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RESEARCH ARTICLE

In vitro and in vivo study of butyrylfentanyl and 4-fluorobutyrylfentanyl in female and male mice: Role of the CRF_1 receptor in cardiorespiratory impairment

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Abstract

Background and Purpose: Fentanyl analogues have been implicated in many cases of intoxication and death with overdose worldwide. The aim of this study is to investigate the pharmaco-toxicology of two fentanyl analogues: butyrylfentanyl (BUF) and 4-fluorobutyrylfentanyl (4F-BUF).

Experimental Approach: In vitro, we measured agonist opioid receptor efficacy, potency, and selectivity and ability to promote interaction of the μ receptor with G protein and β-arrestin 2. In vivo, we evaluated thermal antinociception, stimulated motor activity and cardiorespiratory changes in female and male CD-1 mice injected with BUF or 4F-BUF (0.1–6 $mg \cdot kg^{-1}$). Opioid receptor specificity was investigated using naloxone (6 $mg \cdot kg^{-1}$). We investigated the possible role of stress in increasing cardiorespiratory toxicity using the corticotropin-releasing factor 1 (CRF_1) antagonist antalarmin (10 $mg \cdot kg^{-1}$).

Key Results: Agonists displayed the following rank of potency at μ receptors: fentanyl > 4F-BUF > BUF. Fentanyl and BUF behaved as partial agonists for the β-arrestin 2 pathway, whereas 4F-BUF did not promote β-arrestin 2 recruitment. In vivo, we revealed sex differences in motor and cardiorespiratory impairments but not antinociception induced by BUF and 4F-BUF. Antalarmin alone was effective in blocking respiratory impairment induced by BUF in both sexes but not 4F-BUF. The combination of naloxone and antalarmin significantly enhanced naloxone reversal of the cardiorespiratory impairments induced by BUF and 4F-BUF in mice.

Conclusion and Implications: In this study, we have uncovered a novel mechanism by which synthetic opioids induce respiratory depression, shedding new light on the role of CRF_1 receptors in cardiorespiratory impairments by μ agonists.

Abbreviations: 4F-BUF, 4-fluorobutyrylfentanyl or para-fluorobutyrylfentanyl; ANT, antalarmin; BB_ie, breath length, computed by adding inspiration + expiration duration. Expressed in ms; BRET, bioluminescence resonance energy transfer; BUF, butyrylfentanyl; FR ie, breathing frequency derived from BB_ie. Expressed in bpm; MV, minute volume; NPSs, novel psychoactive substances; NSOs, novel synthetic opioids; PLETH, plethysmography.

Chemical compounds studied in this article: fentanyl (PubChem CID: 3345), butyrylfentanyl (PubChem CID: 621174), 4-fluorobutyrylfentanyl (PubChem CID: 86280430), naloxone (PubChem CID: 5284596) and antalarmin (PubChem CID: 177990).

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KEYWORDS
4-fluorobutyrylfentanyl, antalarmin, butyrylfentanyl, $_{CRF1}$, fentanyl, mu opioid receptor, naloxone, novel psychoactive substances, respiratory depression, β-arrestin 2

1 | INTRODUCTION

The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) has been monitoring more than 930 novel psychoactive substances (NPSs) since 2009, 41 of which were first reported in Europe in 2022 (EMCDDA, 2022). Novel synthetic opioids (NSOs) are a small class of NPSs that includes about 74 new opioids identified in the European drug market since 2009 (EMCDDA, 2022).

Although the number of NSOs is low with respect to the other classes such as cathinones, recent signals, mostly from Baltic countries, suggest increased availability and harms (including intoxications and death) linked to **fentanyl** (fentanyl) derivatives and benzimidazole opioids. Recent reports from Estonia and Lithuania revealed an increase in lethal NSO overdose, which reveals the risk of life-threatening poisoning by these compounds (EMCDDA, 2022).

Butyrylfentanyl (BUF) is a fentanyl analogue, with an *N*-butyryl group replacing the *N*-propionyl group of fentanyl (Figure 1). It is sold on the NPS market as a substitute for heroin or prescription opioids. Seven case reports on BUF intoxication and deaths were reported in 2014, and this number increased to 81 reports in 2015. This compound was designated as a Schedule I substance under the Controlled Substances Act in 2016 (Drug Enforcement Administration, Department of Justice, 2016). BUF showed lower pharmacological efficacy with respect to fentanyl (Varshneya et al., 2023). Carboxy- and hydroxy-BUF metabolites were majorly found in the blood, urine and organs of a fatal case (Staeheli et al., 2016).

4-Fluorobutyrylfentanyl or para-fluorobutyrylfentanyl (4F-BUF) is a structural analogue of BUF. 4F-BUF and BUF share a common basic chemical structure characterized by a phenethylamine core (Figure 1). While BUF features a butyryl group, 4F-BUF contains a para-fluoro substitution on the phenethylamine backbone (Figure 1). This structural variance can significantly impact pharmacodynamic properties (Varshneya et al., 2023). 4F-BUF was first detected in 2015 (Bäckberg et al., 2015). The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) reported the seizure of this drug in different forms including powders, tablets, electronic cigarette liquid and nasal spray formulations (EMCDDA, 2015). One case of intoxication by 4F-BUF was reported in Sweden, and two deaths were reported from Poland (WHO, 2017). Since September 2017, 4F-BUF has been controlled in Cyprus, Finland, Czech Republic, Sweden, Poland, Denmark and France (WHO, 2017). Functional studies using the [^{35}S]GTPγS binding assay demonstrated that 4F-BUF showed a reduced efficacy for **μ** and **κ opioid** receptor activation compared with fentanyl (WHO, 2017), and in vivo, this drug showed a lower analgesic efficacy with respect to fentanyl (WHO, 2017). The metabolic pathway of 4F-BUF was investigated in vitro using human liver microsomes and suggests three major metabolites: nor-4F-BUF, 4-fluorofentanyl (4F-fentanyl) and hydroxyl-4F-BUF (Richeval et al., 2019). Similar to fentanyl, the most common adverse effects reported with BUF and 4F-BUF were disorientation, miosis, bradycardia, respiratory depression and apnoea, which may lead to death in some cases (Bäckberg et al., 2015). Since the appearance of fentanyl analogues in the NPS drug market, the number of cases of intoxication and death due to these substances increased exponentially. The recent world drug report revealed an important increase in female non-medical pharmaceutical opioids users (increase of 47%) as compared to opiates users (increase of 25%). This may explain the increasing rate of opioid use disorders in the last few years (United Nations Office on Drugs and Crime [UNDOC], 2023, executive summary).

Sex differences in opioid use, pharmacology and consequences are an important area of study in medical and scientific research (Fattore et al., 2020). While opioids affect both males and females, there are notable differences in how these drugs impact each gender (Fattore et al., 2020). Indeed, research on sex-based treatment differences in opioid use disorders has increased; however, studies focusing on sex-dependent differences in the pharmacotoxicological aspects of these potent synthetic opioids have not yet been reported.

Therefore, the aim of this study was to investigate the pharmacodynamic profile of the BUF and 4-fluoro-furanylfentanyl (4F-FUF). To evaluate the in vitro basic pharmacological profile of these

What is already known

- Fentanyl analogues induce cardiorespiratory impairments that are reverted by naloxone.

What does this study add

- CRF_1 receptors are involved in cardiorespiratory impairments induced by fentanyl analogues.

What is the clinical significance

- This study uncovers a novel mechanism underlying fentanyl analogue-induced respiratory depression.
- This potentially paves the way for development of more effective antidotes for synthetic opioid overdoses.

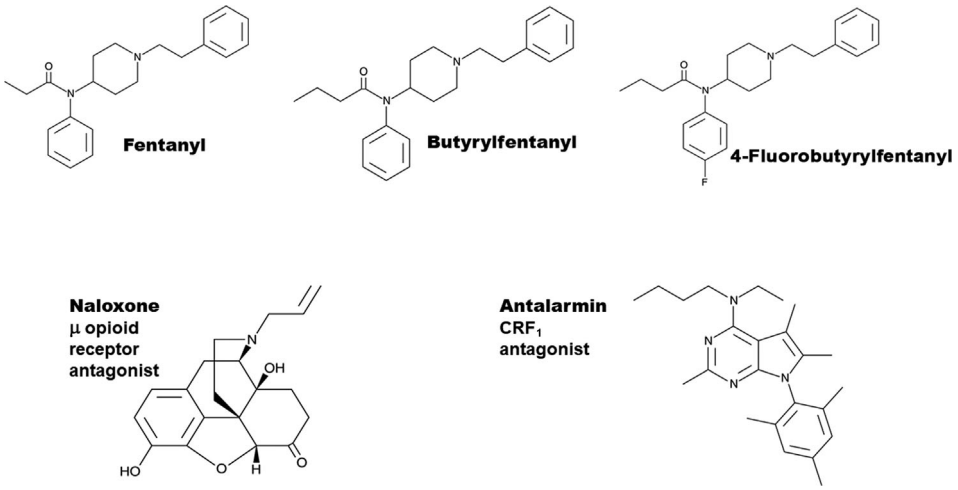

FIGURE 1 Chemical structures of fentanyl, butyrylfentanyl, 4-fluorobutyrylfentanyl, naloxone and antalarmin.

compounds, a calcium mobilization assay was performed using cells expressing **opioid receptors** and chimeric **G proteins** (Camarda & Calo', 2013). Moreover, a bioluminescence resonance energy transfer (BRET) assay was also used to investigate the ability of the compounds to promote μ receptor interaction with G protein and β-arrestin 2 (Molinari et al., 2010). We investigated the acute effects of BUF and 4F-BUF in vivo by evaluating thermal antinociception using the tail withdrawal test, the motor impairments using the rotarod test and by measuring cardiorespiratory changes using non-invasive electrocardiogram (ECG) (heart rate [HR], RR interval and QRS complex) and plethysmography (PLETH) (breath rate, breath length and minute volume [MV]) in CD-1 female and male mice. **Naloxone** pre-treatment was applied to investigate receptor specificity in the cardiorespiratory responses of male and female mice. Moreover, many studies revealed the role of functional antagonism of corticotropin-releasing factor (CRF) neurotransmission, in particular **CRF_1** receptors, in reducing stress and ameliorating negative affective-like states associated with opioid withdrawal and dependence (Heinrichs et al., 1995; Shaham et al., 1998; Stinus et al., 2005). Yet the role of CRF receptors in reducing the acute effects of opioids, especially the cardiorespiratory impairments, is unknown. To fill the gap on this issue, we investigated the possible involvement of this stress factor in worsening cardiorespiratory impairments related to opioids in female and male animals using the CRF_1 receptor antagonist **antalarmin** (ANT) alone and in combination with naloxone.

2 | METHODS

2.1 | In vitro studies

2.1.1 | Drugs and reagents

All cell culture media and supplements were from Invitrogen (Thermo Fisher Scientific Inc., MA, USA). Dermorphin, (D-Pen^2,D-Pen^5)-enkephalin (DPDPE) and dynorphin A were synthetized in the laboratory of Prof. Remo Guerrini (University of Ferrara, Italy). fentanyl, BUF, 4F-BUF and ANT were purchased from LGC Standards (LGC Standards S.r.l., Sesto San Giovanni, Milan, Italy) (authorization SP/141 24/11/2021 to MM), while naloxone was purchased from Sigma-Aldrich (St. Louis, MO, USA). Opioid peptides were solubilized in bidistilled water, whereas all other compounds were solubilized in dimethyl sulfoxide (DMSO) at a final concentration of 10 mM. Stock solutions of ligands were stored at −20°C. Serial dilutions were made in each assay buffer.

2.1.2 | Calcium mobilization assay

Chinese hamster ovary (CHO) cells (IZSLER Cat# BS CL 15, Brescia, Italy, RRID:CVCL_0214) stably co-expressing the human recombinant μ or κ opioid receptors with the C-terminally modified $G\alpha_{qi5}$ and CHO cells co-expressing the δ opioid receptor and the $G\alpha_{qG66Di5}$ chimeric protein were generated as previously described (Camarda & Calo, 2013). Cells were cultured in medium consisting of Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (1:1) supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU·ml^{-1}), streptomycin (100 mg·ml^{-1}), geneticin (G418; 200 μg·ml^{-1}) and hygromycin B (100 μg·ml^{-1}). Cell cultures were kept at 37°C in 5% CO_2/humidified air. When confluence was reached (3–4 days), cells were sub-cultured as required using trypsin/ethylenediaminetetraacetic acid (EDTA) and used for experimentation. Cells were seeded at a density of 50,000 cells per well into 96-well black, clear-bottom plates. After 24 h of incubation, the cells were incubated with Hank's balanced salt solution (HBSS) supplemented with 2.5-mM probenecid, 3 μM of the calcium-sensitive fluorescent dye Fluo-4 AM, 0.01% pluronic acid and 20-mM HEPES (pH 7.4) for 30 min at 37°C. Afterwards, the loading solution was aspirated, followed by a washing step with 100 μl per well of HBSS, HEPES (20 mM, pH 7.4), 2.5-mM probenecid and 500-μM Brilliant Black. Subsequently, 100 μl per well of the same buffer was added. After placing cell culture and compound plates into the FlexStation II (Molecular Devices, Sunnyvale, CA, USA), the changes in fluorescence of the cell-loaded calcium-sensitive dye Fluor-4 AM were measured.

2.1.3 | BRET assay

SH-SY5Y cells (RRID:CVCL_0019) stably co-expressing different pairs of fusion proteins, that is, μ-RLuc/Gβ1-RGFP or mu-RLuc/β-arrestin 2-RGFP, were prepared using a pantropic retroviral expression system as described previously (Malfacini et al., 2015; Molinari et al., 2010). Cells were grown in DMEM/Ham's F12 (1:1) supplemented with 10% FBS, penicillin G (100 $\mu g \cdot ml^{-1}$), streptomycin (100 $\mu g \cdot ml^{-1}$), l-glutamine (2 mM), fungizone (1 $\mu g \cdot ml^{-1}$), geneticin (G418; 400 $\mu g \cdot ml^{-1}$) and hygromycin B (100 $\mu g \cdot ml^{-1}$) in a humidified atmosphere of 5% CO_2 at 37°C. For G-protein experiments, enriched plasma membrane aliquots from μ-RLuc/Gβ1-RGFP cells were prepared by differential centrifugation (Beckman Coulter, CA, USA); cells were detached with phosphate-buffered saline (PBS)/EDTA solution (1 mM, pH 7.4, NaOH) and then, after 5 min 200 *g* of centrifugation, Dounce-homogenized (30 strokes) in cold homogenization buffer (Tris 5 mM, ethylene glycol-bis(β-amino ethyl ether)tetraacetic acid [EGTA] 1 mM, dithiothreitol [DTT] 1 mM, pH 7.4, HCl) in the presence of sucrose (0.32 M). Threeconsecutive centrifugation steps were performed for 10 min at 1000 *g* (4°C), and the supernatants were kept. Membrane enrichment was achieved through two consecutive centrifugation steps at 24,000 *g* for 20 minutes each, at 4°C. The second step was performed without sucrose. Following centrifugation, the supernatant was discarded, and the enriched membranefractions were resuspended in ultrapure water and stored at -80°C (Vachon et al., 1987). Membrane protein was determined using the QPRO-BCA kit (Cyanagen Srl, Bologna, Italy) and the multimode EnSight plate reader (PerkinElmer, Waltham, MA, USA). Luminescence in membranes and cells was recorded in 96-well white opaque microplates (PerkinElmer) using the Victor 2030 luminometer (PerkinElmer). For the determination of receptor/G-protein interaction, membranes (3 μg of protein) prepared from cells co-expressing μ-RLuc/Gβ1-RGFP were added to wells in Dulbecco's PBS (DPBS). For the determination of receptor/β-arrestin 2 interaction, whole cells co-expressing μ-RLuc/β-arrestin 2-RGFP were plated 24 h before the experiment (100,000 cells per well). The cells were prepared for the experiment by substituting the medium with PBS with $MgCl_2$ (0.5 mM) and $CaCl_2$ (0.9 mM). Coelenterazine at a final concentration of 5 μM was injected 15 min prior to reading the cell plate. Different concentrations of ligands in 20 μl of PBS–bovine serum albumin (BSA) 0.01% were added and incubated for 5 min before reading luminescence. All experiments were performed at room temperature.

