jpet.120.265561

Torralva, R., & Janowsky, A. (2019). Noradrenergic mechanisms in fentanyl-mediated rapid death explain failure of naloxone in the opioid crisis. *Journal of Pharmacology and Experimental Therapeutics*, *371*(2), 453–475. https://doi.org/10.1124/jpet.119.258566

Travagli, R. A., Dunwiddie, T. V., & Williams, J. T. (1995). Opioid inhibition in locus coeruleus. *Journal of Neurophysiology*, *74*(2), 519–528. https://doi.org/10.1152/jn.1995.74.2.519

Traynor, J. R., & Nahorski, S. R. (1995). Modulation by μ-opioid agonists of guanosine-5′-O-(3-[^{35}S]thio)triphosphate binding to membranes from human neuroblastoma SH-SY5Y cells. *Molecular Pharmacology*, *47*(4), 848–854. PMID: 7723747

Tsou, M. Y., Lui, P. W., Lee, T. Y., Pan, J. T., & Chan, S. H. (1989). Differential effects of prazosin and yohimbine on fentanyl-induced muscular rigidity in rats. *Neuropharmacology*, *28*(11), 1163–1168. https://doi.org/10.1016/0028-3908(89)90206-2

Vankova, M. E., Weinger, M. B., Chen, D. Y., Bronson, J. B., Motis, V., & Koob, G. F. (1996). Role of central μ, δ-1, and κ-1 opioid receptors in opioid-induced muscle rigidity in the rat. *Anesthesiology*, *85*(3), 574–583. https://doi.org/10.1097/00000542-199609000-00017

Vaughn, R. L., & Bennett, C. R. (1981). Fentanyl chest wall rigidity syndrome—A case report. *Anesthesia Progress*, *28*(2), 50–51. PMID: 6943947

Vo, Q. N., Mahinthichaichan, P., Shen, J., & Ellis, C. R. (2021). How μ-opioid receptor recognizes fentanyl. *Nature Communications*, *12*(1), 984. https://doi.org/10.1038/s41467-021-21262-9

Walentiny, D. M., Moisa, L. T., & Beardsley, P. M. (2019). Oxycodone-like discriminative stimulus effects of fentanyl-related emerging drugs of abuse in mice. *Neuropharmacology*, *150*, 210–216. PMID: 30735691

Walker, E. A., Makhay, M. M., House, J. D., & Young, A. M. (1994). In vivo apparent pA_2 analysis for naltrexone antagonism of discriminative stimulus and analgesic effects of opiate agonists in rats. *The Journal of Pharmacology and Experimental Therapeutics*, *271*(2), 959–968. PMID: 7965818

Walker, E. A., Richardson, T. M., & Young, A. M. (1997). Tolerance and cross-tolerance to morphine-like stimulus effects of μ opioids in rats. *Psychopharmacology*, *133*(1), 17–28. PMID: 9335076

Weinger, M. B., Chen, D. Y., Lin, T., Lau, C., Koob, G. F., & Smith, N. T. (1995). A role for CNS α-2 adrenergic receptors in opiate-induced muscle rigidity in the rat. *Brain Research*, *669*(1), 10–18. https://doi.org/10.1016/0006-8993(94)01216-5

Weinger, M. B., Smith, N. T., Blasco, T. A., & Koob, G. F. (1991). Brain sites mediating opiate-induced muscle rigidity in the rat: Methylnaloxonium mapping study. *Brain Research*, *544*(2), 181–190. https://doi.org/10.1016/0006-8993(91)90052-w

Weinger, M. B., & Taurek, D. L. (1990). The effects on muscle tone of selective alpha-2 adrenergic agonists and antagonists during high-dose alfentanil anesthesia in the rat. *Anesthesia and Analgesia*, *70*(2), S425.

White, J. M., & Irvine, R. J. (1999). Mechanisms of fatal opioid overdose. *Addiction*, *94*(7), 961–972. PMID: 10707430

Widdowson, P. S., Griffiths, E. C., & Slater, P. (1986). The effects of opioids in the periaqueductal grey region of rat brain on hind-limb muscle tone. *Neuropeptides*, *7*(3), 251–258. https://doi.org/10.1016/0143-4179(86)90019-3

Wilcox, R. E., & Levitt, R. A. (1978). Naloxone reversal of morphine catatonia: Role of caudate and periaqueductal gray. *Pharmacology, Biochemistry and Behaviour*, *9*(4), 425–428. https://doi.org/10.1016/0091-3057(78)90035-7

Willette, R. N., Krieger, A. J., & Sapru, H. N. (1982). Opioids increase laryngeal resistance and motoneuron activity in the recurrent laryngeal nerve. *European Journal of Pharmacology*, *80*(1), 57–63. https://doi.org/10.1016/0014-2999(82)90177-7

Williams, J. T., Egan, T. M., & North, R. A. (1982). Enkephalin opens potassium channels on mammalian central neurones. *Nature*, *299*(5878), 74–77. https://doi.org/10.1038/299074a0

Williams, J. T., Ingram, S. L., Henderson, G., Chavkin, C., von Zastrow, M., Schulz, S., Koch, T., Evans, C. J., & Christie, M. J. (2013). Regulation of μ-opioid receptors: Desensitization, phosphorylation, internalization, and tolerance. *Pharmacological Reviews*, *65*(1), 223–254. https://doi.org/10.1124/pr.112.005942

Yang, P. K., Weinger, M. B., & Negus, S. S. (1992). Elucidation of dose–effect relationships for different opiate effects using alfentanil in the spontaneously ventilating rat. *Anesthesiology*, *77*(1), 153–161. https://doi.org/10.1097/00000542-199207000-00022

Zaki, P. A., Keith, D. E. Jr., Brine, G. A., Carroll, F. I., & Evans, C. J. (2000). Ligand-induced changes in surface μ-opioid receptor number: Relationship to G protein activation? *Journal of Pharmacology and Experimental Therapeutics*, *292*(3), 1127–1134. PMID: 10688632

Zebala, J. A., Schuler, A. D., Kahn, S. J., & Maeda, D. Y. (2020). Desmetramadol is identified as a G-protein biased μ opioid receptor agonist. *Frontiers in Pharmacology*, *10*, 1680. https://doi.org/10.3389/fphar.2019.01680

How to cite this article: Kelly, E., Sutcliffe, K., Cavallo, D., Ramos-Gonzalez, N., Alhosan, N., & Henderson, G. (2023). The anomalous pharmacology of fentanyl. *British Journal of Pharmacology*, *180*(7), 797–812. https://doi.org/10.1111/bph.15573