2.1.4 | Data analysis and terminology

The pharmacological terminology adopted in this report is consistent with the International Union of Basic and Clinical Pharmacology (IUPHAR) recommendations. All data are expressed as the mean ± standard error of the mean (SEM) of n experiments. For potency values, 95% confidence limits ($CL_{95\%}$) were indicated. In calcium mobilization studies, agonist effects were expressed as the maximum change in per cent over the baseline fluorescence. Baseline fluorescence was measured in wells treated with saline. In BRET studies, agonist effects were calculated as the BRET ratio between counts per second (CPS) measured for the RGFP and RLuc light emitted using 510(10) and 460(25) filters (PerkinElmer), respectively. Maximal agonist effects were expressed as a fraction of the dermorphin maximal effect, which was determined in every assay plate (dermorphin $E_{max} = 1.00$). The concentration–response curve to agonists was fitted with the four-parameter logistic nonlinear regression model:

$$\text{Effect} = \text{Baseline} + \frac{E_{max} - \text{Baseline}}{1 + 10^{(\text{LogEC}_{50} - \text{Log}_{(\text{compound})}\text{Hillslope})}}$$

For all assays, agonist potencies are given as pEC_{50}, that is, the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal effect of that agonist. The potency of antagonists was expressed as pA_2 derived from the following equation: $pA_2 = -\log[(CR - 1)/[A]]$, assuming a slope value equal to unity, where concentration ratio (*CR*) indicates the ratio between agonist potency in the presence and absence of antagonist and [*A*] is the molar concentration of the antagonist (Kenakin, 2004).

2.2 | In vivo studies

2.2.1 | Animals

Four hundred eighty female and male Institute of Cancer Research (ICR) (CD-1®) adult mice weighing 30–35 g (Centralized Preclinical Research Laboratory, University of Ferrara, Italy) were group housed (five mice per cage; floor area per animal was 80 cm^2; minimum enclosure height was 12 cm), exposed to a 12:12-h light–dark cycle (light period from 6:30 AM to 6:30 PM) at a temperature of 20–22°C and humidity of 45%–55%, and were provided ad libitum access to food (Diet 4RF25 GLP; Mucedola, Settimo Milanese, Milan, Italy) and water. The experimental protocols performed in the present study were in accordance with the UK Animals (Scientific Procedures) Act of 1986 and associated guidelines and the new European Communities Council Directive of September 2010 (2010/63/EU). Experimental protocols were approved by the Italian Ministry of Health (Licence No. 223/2021-PR, CBCC2.46.EXT.21) and by the Animal Welfare Body of the University of Ferrara. Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020).

2.2.2 | Drug preparation and dose selection

Drugs were initially dissolved in absolute ethanol (final concentration: 5%) and Tween 80 (2%) and brought to the final volume with saline (0.9% NaCl). Drugs were administered by intraperitoneal (i.p.) injection at a volume of 4 $\mu l \cdot g^{-1}$. The dose range of BUF and 4F-BUF (0.1–6 $mg \cdot kg^{-1}$ i.p.) was chosen based on our previous studies (Bilel

et al., 2022). In antagonist experiments, the opioid receptor antagonist naloxone (6 mg·kg^{-1} i.p.) was administered 15 min before test compound injections, similar to our previous study (Bilel et al., 2022). The dose of ANT was chosen based on a previous study (Ducottet et al., 2003). Also, ANT was given intraperitoneally 15 min before the administration of the agonists (Broadbear et al., 2004). Animals were also injected with the combination ANT (10 mg·kg^{-1}) and naloxone (6 mg·kg^{-1}) 15 min before the administration of the test agonists. Female and male mice treated with naloxone alone (6 mg·kg^{-1}), ANT alone (10 mg·kg^{-1}) or their combination did not show any cardiorespiratory alterations during the 60-min period of measurements (data not shown).

2.2.3 | Tail withdrawal test

The mouse was restrained in a dark plastic cylinder, and half of its tail was dipped in 48°C water. Then, the length of time (s) the tail was kept in the water was recorded. The test was repeated three times (the final value represents the average), and a cut-off of 15 s was set to avoid tissue damage (Bilel et al., 2020). Acute thermal nociception was measured at 0, 5, 30, 60 and 120 min after injection.

2.2.4 | Rotarod test

Animals were placed on a rotating cylinder (Ugo Basile, Italy) that automatically increases in velocity in a constant manner (0–60 rotations/min in 5 min). The time spent on the cylinder was measured (Bilel et al., 2020). The accelerod test was performed at 0, 5, 30, 60 and 120 min after injection.

2.2.5 | Cardiorespiratory analysis

As previously reported by Marchette et al. (2023), ECG and PLETH were performed in conscious animals using a non-invasive ECG and PLETH-TUNNEL system with an acquisition frequency of 1000 Hz (emka TECHNOLOGIES, Paris, France). All PLETH recording sessions were performed during the daytime, and data were analysed using the IOX2 data acquisition analysis software (emka TECHNOLOGIES). A cylinder-shaped tube (i.e., restraining tunnel) restrains the animal firmly but gently, without causing trauma. Animals were trained to stay in the restraining system 1 week before the experimentation. In particular, in the habituation protocol, each mouse was allowed inside the dome for 15 min and then liberated and reinserted for 65 min. This process was repeated for three to four consecutive days. On the day of the experiments, animals were inserted inside domes for 1 min before the starting of measurements. The experiment provides baseline recording lasting for 15 min, followed by a recording session of 65 min immediately after vehicle or drug administration (Marchette et al., 2023).

2.2.6 | Data and statistical analysis

Antinociception (tail withdrawal test) is calculated as the per cent of maximal possible effect. Data are expressed as E_{max}%: {E_{max}% = [(test − control latency)/(cut-off time − control)] × 100}.

For the rotarod test, data are expressed as percentage of basal value (% of basal).

Emka TECHNOLOGIES ECG and PLETH analyser software were used to analyse tracings recorded during data acquisition. Six leads of ECG were recorded using specific software (IOX®, emka TECHNOLOGIES): Three leads were measured (L1–L3), and three were calculated (aVR, aVL and aVF). The parameters reported in this work were obtained from L1 and L2.

For the ECG analysis, HR (bpm), RR interval (ms; time between two consecutive peaks) and QRS (ms; time from the start to the end of the QRS complex) were expressed as % change of basal value.

For PLETH, BB_ie (ms; breath length, sum of the duration of inspiration and the duration of expiration), FR_ie (bpm; breathing frequency derived from BB_ie) and MV (F/min; computed from tidal volume and breathing frequency FR_ie) were expressed as % change of basal value.

Statistical analysis of the effects of the substances at different doses over time and of antagonism studies was done via repeated measures two-way analysis of variance (ANOVA) followed by a Bonferroni test for multiple comparisons (GraphPad Prism, Version 9, San Diego, CA, USA). Post-hoc tests were run only if F achieved $P<0.05$ and there was no significant variance inhomogeneity. The mean effect values represent the average of the effects induced by each compound at each dose over the time course of the experiment.

For all tests, a P value of <0.05 was considered statistically significant. The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2022).

2.2.7 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander Christopoulos, et al., 2023; Alexander, Fabbro et al., 2023).

3 | RESULTS

3.1 | In vitro studies

3.1.1 | Calcium mobilization studies

In CHO cells stably transfected with the human μ opioid receptor, the standard agonist dermorphin evoked a robust concentration-dependent stimulation of calcium release displaying high potency (pEC_{50} of 8.19) and maximal effects (319 ± 13% over the basal values).

fentanyl and its derivatives were able to activate the μ opioid receptor in a concentration-dependent manner with the following rank order of potency: fentanyl > 4F-BUF > BUF. Regarding ligand efficacy, fentanyl and BUF were able to elicit maximal effects similar to those of dermorphin, while 4F-BUF displayed statistically significantly lower maximal effects, thus behaving as a partial agonist (Table 1).

In CHO_{delta} cells, the standard agonist DPDPE evoked a robust concentration-dependent stimulation of calcium release with high potency (pEC_{50} of 7.47) and maximal effects (230 ± 18% over the basal values). fentanyl, BUF and 4F-BUF were inactive or displayed an incomplete concentration–response curve, stimulating calcium mobilization only at micromolar concentrations.

In CHO_{kappa} cells, the standard agonist dynorphin A evoked a robust concentration-dependent stimulation of calcium release with very high potency (pEC_{50} of 8.81) and maximal effects (257 ± 34% over the basal values). fentanyl, BUF and 4F-BUF were either inactive or displayed an incomplete concentration–response curve, stimulating calcium mobilization only at micromolar concentrations.

3.1.2 | BRET studies

In the BRET G-protein assay, dermorphin promoted μ/G-protein interaction in a concentration-dependent manner with pEC_{50} of 7.71 (7.41–8.01) and maximal effect of 0.96 ± 0.11 stimulated BRET ratio. fentanyl, BUF and 4F-BUF mimicked stimulatory effects elicited by dermorphin, showing similar maximal effects and slightly higher potency (Figure 2 and Table 2).

In the BRET β-arrestin assay, dermorphin stimulated the interaction of the μ receptor with β-arrestin 2 in a concentration-dependent manner with pEC_{50} 6.96 (6.56–7.37) and maximal effects corresponding to 0.24 ± 0.09 stimulated BRET ratio. Fentanyl and BUF were able to promote the interaction of the μ receptor with β-arrestin 2, showing similar potency but significantly reduced efficacy; in fact, the two compounds behaved as partial agonists. In the BRET β-arrestin assay, 4F-BUF was completely inactive as an agonist (Figure 2d). Thus, 4F-BUF was further investigated as an antagonist of fentanyl-induced β-arrestin 2 recruitment. At 0.1 μM, 4F-BUF was able to shift the concentration–response curve to fentanyl to the right with no modifications of the agonist maximal effect (see Figure S1); a pA_2 of 7.81 was derived from these experiments for 4F-BUF.

3.2 | In vivo studies

3.2.1 | Tail withdrawal test

Acute thermal pain stimuli were not affected in female and male mice treated with the vehicle (Figures 3 and 4). Systemic administration of BUF and 4F-BUF (0.1–6 $mg \cdot kg^{-1}$ i.p.) increased the threshold to the acute thermal pain stimulus in female and male mice in the tail withdrawal test (Figures 3 and 4). In particular, after the administration of BUF, thermal antinociception was significantly affected by treatment (Figure 3). Maximal antinociception threshold was reached at the dose of 6 $mg \cdot kg^{-1}$ after 5 min of injection in both sexes (Figure 3a–c). The antinociceptive effect of BUF (6 $mg \cdot kg^{-1}$) was long-lasting in female mice, while it disappeared after 120 min from the injection in males (Figure 3a–c).

Pre-treatment with naloxone alone (6 $mg \cdot kg^{-1}$ i.p.) fully prevented the antinociception induced by BUF (6 $mg \cdot kg^{-1}$ i.p.) in male mice (Figure 3d), while in female mice, it reappeared at 120 min (Figure 3b). Moreover, pre-treatment with ANT (6 $mg \cdot kg^{-1}$ i.p.) did not prevent antinociception in both sexes (Figure 3b–d).

Systemic administration of 4F-BUF (0.1–6 $mg \cdot kg^{-1}$ i.p.) increased the threshold to the acute thermal pain stimulus in female and male mice in the tail withdrawal test (Figure 4). In particular, after the administration of 4F-BUF, thermal antinociception was significantly affected by treatment (Figure 4). Maximal antinociception was reached at the dose of 6 $mg \cdot kg^{-1}$ after 5 min of injection in both sexes (Figure 4a–c), and the effect persisted until the end of the test

TABLE 1 Effects of dermorphin, fentanyl, BUF and 4F-BUF in calcium mobilization experiments performed in CHO cells co-expressing opioid receptors and chimeric G proteins.

	μ		δ		κ	
	pEC_{50} ($CL_{95\%}$)	E_{max} ± SEM %	pEC_{50} ($CL_{95\%}$)	E_{max} ± SEM %	pEC_{50} ($CL_{95\%}$)	E_{max} ± SEM %
Standard agonists	8.19 (8.02–8.36)	319 ± 13	7.47 (7.09–7.85)	230 ± 18	8.81 (8.22–9.40)	257 ± 34
Fentanyl	8.13 (7.73–8.52)	326 ± 13	CRC incomplete		CRC incomplete	
BUF	7.32 (6.70–7.93)	283 ± 18	CRC incomplete		CRC incomplete	
4F-BUF	7.50 (6.92–8.09)	258 ± 8*	Inactive		Inactive	

Note: Data are mean of at least three separate experiments made in duplicate. Standard agonists were dermorphin, DPDPE and dynorphin A for μ, δ and κ opioid receptors, respectively (data on standard agonists and fentanyl were reported in Bilel et al., 2022).

Abbreviations: 4F-BUF, 4-fluorobutyrylfentanyl or para-fluorobutyrylfentanyl; ANOVA, analysis of variance; BUF, butyrylfentanyl; CHO, Chinese hamster ovary; $CL_{95\%}$, 95% confidence limits; CRC: concentration- response curve; DPDPE, (D-Pen^2,D-Pen^5)-enkephalin.

*$P < 0.05$ versus dermorphin according to ANOVA followed by Dunnett's test.

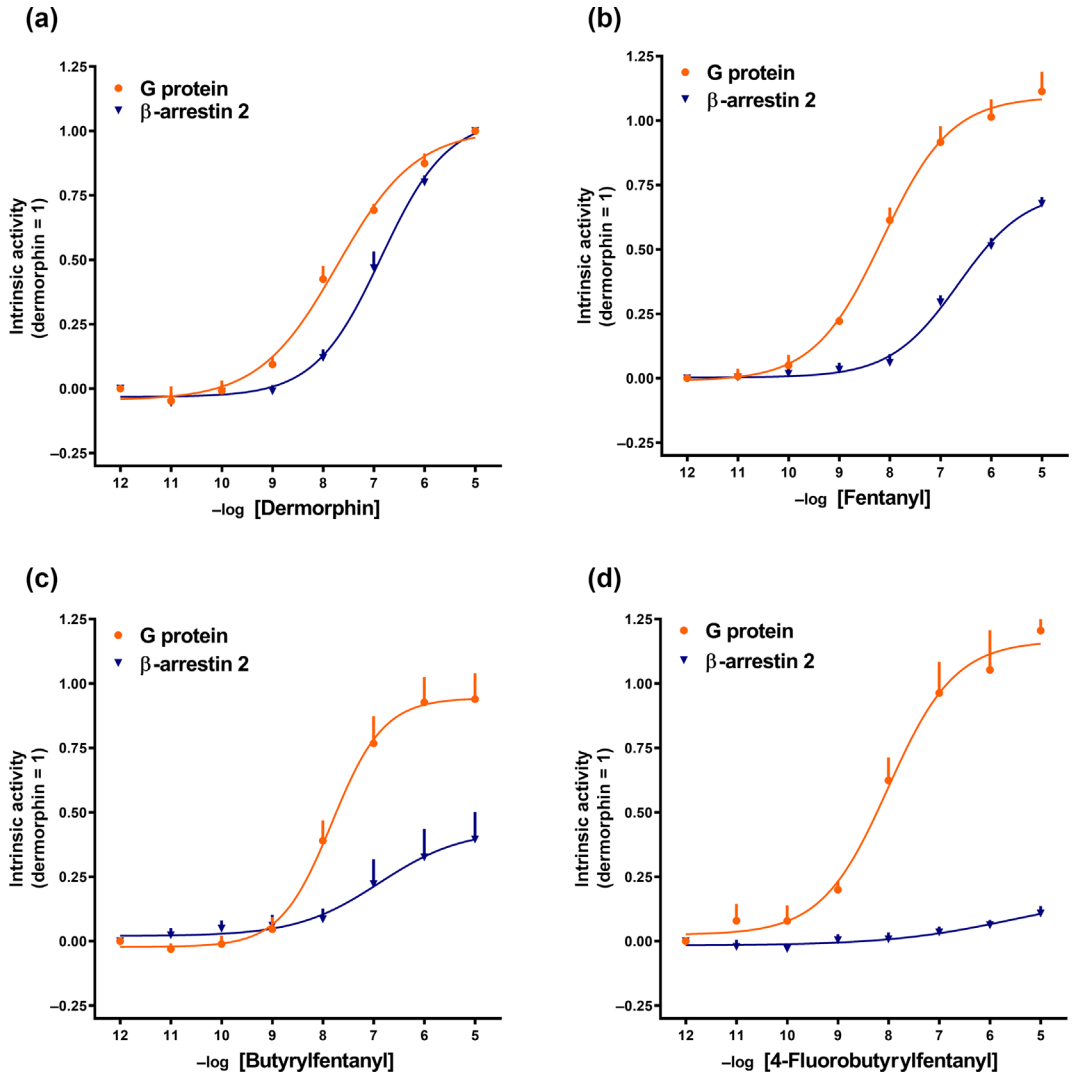

FIGURE 2 Bioluminescence resonance energy transfer assay. Concentration–response curve to (a) dermorphin, (b) fentanyl, (c) butyrylfentanyl and (d) 4-fluorobutyrylfentanyl for μ receptor/G-protein and μ receptor/β-arrestin 2 interaction. Data are the mean ± SEM of five separate experiments made in duplicate.

	μ receptor/G protein		μ receptor /β-arrestin2	
	pEC_{50} ($CL_{95\%}$)	α ± SEM	pEC_{50} ($CL_{95\%}$)	α ± SEM
Dermorphin	7.71 (7.41–8.01)	1.00	6.96 (6.56–7.37)	1.00
Fentanyl	8.28 (8.06–8.49)	1.11 ± 0.07	6.86 (6.54–7.18)	0.67 ± 0.02[a]
BUF	7.87 (7.34–8.40)	0.94 ± 0.10	6.54 (5.83–7.25)	0.39 ± 0.10*
4F-BUF	8.02 (7.65–8.40)	1.20 ± 0.19	Inactive	

TABLE 2 Effects of dermorphin, fentanyl and its derivatives in BRET experiments investigating mu/G-protein and mu/β-arrestin 2 interaction.

Note: Data on standard agonists and fentanyl were reported in Bilel et al. (2022).
Abbreviations: 4F-BUF, 4-fluorobutyrylfentanyl or para-fluorobutyrylfentanyl; BRET, bioluminescence resonance energy transfer; BUF, butyrylfentanyl; $CL_{95\%}$, 95% confidence limit. [a]The inclusion of the standard's E_{max} within the $CL_{95\%}$ of a ligand was used as a criterion to determine whether there was a significant difference from the standard.

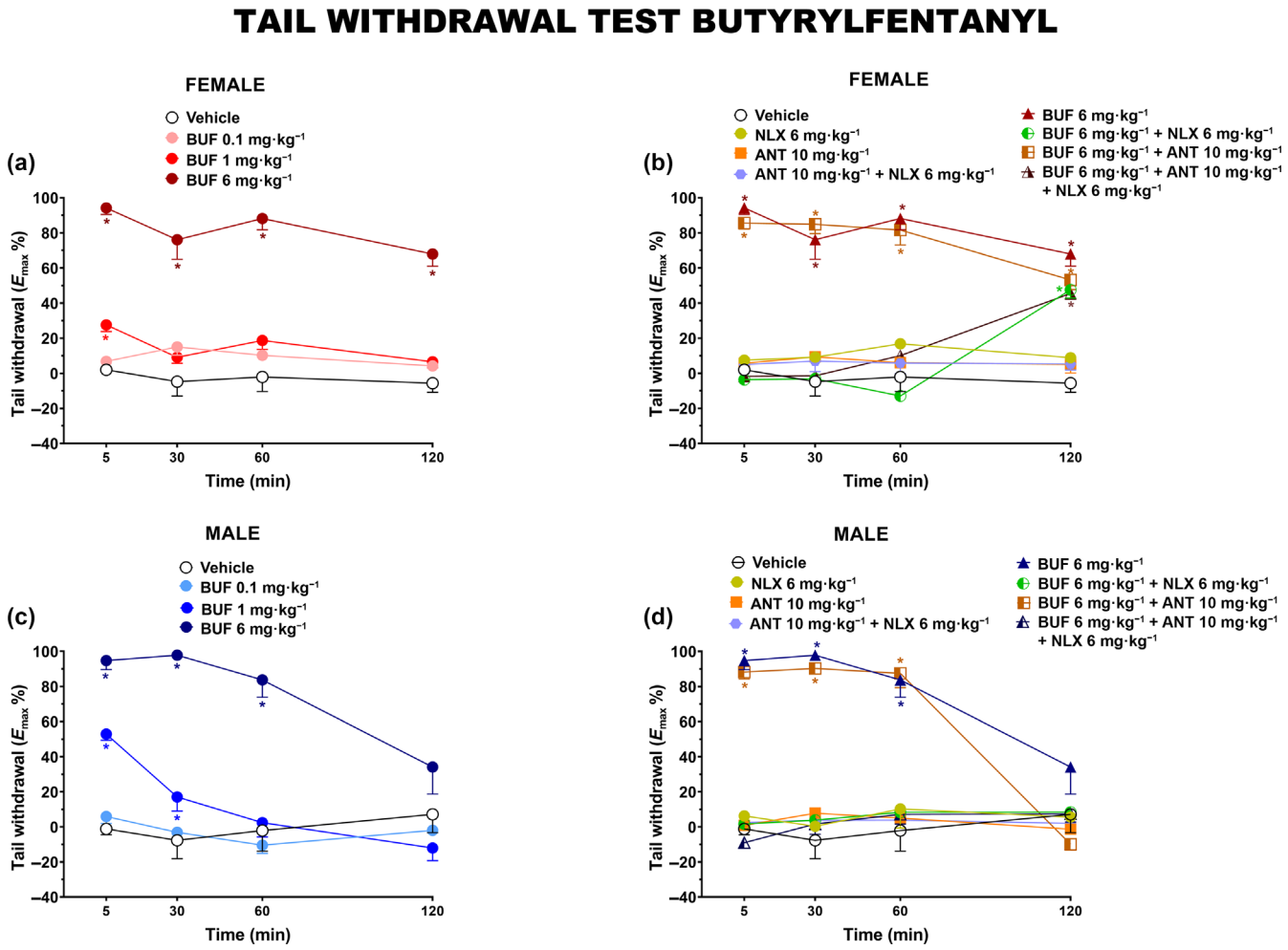

FIGURE 3 Tail withdrawal test: effect of the systemic administration of butyrylfentanyl (BUF) (0.1–6 mg·kg^{-1} i.p.) in (a) female and (c) male mice. (b, d) Interaction of the effective dose of BUF (6 mg·kg^{-1}) with the opioid receptor antagonist naloxone (NLX) (6 mg·kg^{-1}) or the corticotropin-releasing factor 1 antagonist antalarmin (ANT) (10 mg·kg^{-1}) or their combination (ANT 10 mg·kg^{-1} + NLX 6 mg·kg^{-1}). Data are expressed as percentage of maximum (see Section 2) and represent the mean ± SEM of 6 determinations for each treatment. Statistical analysis was performed by two-way analysis of variance followed by Bonferroni's test for multiple comparisons for the dose–response curve of the compounds at different times (a–d). *P < 0.05 versus vehicle.

(Figure 4a–c). In contrast to females, 4F-BUF (1 mg·kg^{-1}) induced a significant and moderate antinociception in male mice only after 5 min of injection (Figure 4c).

Pre-treatment with naloxone alone (6 mg·kg^{-1} i.p.) totally prevented the antinociception induced by 4F-BUF (6 mg·kg^{-1} i.p.) in female and male mice (Figure 4b–d). Moreover, pre-treatment with ANT (6 mg·kg^{-1} i.p.) did not significantly modify antinociception in both sexes (Figure 4b–d).

The comparison of the dose–response curves of BUF and 4F-BUF on female and male mice (Figure S2A,B) did not reveal sex differences in terms of thermal antinociception. The extrapolation based on the slope of the dose–response curves (Figure S2A–D) reveals a similar value of ED_{50} ± 3 mg·kg^{-1} of the two compounds and in both sexes.

The comparison of the dose–response curves of BUF and 4F-BUF on male mice revealed significant differences of effectiveness between the two compounds at the dose of 6 mg·kg^{-1} (Figure S2D).

All the statistical analyses are illustrated in Table S1.

3.2.2 | Rotarod test

Female and male mice treated with a vehicle did not change their performance in the rotarod test (Figures 5 and 6). Systemic administration of BUF and 4F-BUF (0.1–6 mg·kg^{-1}) significantly modified animal performance in the rotarod test. In particular, BUF produced monophasic inhibitory effects in female mice (Figure 5a), while in males, the effect was biphasic with transient inhibitory effects at 1 mg·kg^{-1} and persistent facilitatory effects at 6 mg·kg^{-1} (Figure 5c).

Pre-treatment with naloxone (6 mg·kg^{-1} i.p.), ANT (6 mg·kg^{-1} i.p.) or their combination totally prevented motor impairments induced by BUF 6 mg·kg^{-1} i.p. in female mice (Figure 5b). Pre-treatment with naloxone (6 mg·kg^{-1} i.p.), ANT (6 mg·kg^{-1} i.p.) or their combination totally prevented the motor facilitatory effects of BUF (6 mg·kg^{-1} i.p.) in male mice (Figure 5d).

Systemic administration of 4F-BUF (0.1–6 mg·kg^{-1}) significantly modified female and male mice performances in rotarod test

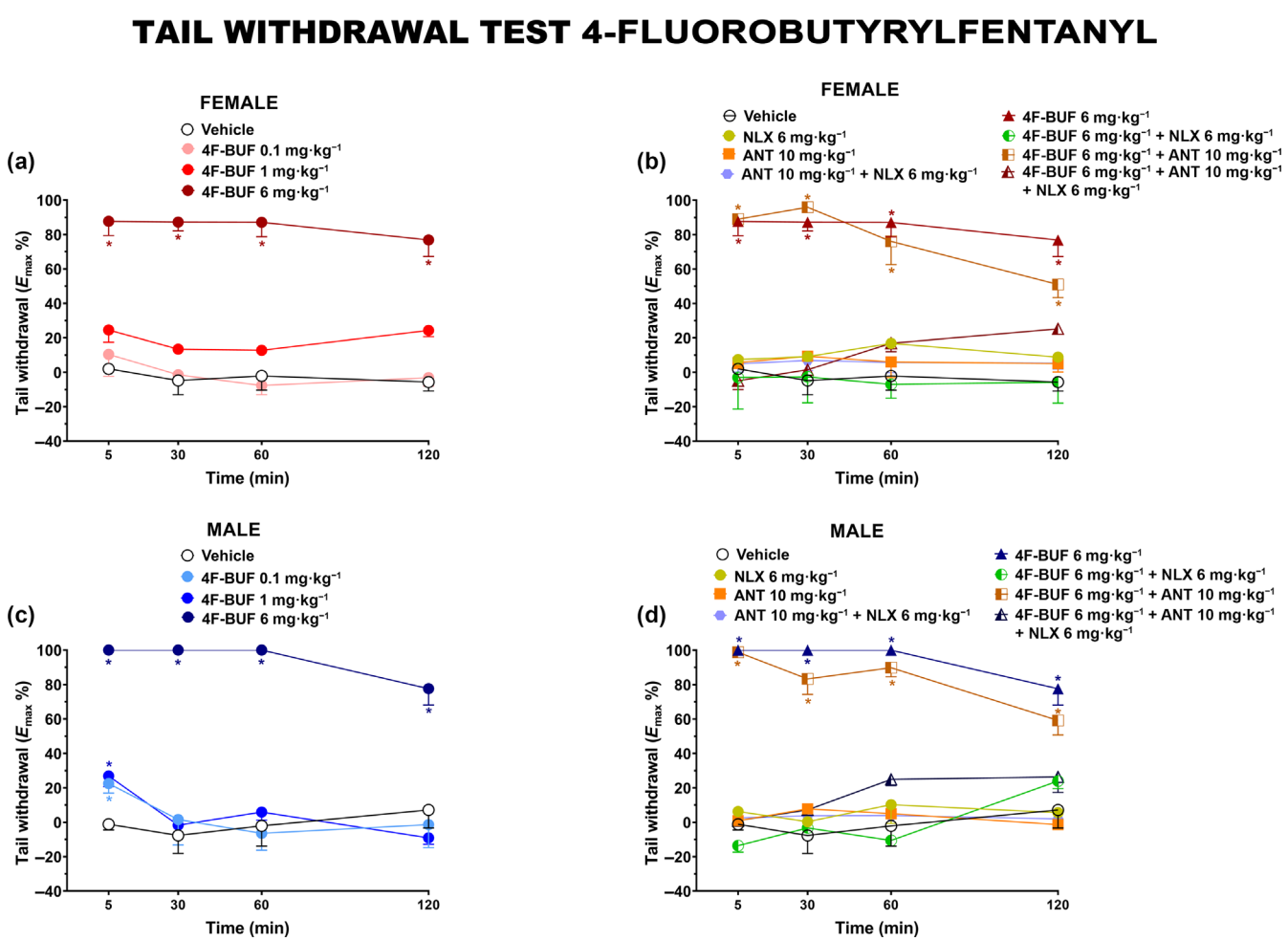

FIGURE 4 Tail withdrawal test: effect of the systemic administration of 4-fluorobutyrylfentanyl (4F-BUF) (0.1–6 mg·kg^{-1} i.p.) in (a) female and (c) male mice. (b, d) Interaction of the effective dose of butyrylfentanyl (BUF) (6 mg·kg^{-1}) with the opioid receptor antagonist naloxone (NLX) (6 mg·kg^{-1}) or the corticotropin-releasing factor 1 (CRF_1) antagonist antalarmin (ANT) (10 mg·kg^{-1}) or their combination (ANT 10 mg·kg^{-1} + NLX 6 mg·kg^{-1}). Data are expressed as percentage of maximum (see Section 2) and represent the mean ± SEM of 6 determinations for each treatment. Statistical analysis was performed by two-way analysis of variance followed by Bonferroni's test for multiple comparisons for the dose–response curve of the compounds at different times (a–d). *P < 0.05 versus vehicle.

(Figure 6). In particular, the administration of 4F-BUF produced biphasic effects in female mice (Figure 6a) with transient stimulatory effects at 1 mg·kg^{-1} and inhibitory effects at 6 mg·kg^{-1} (Figure 6c), while in males, the effect was monophasic and stimulatory at the doses of 1 and 6 mg·kg^{-1}.

Pre-treatment with naloxone alone (6 mg·kg^{-1} i.p.), ANT alone (6 mg·kg^{-1} i.p.) or the combination of ANT (10 mg·kg^{-1} i.p.) and naloxone (6 mg·kg^{-1} i.p.) totally prevented motor impairments induced by 4F-BUF (6 mg·kg^{-1} i.p.) in female and male mice (Figure 6b–d).

The comparison of the dose–response curves of BUF and 4F-BUF on female and male mice (Figure S3A,B) reveals sex differences in female and male performances at the dose of 6 mg·kg^{-1}.

The comparison of the dose–response curves of BUF and 4F-BUF on female and male mice revealed significant differences of effectiveness between the two compounds at the dose of 1 mg·kg^{-1} (Figure S3C) between females and 1 and 6 mg·kg^{-1} between males (Figure S3D). All the statistical analyses are illustrated in Table S1.

3.2.3 | Cardiorespiratory analysis

The vehicle used in this experiment showed a stable profile during the 80 min of the ECG (HR, RR and QRS) and PLETH (FR_ie, BB_ie and MV) parameter measurement in both sexes (Figures 7–14). Systemic administration of BUF and 4F-BUF (0.1–6 mg·kg^{-1} i.p.) induced important variations in cardiorespiratory parameters in female and male mice, especially at the highest dose tested (see Figure S4). All the statistical analyses are illustrated in Table S2.

3.2.4 | ECG: BUF

Female ECG measurements

HR (Figure 7) was immediately and significantly affected by BUF treatment (Figure 7a). In addition, RR interval increased immediately and significantly in female BUF-treated mice (Figure 7c). The QRS

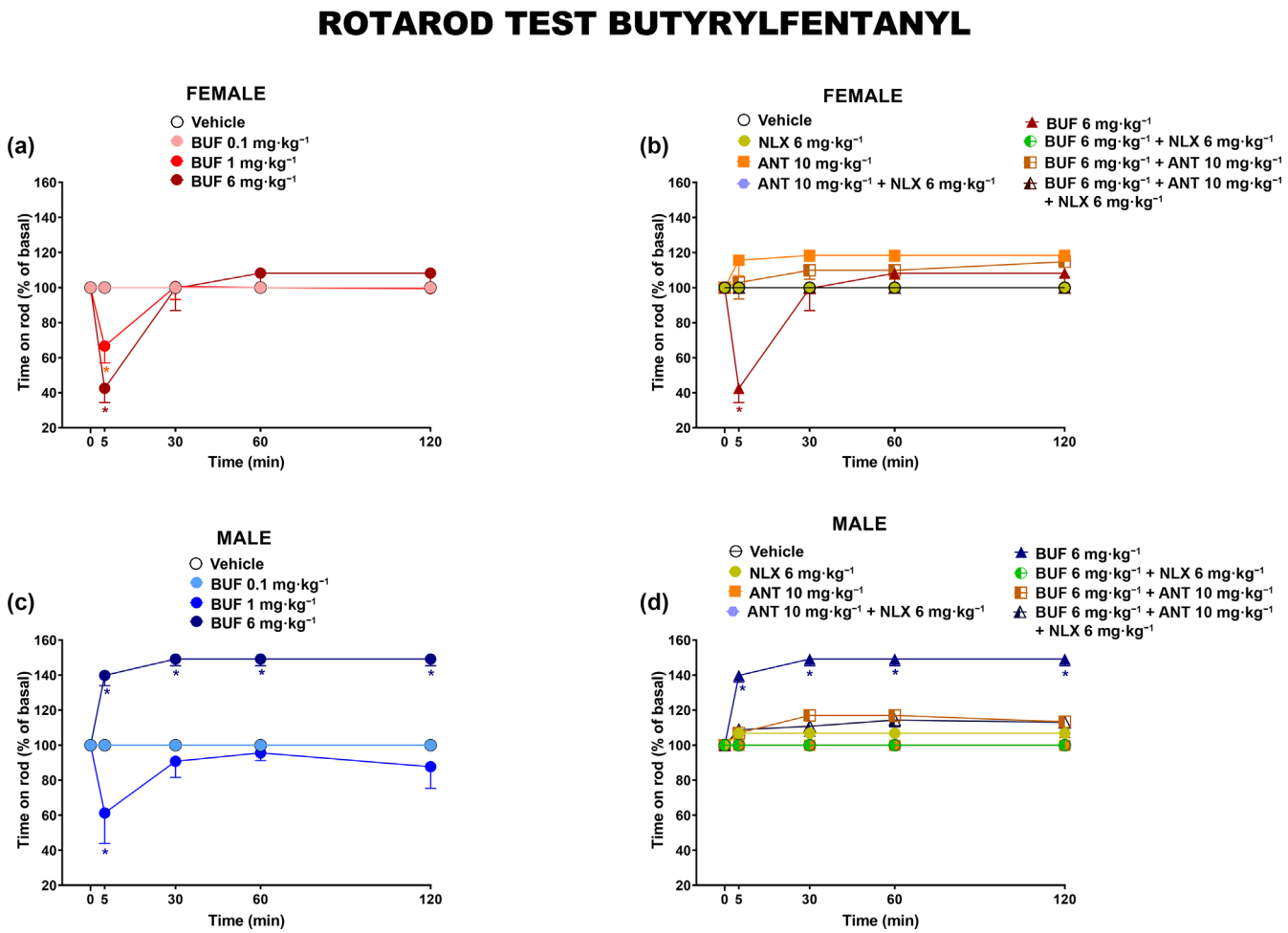

FIGURE 5 Rotarod test: effect of the systemic administration of butyrylfentanyl (BUF) (0.1–6 mg·kg^{-1} i.p.) in (a) female and (c) male mice. (b, d) Interaction of the effective dose of BUF (6 mg·kg^{-1}) with the opioid receptor antagonist naloxone (NLX) (6 mg·kg^{-1}) or the corticotropin-releasing factor 1 (CRF_1) antagonist antalarmin (ANT) (10 mg·kg^{-1}) or their combination (ANT 10 mg·kg^{-1} + NLX 6 mg·kg^{-1}). Data are expressed as percentage of basal value (see Section 2) and represent the mean ± SEM of 6 determinations for each treatment. Statistical analysis was performed by two-way analysis of variance followed by Bonferroni's test for multiple comparisons for the dose–response curve of the compounds at different times (a–d). *P < 0.05 versus vehicle.

complex increased immediately and significantly with the dose of 6 mg·kg^{-1} of BUF (Figure 7e).

Pre-treatment with naloxone (6 mg·kg^{-1} i.p.; Figure 7b) partially prevented female BUF bradycardia. The pre-treatment with ANT (10 mg·kg^{-1} i.p.) was not effective in preventing the effects of BUF on HR (Figure 7b), RR interval (Figure 7d) and QRS complex (Figure 7f).

Male ECG measurements

HR (Figure 8) was immediately and significantly affected by BUF treatment (Figure 8a). In addition, RR interval increased immediately and significantly in male BUF-treated mice (Figure 8c). The QRS complex increased immediately and significantly with the doses of 1 and 6 mg·kg^{-1} of BUF.

Pre-treatment with naloxone (6 mg·kg^{-1} i.p.; Figure 8b) partially prevented female BUF bradycardia, while ANT (10 mg·kg^{-1} i.p.) was not effective in preventing the effects of BUF on HR (Figure 8b), RR interval (Figure 8d) and QRS complex (Figure 8f).

3.2.5 | PLETH: BUF

Female PLETH measurements

Respiratory rate (Figure 9) was immediately and significantly affected by BUF treatment (Figure 9a). In addition, breath length increased immediately and significantly in female BUF-treated mice (Figure 9c). MV also decreased immediately and significantly after the administration of BUF (Figure 9e).

Pre-treatment with naloxone alone (6 mg·kg^{-1} i.p.; Figure 9b) partially prevented BUF bradypnoea but did not prevent MV reduction (Figure 9f). However, ANT (10 mg·kg^{-1} i.p.) alone or ANT (10 mg·kg^{-1} i.p.) in combination with naloxone (6 mg·kg^{-1} i.p.) was effective in preventing the effects of BUF on FR_ie (Figure 9b), BB_ie (Figure 9d) and MV (Figure 9f).

Male PLETH measurements

Respiratory rate (Figure 10) was immediately and significantly affected by BUF treatment (Figure 10a). In addition, breath length increased

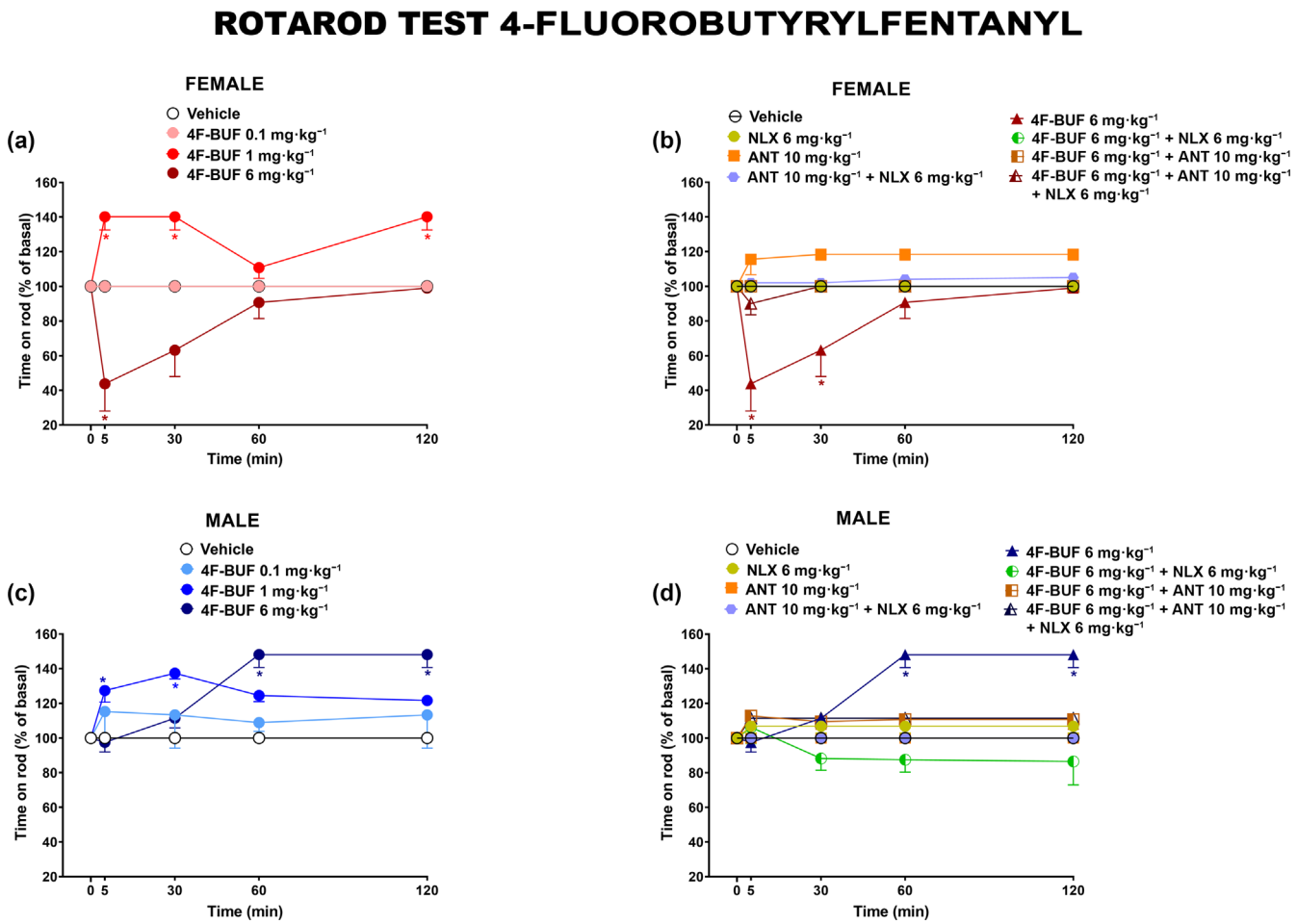

FIGURE 6 Rotarod test: effect of the systemic administration of 4-fluorobutyrylfentanyl (4F-BUF) (0.1–6 mg·kg^{-1} i.p.) in (a) female and (c) male mice. (b, d) Interaction of the effective dose of butyrylfentanyl (BUF) (6 mg·kg^{-1}) with the opioid receptor antagonist naloxone (NLX) (6 mg·kg^{-1}) or the corticotropin-releasing factor 1 (CRF_1) antagonist antalarmin (ANT) (10 mg·kg^{-1}) or their combination (ANT 10 mg·kg^{-1} + NLX 6 mg·kg^{-1}). Data are expressed as percentage of basal value (see Section 2) and represent the mean ± SEM of 6 determinations for each treatment. Statistical analysis was performed by two-way analysis of variance followed by Bonferroni's test for multiple comparisons for the dose–response curve of the compounds at different times (a–d). *$P < 0.05$ versus vehicle.

immediately and significantly in BUF-treated mice (Figure 10c). MV also decreased immediately and significantly after the administration of BUF (Figure 10e).

Pre-treatment with naloxone alone (6 mg·kg^{-1} i.p.; Figure 10b) partially prevented male BUF respiratory depression and MV reduction (Figure 10f). However, ANT (10 mg·kg^{-1} i.p.) alone or ANT (10 mg·kg^{-1} i.p.) in combination with naloxone (6 mg·kg^{-1} i.p.) was effective in preventing the effects of BUF on FR_ie (Figure 10b), BB_ie (Figure 10d) and MV (Figure 10f).

3.2.6 | ECG: 4F-BUF

Female ECG measurements

HR (Figure 11) was immediately and significantly affected by 4F-BUF treatment (Figure 11a). In addition, RR interval increased immediately and significantly in female BUF-treated mice (Figure 11c). The QRS complex increased immediately and significantly with the dose of 6 mg·kg^{-1} of BUF (Figure 11e).

Pre-treatment with naloxone (6 mg·kg^{-1} i.p.; Figure 11b) partially prevented female 4F-BUF bradycardia. ANT (10 mg·kg^{-1} i.p.) alone was not effective in preventing the effects of BUF on HR (Figure 11b), RR interval (Figure 11d) and QRS complex (Figure 11f). However, the pre-treatment with ANT (10 mg·kg^{-1} i.p.) in combination with naloxone (6 mg·kg^{-1} i.p.) reduced significantly the cardiorespiratory impairments with respect to their single administration (Figure 11b–d,f), and the transitory effect of naloxone became more persistent in the presence of ANT.

Male ECG measurements

HR (Figure 12) was immediately and significantly affected by 4F-BUF treatment (Figure 12a). In addition, RR interval increased immediately and significantly in 4F-BUF-treated mice (Figure 12c). The QRS complex increased immediately and significantly with the doses of 1 and 6 mg·kg^{-1} of 4F-BUF (Figure 12e).

Pre-treatment with naloxone alone (6 mg·kg^{-1} i.p.; Figure 12b) partially prevented male 4F-BUF bradycardia. ANT (10 mg·kg^{-1} i.p.) alone was not effective in preventing the effects of 4F-BUF on HR

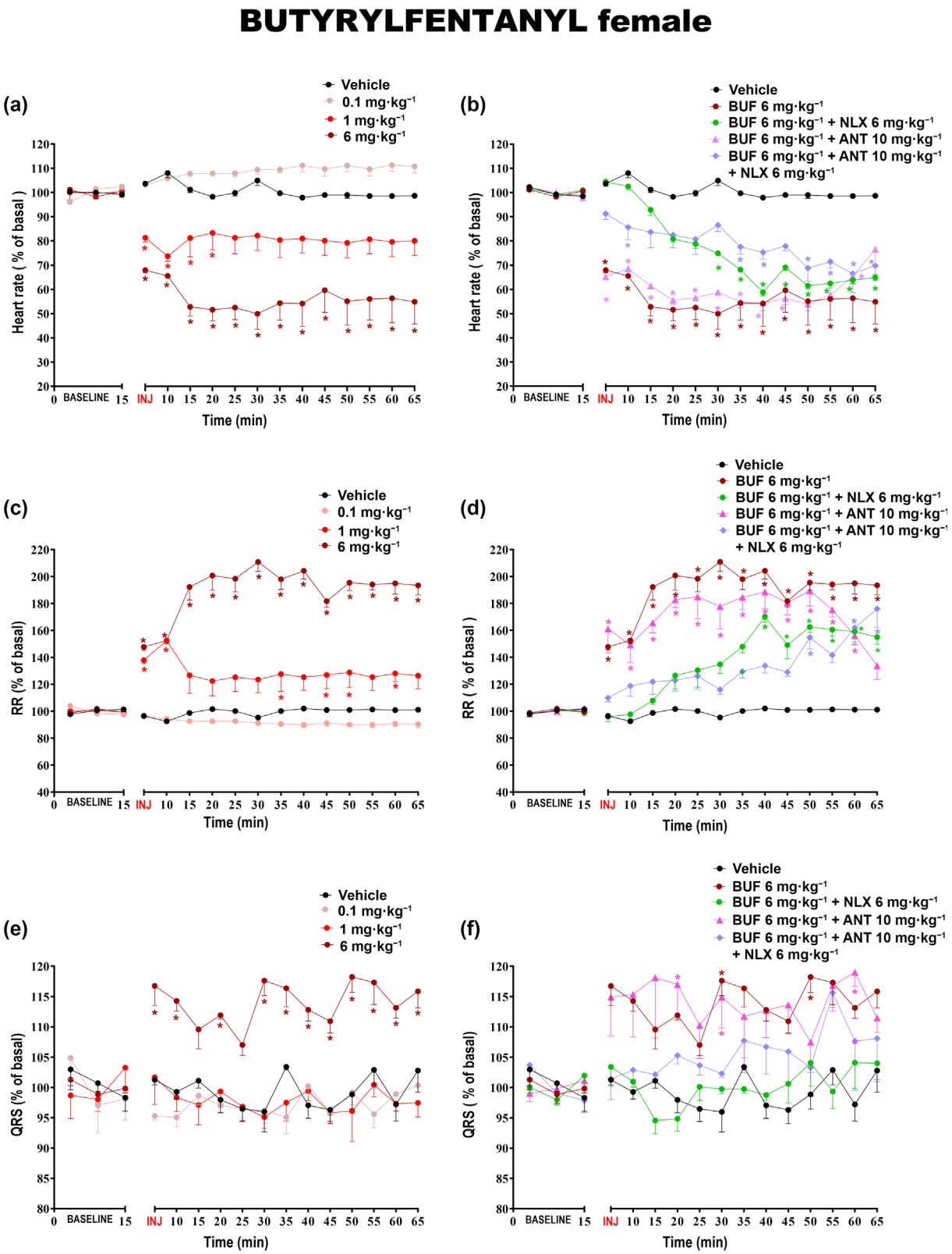

FIGURE 7 Electrocardiogram measurements: systemic administration of butyrylfentanyl (BUF) (0.1–6 mg·kg^{-1} i.p.) in (a) heart rate, (c) RR interval and (e) QRS complex in female mice. (b, d, f) Interaction of the effective dose of BUF (6 mg·kg^{-1}) with the opioid receptor antagonist naloxone (NLX) (6 mg·kg^{-1}) or the corticotropin-releasing factor 1 (CRF_1) antagonist antalarmin (ANT) (10 mg·kg^{-1}) or their combination (ANT 10 mg·kg^{-1} + NLX 6 mg·kg^{-1}). Data are expressed as percentage of baseline (see Section 2) and represent the mean ± SEM of 6 determinations for each treatment. Statistical analysis was performed by two-way analysis of variance followed by Bonferroni's test for multiple comparisons for the dose–response curve of the compounds at different times (a–f). *P < 0.05 versus vehicle.

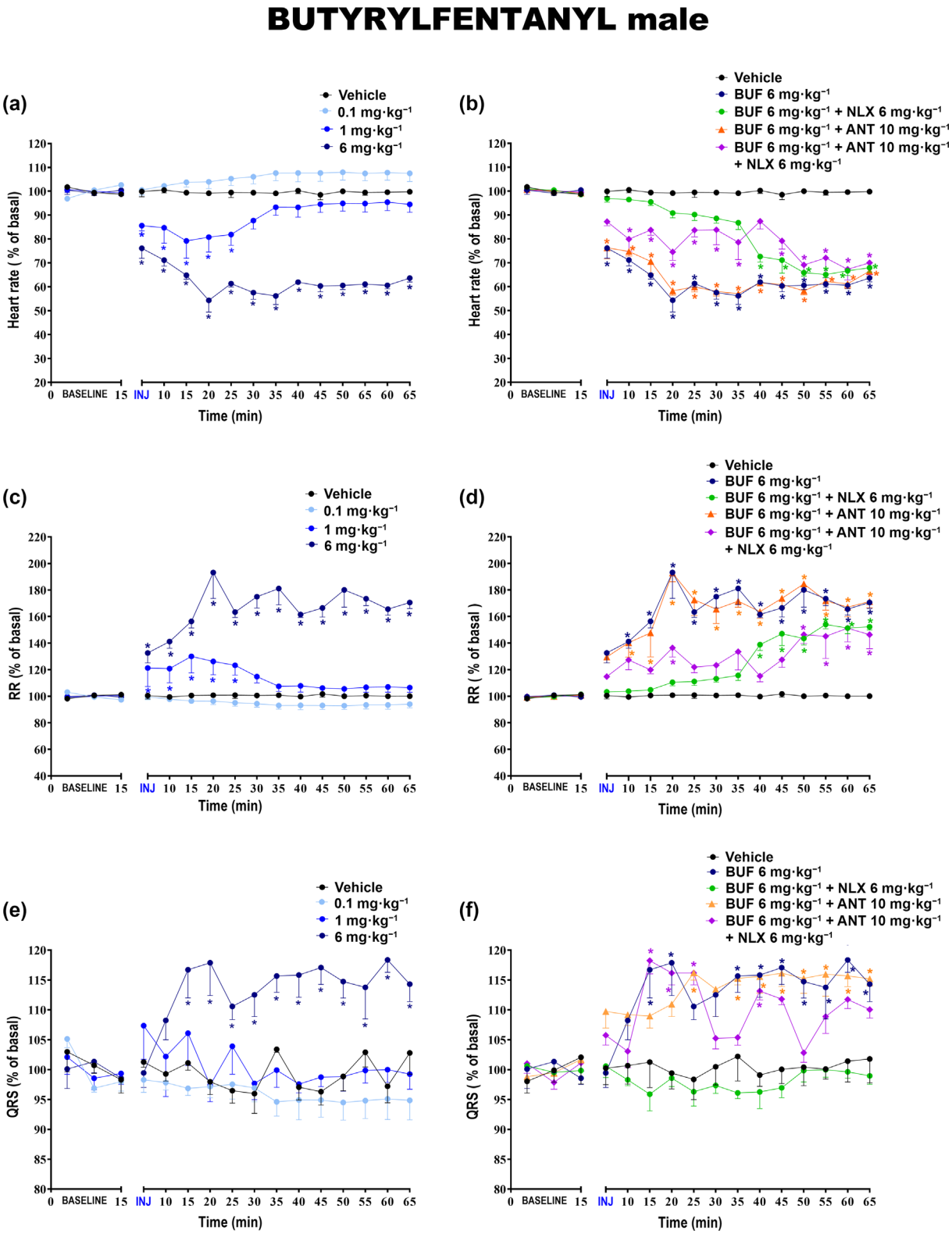

FIGURE 8 Electrocardiogram measurements: systemic administration of butyrylfentanyl (BUF) (0.1–6 mg·kg^{-1} i.p.) in (a) heart rate, (c) RR interval and (e) QRS complex in male mice. (b, d, f) Interaction of the effective dose of BUF (6 mg·kg^{-1}) with the opioid receptor antagonist naloxone (NLX) (6 mg·kg^{-1}) or the corticotropin-releasing factor 1 (CRF_1) antagonist antalarmin (ANT) (10 mg·kg^{-1}) or their combination (ANT 10 mg·kg^{-1} + NLX 6 mg·kg^{-1}). Data are expressed as percentage of baseline (see Section 2) and represent the mean ± SEM of 6 determinations for each treatment. Statistical analysis was performed by two-way analysis of variance followed by Bonferroni's test for multiple comparisons for the dose–response curve of the compounds at different times (a–f). *P < 0.05 versus vehicle.

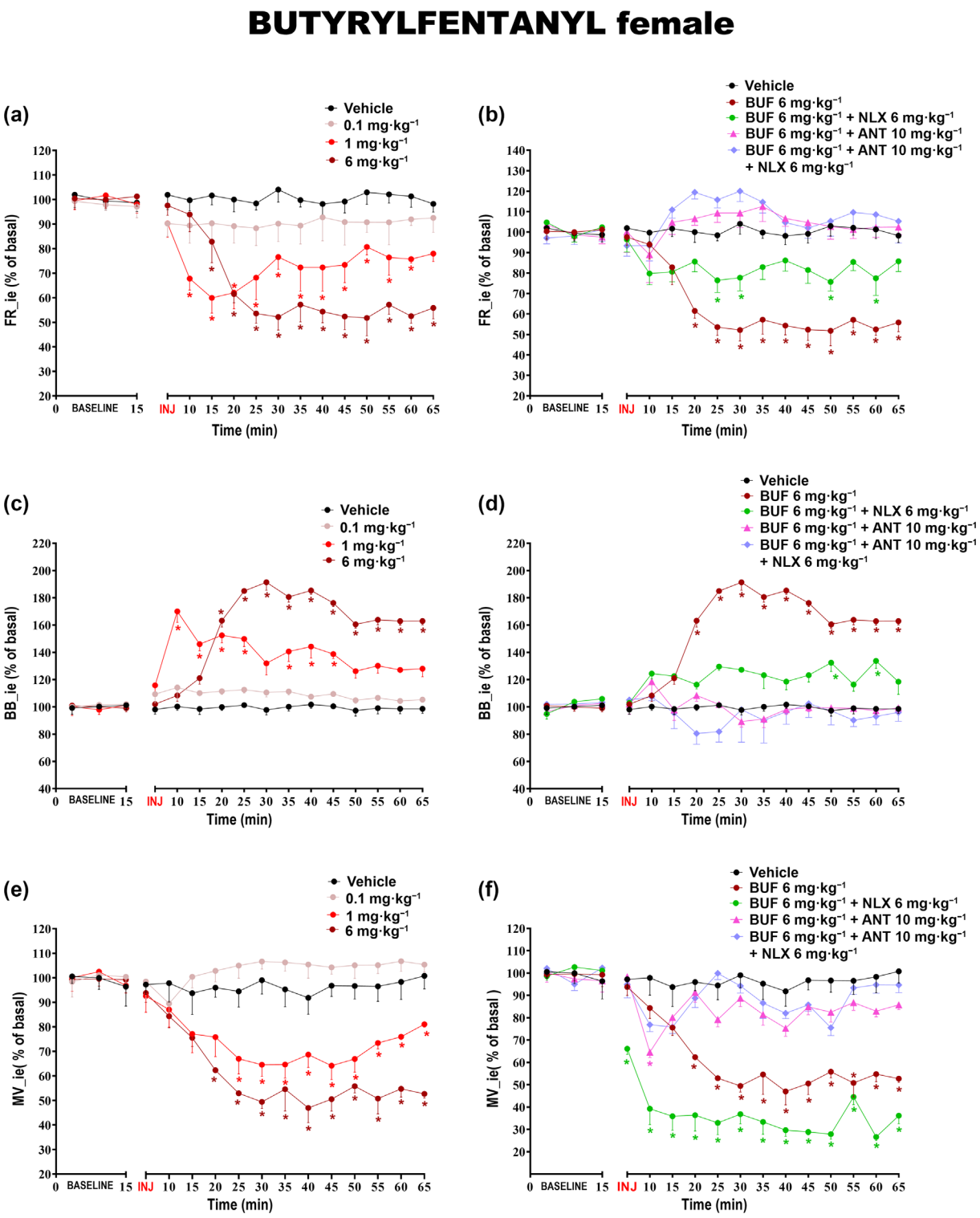

FIGURE 9 Plethysmography measurements: systemic administration of butyrylfentanyl (BUF) (0.1–6 mg·kg^{-1} i.p.) in (a) breath rate, (c) breath length and (e) minute volume in female mice. (b, d, f) Interaction of the effective dose of BUF (6 mg·kg^{-1}) with the opioid receptor antagonist naloxone (NLX) (6 mg·kg^{-1}) or the corticotropin-releasing factor 1 (CRF_1) antagonist antalarmin (ANT) (10 mg·kg^{-1}) or their combination (ANT 10 mg·kg^{-1} + NLX 6 mg·kg^{-1}). Data are expressed as percentage of baseline (see Section 2) and represent the mean ± SEM of 6 determinations for each treatment. Statistical analysis was performed by two-way analysis of variance followed by Bonferroni's test for multiple comparisons for the dose–response curve of the compounds at different times (a–f). *$P < 0.05$ versus vehicle.

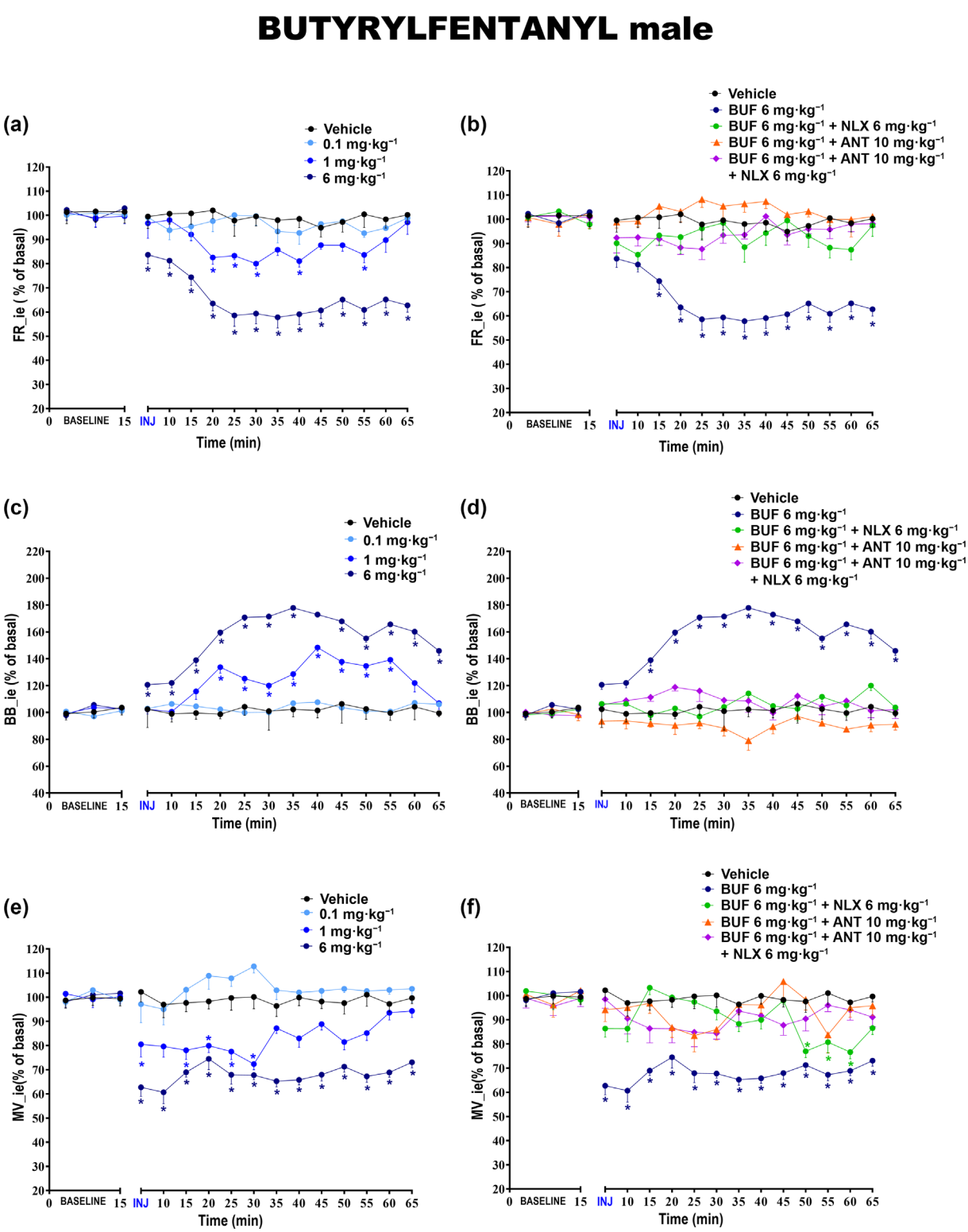

FIGURE 10 Plethysmography measurements: systemic administration of butyrylfentanyl (BUF) (0.1–6 mg·kg^{-1} i.p.) in (a) breath rate (FR ie), (c) breath length (BB ie) and (e) minute volume (MV) in male mice. (b, d, f) Interaction of the effective dose of BUF (6 mg·kg^{-1}) with the opioid receptor antagonist naloxone (NLX) (6 mg·kg^{-1}) or the corticotropin-releasing factor 1 (CRF_1) antagonist antalarmin (ANT) (10 mg·kg^{-1}) or their combination (ANT 10 mg·kg^{-1} + NLX 6 mg·kg^{-1}). Data are expressed as percentage of baseline (see Section 2) and represent the mean ± SEM of 6 determinations for each treatment. Statistical analysis was performed by two-way analysis of variance followed by Bonferroni's test for multiple comparisons for the dose–response curve of the compounds at different times (a–f). *P < 0.05 versus vehicle.

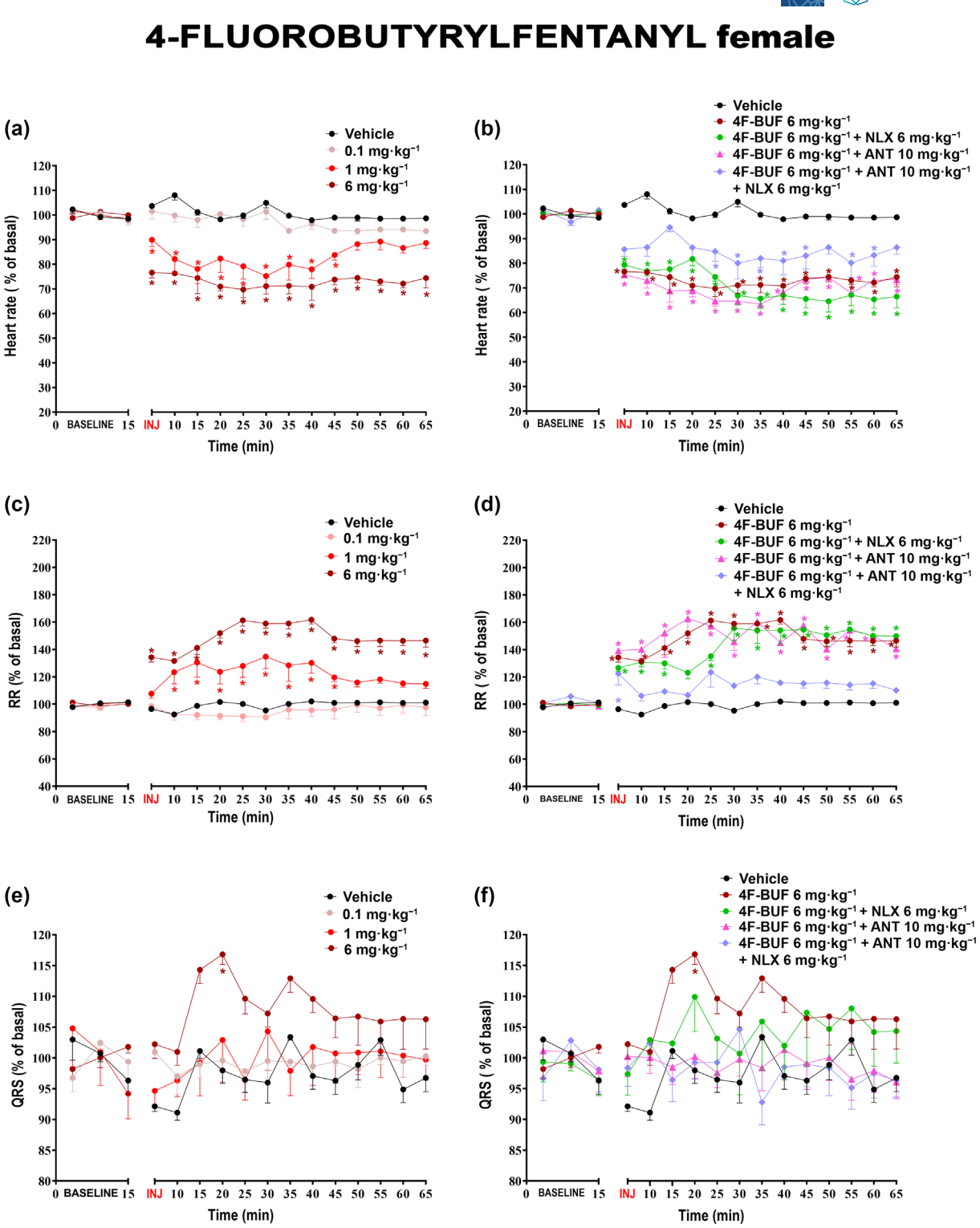

FIGURE 11 Electrocardiogram measurements: systemic administration of 4-fluorobutyrylfentanyl (4F-BUF) (0.1–6 mg·kg^{-1} i.p.) in (a) heart rate, (c) RR interval and (e) QRS complex in female mice. (b, d, f) Interaction of the effective dose of 4F-BUF (6 mg·kg^{-1}) with the opioid receptor antagonist naloxone (NLX) (6 mg·kg^{-1}) or the corticotropin-releasing factor 1 (CRF_1) antagonist antalarmin (ANT) (10 mg·kg^{-1}) or their combination (ANT 10 mg·kg^{-1} + NLX 6 mg·kg^{-1}). Data are expressed as percentage of baseline (see Section 2) and represent the mean ± SEM of 6 determinations for each treatment. Statistical analysis was performed by two-way analysis of variance followed by Bonferroni's test for multiple comparisons for the dose–response curve of the compounds at different times (a–f). *P < 0.05 versus vehicle.

(Figure 12b), RR interval (Figure 12d) and QRS complex (Figure 12f). However, the pre-treatment with ANT (10 mg·kg^{-1} i.p.) in combination with naloxone (6 mg·kg^{-1} i.p.) reduced significantly the cardiorespiratory impairment with respect to their single administration (Figure 12b–d,f), and the transitory effect of naloxone became more persistent in the presence of ANT.

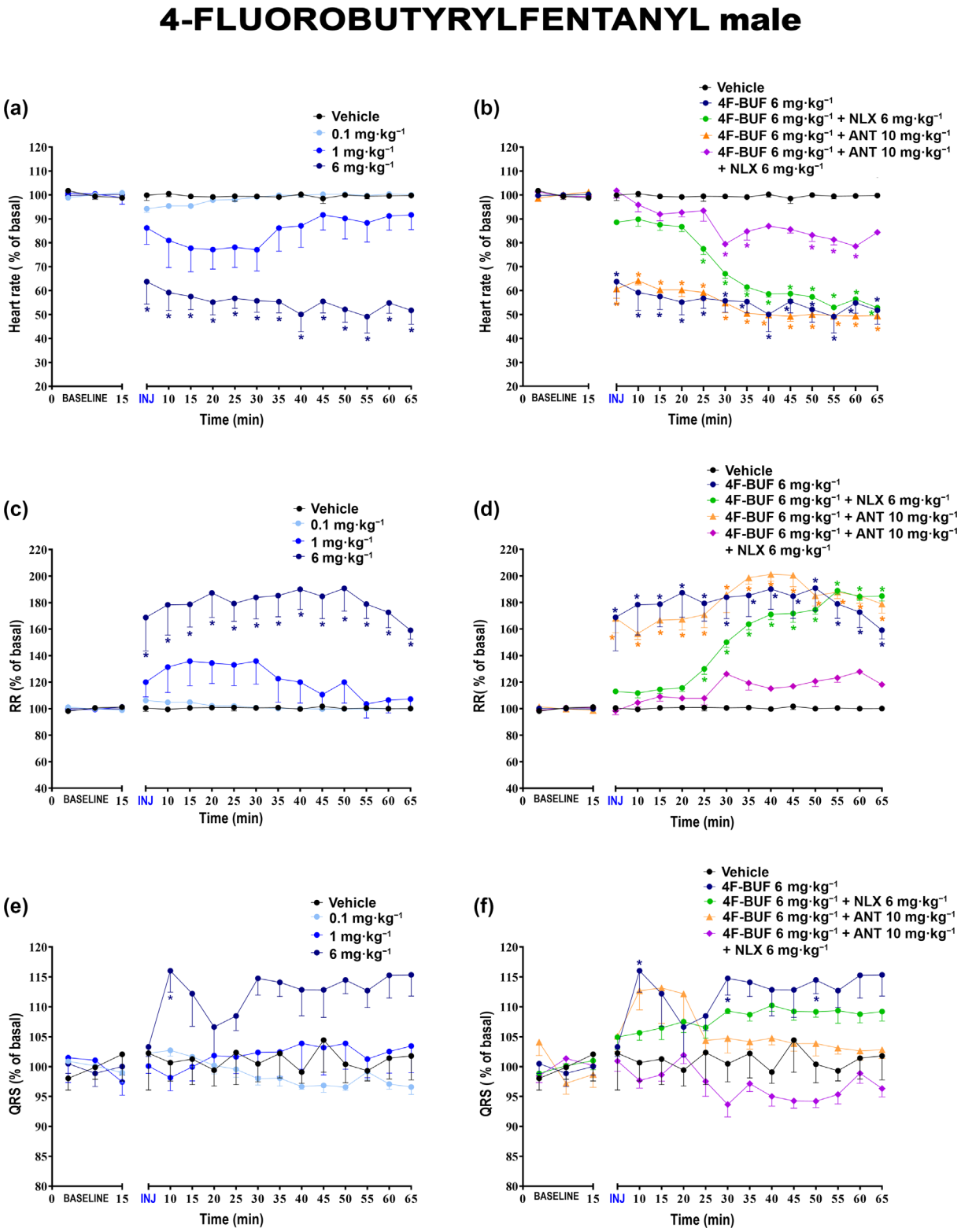

FIGURE 12 Electrocardiogram measurements: systemic administration of 4-fluorobutyrylfentanyl (4F-BUF) (0.1–6 mg·kg^{-1} i.p.) in (a) heart rate, (c) RR interval and (e) QRS complex in male mice. (b, d, f) Interaction of the effective dose of 4F-BUF (6 mg·kg^{-1}) with the opioid receptor antagonist naloxone (NLX) (6 mg·kg^{-1}) or the corticotropin-releasing factor 1 (CRF_1) antagonist antalarmin (ANT) (10 mg·kg^{-1}) or their combination (ANT 10 mg·kg^{-1} + NLX 6 mg·kg^{-1}). Data are expressed as percentage of baseline (see Section 2) and represent the mean ± SEM of 6 determinations for each treatment. Statistical analysis was performed by two-way analysis of variance followed by Bonferroni's test for multiple comparisons for the dose–response curve of the compounds at different times (a–f). *P < 0.05 versus vehicle.

3.2.7 | PLETH: 4F-BUF

Female PLETH measurements

Respiratory rate (Figure 13) was immediately and significantly affected by 4F-BUF treatment (Figure 13a).

In addition, breath length increased immediately and significantly in female 4F-BUF-treated mice (Figure 13c). MV also decreased immediately and significantly after the administration of 4F-BUF (Figure 13e).

Pre-treatment with naloxone alone (6 mg·kg^{-1} i.p.; Figure 13b) partially prevented female 4F-BUF-induced bradypnoea, while MV reduction was potentiated with naloxone (Figure 13e). However, ANT (10 mg·kg^{-1} i.p.) alone or ANT (10 mg·kg^{-1} i.p.) in combination with naloxone (6 mg·kg^{-1} i.p.) was effective in preventing the effects of BUF on FR_ie (Figure 13b), BB_ie (Figure 13d) and MV (Figure 13f).

Male PLETH measurements

Respiratory rate (Figure 14) was immediately and significantly affected by 4F-BUF treatment (Figure 14a). In addition, breath length increased immediately and significantly in male 4F-BUF-treated mice (Figure 14c). MV also decreased immediately and significantly after the administration of 4F-BUF (Figure 14e).

Pre-treatment with naloxone alone (6 mg·kg^{-1} i.p.; Figure 14b) partially prevented male 4F-BUF respiratory depression, while MV reduction was potentiated with naloxone (Figure 14f). However, ANT (10 mg·kg^{-1} i.p.) alone or ANT (10 mg·kg^{-1} i.p.) in combination with naloxone (6 mg·kg^{-1} i.p.) was effective in preventing the effects of BUF on FR_ie (Figure 14b), BB_ie (Figure 14d) and MV (Figure 14f).

3.3 | Comparison of cardiorespiratory response to BUF and 4F-BUF on female and male mice

The comparison of the dose–response curves of BUF and 4F-BUF on female mice revealed significant differences of effectiveness between the two compounds on the HR (doses of 0.1 and 6 mg·kg^{-1}) and the RR interval (6 mg·kg^{-1}) but no differences on the breath rate and the breath length (see Figure S5).

The comparison of the dose–response curves of BUF and 4F-BUF on male mice did not show significant differences of the cardiorespiratory parameters with the two compounds similarly acting on ECG and PLETH in the dose range examined (see Figure S6).

3.4 | Sex differences on the cardiorespiratory responses of BUF and 4F-BUF

The comparison of the dose–response curves of BUF on female and male mice (Figure S7) revealed sex differences in responses to RR interval (Figure S7B), respiratory rate (Figure S7C) and breath length (Figure S7D). However, no sex difference was revealed with BUF on the HR.

The comparison of the dose–response curves of 4F-BUF on female and male mice (Figure S8) revealed sex differences in responses to HR (Figure S8A), RR interval (Figure S8B), respiratory rate (Figure S8C) and breath length (Figure S8D).

The antagonistic profile of naloxone and ANT on cardiorespiratory responses of female and male mice is illustrated in Figure S5.

4 | DISCUSSION

Our study presents novel results regarding the in vitro and in vivo characterization of BUF and 4F-BUF, two fentanyl analogues that have emerged as NSOs. In vitro, in the calcium mobilization assay, BUF and its fluorinated analogue were able to activate the μ opioid receptor in a concentration-dependent manner, and BUF was able to elicit maximal effects similar to that of dermorphin and fentanyl, while 4F-BUF behaved as a partial agonist. In the BRET G-protein assay, the two compounds mimicked the maximal effects of dermorphin. BUF behaved similarly to fentanyl as partial agonist for the β-arrestin 2 pathway, whereas 4F-BUF was inactive in promoting β-arrestin 2 recruitment. In vivo, in mice, the two compounds induced thermal antinociception, produced variable effects on motor functions and impaired cardiorespiratory parameters; moreover, sex differences were detected at the highest dose for the cardiac responses and at the lower dose (1 mg·kg^{-1}) for the respiratory responses. 4F-BUF showed lower potency for inhibiting cardiorespiratory function. Naloxone sensitivity of the actions of the two compounds was variable in cardiac and respiratory responses. Interestingly, the CRF_1 receptor antagonist ANT alone was able to block the respiratory impairment induced by BUF in both sexes but not 4F-BUF. The combination of naloxone and ANT significantly enhanced the action of naloxone reversal of the cardiorespiratory impairments induced by BUF and 4F-BUF in female and male mice.

4.1 | Role of naloxone and ANT in antinociceptive and motor effects of BUF and 4F-BUF

The two fentanyl analogues induced a significant increase in thermal antinociception in female and male mice. The effect was more pronounced and prolonged at the highest dose tested (6 mg·kg^{-1} i.p.). Comparison of the dose–response curves in the tail withdrawal (Figure S4) tests demonstrated no major differences in terms of antinociceptive potency between BUF and 4F-BUF in both sexes (ED_{50} ± 3 mg·kg^{-1}). In contrast to our results, a previous study revealed higher analgesic potency of BUF (ED_{50} = 0.11 mg·kg^{-1}) with respect to 4F-BUF (ED_{50} = 0.91 mg·kg^{-1}); (Varshneya et al., 2022, 2023). The range of doses tested in our study is selected based on our previous study on fentanyl (Bilel et al., 2022). The different results obtained with respect to Varshneya et al. (2023) could be possibly explained by the difference in the animal strain, the route of administration and the intensity of the stimulus. Moreover, no sex differences were evident in that test. In accordance with our data, previous studies on fentanyl in rats showed no sex differences in the tail withdrawal test (Peckham & Traynor, 2006; Schwienteck et al., 2019). The pre-

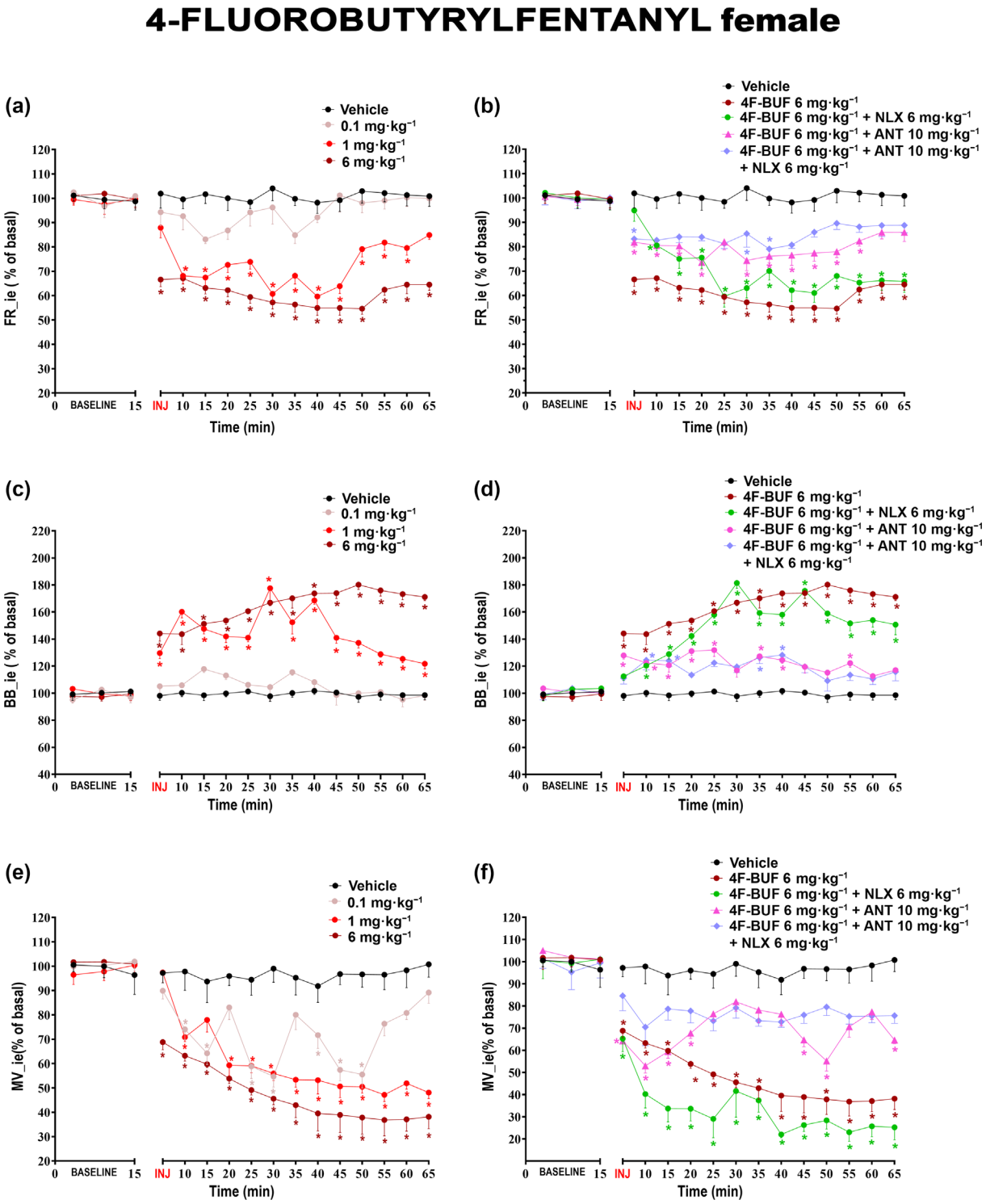

FIGURE 13 Plethysmography measurements: systemic administration of 4-fluorobutyrylfentanyl (4F-BUF) (0.1–6 mg·kg^{-1} i.p.) in (a) breath rate (FR ie.), (c) breath length (BB ie) and (e) minute volume (MV) in female mice. (b, d, f) Interaction of the effective dose of butyrylfentanyl (BUF) (6 mg·kg^{-1}) with the opioid receptor antagonist naloxone (NLX) (6 mg·kg^{-1}) or the corticotropin-releasing factor 1 (CRF_1) antagonist antalarmin (ANT) (10 mg·kg^{-1}) or their combination (ANT 10 mg·kg^{-1} + NLX 6 mg·kg^{-1}). Data are expressed as percentage of baseline (see Section 2) and represent the mean ± SEM of 6 determinations for each treatment. Statistical analysis was performed by two-way analysis of variance followed by Bonferroni's test for multiple comparisons for the dose–response curve of the compounds at different times (a–f). *P < 0.05 versus vehicle.

treatment with naloxone was effective in totally blocking the analgesic effect of 4F-BUF in both sexes (Figure 5a,b). It is worth noting that the effect of 4F-BUF is very similar to furanylfentanyl in the same test (Bilel et al., 2022). The similarity could be related to their similar efficacy on μ opioid receptors (Table 1; Bilel et al., 2022). In female mice treated with BUF, the analgesic effect reappeared at 120 min when

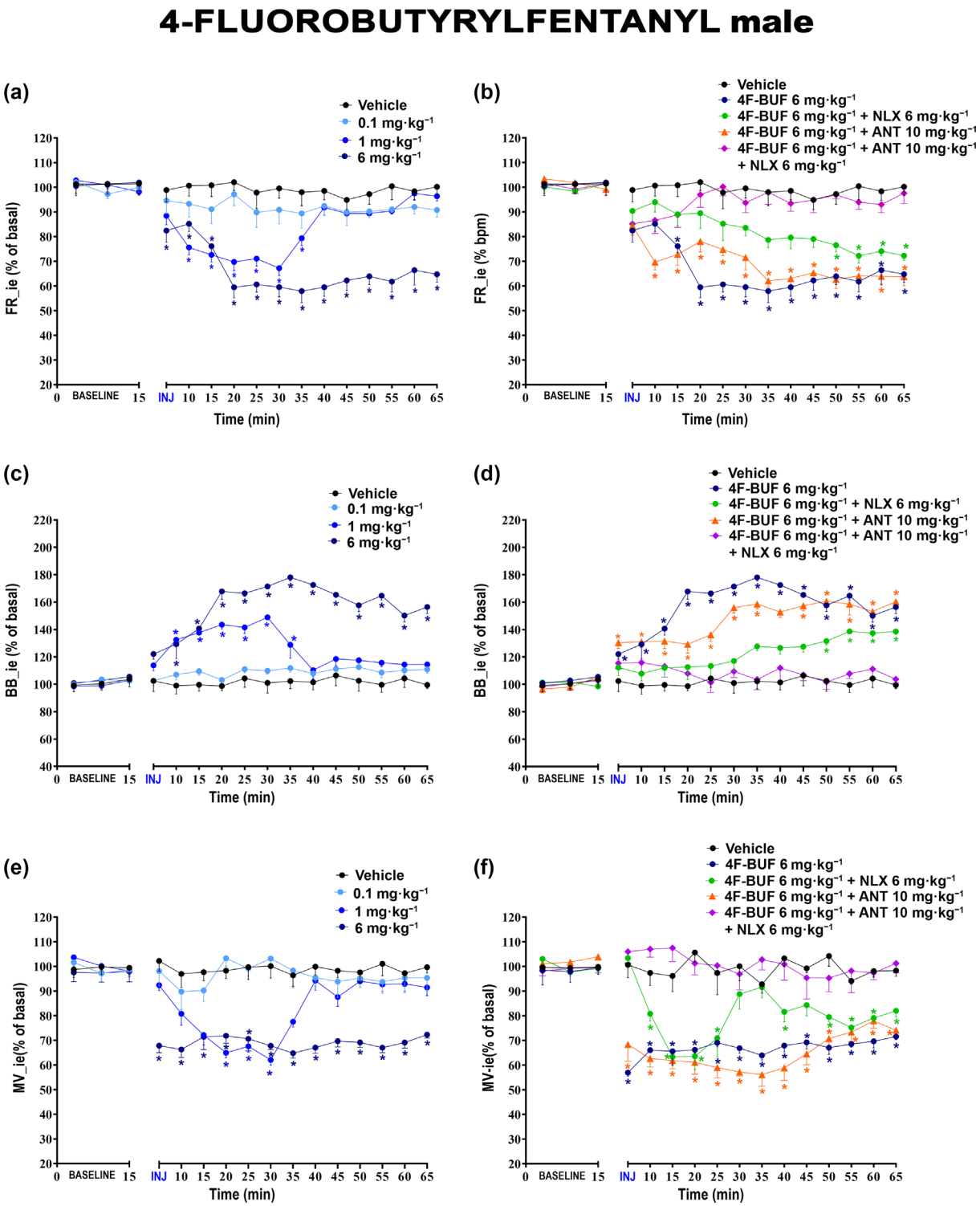

FIGURE 14 Plethysmography measurements: systemic administration of 4-fluorobutyrylfentanyl (4F-BUF) (0.1–6 mg·kg^{-1} i.p.) in (a) breath rate (FR ie.), (c) breath length (BB ie and (e) minute volume (MV in male mice. (b, d, f) Interaction of the effective dose of butyrylfentanyl (BUF) (6 mg·kg^{-1}) with the opioid receptor antagonist naloxone (NLX) (6 mg·kg^{-1}) or the corticotropin-releasing factor 1 (CRF$_1$) antagonist antalarmin (ANT) (10 mg·kg^{-1}) or their combination (ANT 10 mg·kg^{-1} + NLX 6 mg·kg^{-1}). Data are expressed as percentage of baseline (see Section 2) and represent the mean ± SEM of 6 determinations for each treatment. Statistical analysis was performed by two-way analysis of variance followed by Bonferroni's test for multiple comparisons for the dose–response curve of the compounds at different times (a–f). *P < 0.05 versus vehicle.

compared to male mice (Figure 5a,b). This difference is related to the difference in BUF distribution between female and male, as clearly demonstrated in Figure 5c,d, where the analgesic effect has already disappeared at 120 min in male mice. Our results reveal that ANT alone did not induce any effect on mice. Furthermore, pre-treatment with ANT, whether alone or in combination with naloxone, did not

influence the antinociceptive effects of the two agonists. These results suggest that ANT does not have a direct action on μ opioid receptors and that CRF_1 receptors are not involved in mechanisms by which opioids induce antinociception. To the best of our knowledge, there are no studies on the antinociceptive properties of ANT or other pyrrolopyrimidine analogues. However, it was demonstrated in animal studies that various stressors, even non-painful ones, induce analgesia that shows cross-tolerance with morphine and is counteracted by naloxone (Girardot & Holloway, 1984; Rodgers & Randall, 1985).

The systemic administration of BUF and 4F-BUF induced changes in motor performance in female and male mice. BUF induced inhibition of motor activity in females, while in males, its effect was biphasic (Figure 5a–c). Contrastingly, the effect of 4F-BUF was biphasic in females and stimulatory in males. The current results were unexpected, as it has been reported that BUF and 4F-BUF evoke stimulatory effects in male mice (10 $mg{\cdot}kg^{-1}$) using the open field test (Varshneya et al., 2023). Moreover, in our previous study, we demonstrated a biphasic effect in accelerod with fentanyl, ocfentanil and acrylfentanyl but not with furanylfentanyl (Bilel et al., 2022). The current findings confirm what has already been suggested regarding the role played by the chemical substitution of fentanyl analogues in their effects on motor behaviour (Bilel et al., 2022, 2023; Varshneya et al., 2019, 2023). We also confirm that fluorine substitutions on BUF's aniline ring play a key role in μ-mediated effects (Varshneya et al., 2023). Sex differences were revealed with the two drugs only at the dose of 6 $mg{\cdot}kg^{-1}$ (Figure S5A,B). Because previous studies on fentanyl were mostly performed in male mice, the effect on females was ascertained (Bilel et al., 2022, 2023; Varshneya et al., 2019, 2023). Yet our study reveals that the responses of females treated with BUF and 4F-BUF are different in dose and latency, confirming the difference in the pharmacokinetics of the opioid agonist between both sexes (Di Francesco et al., 2024; reviewed in Little & Koston, 2023). The pre-treatment with naloxone totally blocked the motor actions induced by BUF and 4F-BUF in both sexes. These results are consistent with previous studies on the same compounds and with fentanyl and furanylfentanyl, confirming that μ opioid receptors mediate the facilitative and inhibitory effects of both compounds in this test (Bilel et al., 2022; Varshneya et al., 2023). Surprisingly, ANT was also effective in blocking the motor effects of the two compounds in both sexes. To the best of our knowledge, this is the first study evaluating the effect of ANT on blocking the acute motor effects induced by opioid agonists. These results highlight the role played by CRF_1 receptors in the motor effects of opioids. The neurochemical mechanisms by which opioids induce excitatory and inhibitory motor effects are not yet fully understood. One primary mechanism for the facilitatory effects of opioids involves the stimulation of the mesolimbic dopamine (DA) pathway. It has been shown that systemic or direct infusions of μ agonists into the ventral tegmental area (VTA) increase DA release in the dorsal striatum (Di Chiara & Imperato, 1988), thereby enhancing locomotion and motor sensitization. This is likely due to the activation of μ receptors located on GABAergic interneurons in the VTA (Matsui et al., 2014). Moreover, it has been suggested the involvement of the cholinergic system in opioid locomotor effects. In particular, it has been demonstrated that laterodorsal tegmental (LDTg) and/or rostromedial tegmental nucleus (PPTg) cholinergic inputs to VTA mediate opioid-induced locomotion and DA activation via VTA M5 **muscarinic M_5 receptors**. Both cholinergic inputs to the rostromedial tegmental nucleus (RMTg) also modulate opioid-induced locomotion (reviewed in Steidl et al., 2017). It has been demonstrated that more than 90% of cholinergic neurons in the LDTg express CRF_1 receptors (Sauvage & Steckler, 2001). In addition, Fernandez et al. demonstrated that acute activation of CRF_1, but not **CRF_2**, receptors markedly increased the firing of cholinergic LDTg neurons. They also showed that local antagonism of CRF_1 in the LDTg is sufficient to reverse cellular and behavioural markers of stress in their preclinical model of depression (Fernandez et al., 2018). Taken together, this evidence suggests that CRF_1 receptors in the cholinergic system play a role in the locomotor actions of μ opioid agonists (Pintér et al., 2021).

4.2 | Comparison of the cardiorespiratory responses between BUF and 4F-BUF

Many preclinical studies have been recently conducted to evaluate the cardiorespiratory effects of fentanyl analogues (Bilel et al., 2022; Varshneya et al., 2023). BUF and 4F-BUF reduced cardiorespiratory responses at intermediate and high doses (1 and 6 $mg{\cdot}kg^{-1}$). The effect of BUF seemed to be more profound at the highest dose tested in both sexes. Moreover, only with BUF (6 $mg{\cdot}kg^{-1}$) we detected arrhythmia and apnoea at 10 min after injection in female and male mice (Figure S2). 4F-BUF differs from BUF with the para-fluoro substitution on the phenethylamine backbone. Interestingly, this chemical modification could change the potency and efficacy of the compound. Indeed, Varshneya et al. (2023) revealed in their recent elegant study that BUF showed higher effectiveness in reducing MV (whole-body PLETH) when compared to 4F-BUF. Our results confirm the findings of Varshneya et al. (2023) on the higher effects of BUF with respect to 4F-BUF and shed light on the role of fluorine substitution on BUF's μ opioid receptor-mediated cardiorespiratory effects. Additionally, our in vitro data showed that the two compounds display different μ opioid receptor efficacy and ability to promote receptor interaction with arrestin. The lower efficacy of 4F-BUF and inability to recruit arrestin could have an impact on the intensity of the physiological responses triggered, as seen with other fentanyl analogues such as furanylfentanyl (Bilel et al., 2022) and carfentanil (Ramos-Gonzalez et al., 2023). Structurally related BUF substitutes such as isobutyrylfentanyl and para-methoxybutyrylfentanyl showed significant analgesic and higher safety/protective index (PI) than fentanyl and even buprenorphine (Varshneya et al., 2022). Tsai et al. (2023) suggested that the high intrinsic efficacy of NSOs such as fentanyl and non-fentanyl analogues (nitazenes) in G_i protein signalling is a common property that may contribute to their high risk of intoxication and overdose. However, in vitro functional characterization of fentanyl analogues revealed high efficacy of isobutyrylfentanyl ($E_{max} = 94\%$) and para-methoxybutyrylfentanyl ($E_{max} = 55\%$) (Eshleman et al., 2020), which have a greater safety index with respect to fentanyl (Varshneya

et al., 2022). In addition, a recent study on the highly potent nitazenes suggests that these compounds display a superagonism on both G_i and β-arrestin pathways when compared to morphine and fentanyl (Malcolm et al., 2023). It was demonstrated in the same study that transduction coefficients of isotonitazene and *N*-desethyl isotonitazene showed a slight preference for the G-protein pathway over β-arrestin recruitment, and the superagonism exhibited by isotonitazene is related to its ability to induce β-arrestin recruitment even in the absence of **G protein-coupled receptor kinase** (GRK) proteins. These findings suggest that conformational dynamics between μ opioid receptors and the ligand play a key role in opioid actions (Malcolm et al., 2023; Zhao et al., 2024). Collectively, in vitro μ opioid G-protein efficacy and ability to recruit arrestin should not be considered as the only predictive parameter of opioid safety profiles (Varshneya et al., 2022, 2023), suggesting that other in vivo factors, including the administration route, dose, Absorption, Distribution, Metabolism and Excretion (ADME) variables and blood–brain barrier (BBB) permeability, contribute to the behavioural actions of NSOs (Bilel et al., 2022; Eshleman et al., 2020; Vandeputte et al., 2021).

4.3 | Sex differences on the cardiorespiratory responses of BUF and 4F-BUF

The death rate with fentanyl overdose was reported to be two to three times higher for men than women (Butelman et al., 2023); however, the incidence of opioid use disorders and death with overdose by synthetic opioids has increased in women (Barbosa-Leiker et al., 2021; VanHouten et al., 2019). The primary cause of death from fentanyl and its analogue overdose is respiratory depression and hypoxia.

We evaluated the cardiorespiratory effects of two fentanyl analogues in female and male mice. We found a sex difference on the cardiac responses (RR interval) with BUF, where females showed higher responses at the highest dose tested than males. In addition, females showed higher sensitivity to the respiratory depression and hypoventilation at the intermediate dose when compared to males (1 $mg{\cdot}kg^{-1}$). Most of the studies on the respiratory effects of opioids are performed in male rodents, and the respiratory effects of BUF and 4F-BUF measured in males are very similar to fentanyl (Varshneya et al., 2023). In addition, a recent study in female and male rats injected with increasing doses of fentanyl showed a higher respiratory sensitivity of the female compared to male rats. In particular, female rats showed a greater maximum effect on minute ventilation and peak inspiratory flow and longer lasting respiratory depressing effects than male rats (Marchette et al., 2023). These results are in accordance with our findings on the sex-respiratory differences induced by BUF and 4F-BUF (1 $mg{\cdot}kg^{-1}$). While the respiratory responses clearly demonstrate a higher sensitivity of female than male mice, the results obtained on the cardiac responses are somewhat different. In fact, in the case of BUF (6 $mg{\cdot}kg^{-1}$), female mice showed a higher increase of RR interval compared to male mice. Conversely, with 4F-BUF (6 $mg{\cdot}kg^{-1}$), male mice showed slightly higher cardiac sensitivity with respect to female mice. It is noteworthy that differences in cardiac versus respiratory responses between female and male rodents have also been reported with fentanyl. In particular, Haile et al. (2022) measured the physiological effects of fentanyl (0.1 $mg{\cdot}kg^{-1}$) in a set of male and female Sprague–Dawley rats using the pulse oximetry system. They found that fentanyl significantly decreased oxygen saturation, HR and general activity in male rats. However, in female rats, only oxygen saturation was significantly decreased by the same dose of fentanyl. Adding to our findings, these results reveal sex-dependent effects of fentanyl and fentanyl analogues (Kaplovitch et al., 2015; Towers et al., 2022). The sex differences observed in our results might be explained by differences in μ opioid receptor distribution between female and male animals (Zhang et al., 2014) and the influence of circulating ovarian hormones and/or androgens on μ-mediated physiological responses (reviewed in Sharp et al., 2022).

4.4 | Role of naloxone and ANT in μ opioid- and CRF1-mediated cardiorespiratory effects of BUF and 4F-BUF

Following on our previous study on fentanyl analogues (Bilel et al., 2022), we here demonstrated that BUF and 4F-BUF impaired cardiorespiratory activity when administered to female and male mice at the range dose 0.1–6 $mg{\cdot}kg^{-1}$. Both compounds reduced HR, increasing RR interval, and increased the QRS complex, reduced the breath rate, increased breath length and reduced MV. The mechanisms by which opioid agonists induce bradycardia, respiratory depression and hypoventilation have been previously detailed (Bilel et al., 2020, 2022; Varshneya et al., 2023). Naloxone pre-treatment (6 $mg{\cdot}kg^{-1}$) partially prevented the cardiorespiratory impairment induced by BUF and 4F-BUF. These results are in accordance with several preclinical (Bilel et al., 2022; Glatfelter et al., 2023; Haile et al., 2022; Varshneya et al., 2023) and clinical reports (Amaducci et al., 2023; Krantz et al., 2021; Rzasa Lynn & Galinkin, 2018) that prove the necessity of naloxone redosing to reverse cardiorespiratory alterations of fentanyl or its analogues.

In the search of other mechanisms such as stress that might be triggered by the activation of μ receptors, which results in undesired effects, specifically cardiorespiratory depression, we investigated the possible role played by CRF_1 receptors in the physiological responses induced by opioid agonists as seen with opioid withdrawal and dependence (Papaleo et al., 2007). To the best of our knowledge, this is the first study investigating the possible involvement of this stress factor in worsening cardiorespiratory impairments related to opioids in female and male animals using ANT alone or in combination with naloxone. Unexpectedly, our results revealed that ANT alone is able to block the respiratory depression induced by BUF (6 $mg{\cdot}kg^{-1}$) in female and male mice but not in the case of 4F-BUF. In addition, pretreatment with ANT and naloxone enhanced the reversal action of naloxone. One of the mechanical mechanisms by which fentanyl induces respiratory depression is the chest wall rigidity (Haouzi & Tubbs, 2022). It is worth noting that naloxone did not prevent MV reduction induced by the two drugs. Our data suggest a non-opioid

receptor mechanism involved in this effect. Indeed, it was suggested that the decrease of MV may result from rapid closure of the vocal cords, followed by rigidity in the diaphragm, chest wall and upper airway (wooden chest syndrome), involving the noradrenergic and cholinergic systems (Torralva & Janowsky, 2019). It has been demonstrated that fentanyl microinjection into the rat locus coeruleus (LC) increased the electromyographic (EMG) activity of the caudal lateral extensor and gastrocnemius muscles considered as a correlate of opioid-induced muscular rigidity, which was inhibited by the **α_1-adrenoceptor** blocker prazosin (Lui et al., 1990). While the mechanism by which opioids could lead to CRF_1 receptor activation has not been clearly investigated, it is worth highlighting that CRF receptors and m receptors are co-localized in the nucleus LC of rodents (Reyes et al., 2007). This may possibly explain the direct interaction of the opioidergic and noradrenergic systems in response to stress stimulus activated by opioid agonists (Guajardo & Valentino, 2021). Regarding the cholinergic system, it has been demonstrated that the activation of CRF_1 receptors in the hippocampus increased the release of acetylcholine that was significantly reduced by the pre-treatment with ANT (Pintér et al., 2021). These findings strongly support the hypothesis of the involvement of CRF_1 receptor in the deep respiratory depression induced by μ opioid agonists. The effectiveness of ANT alone in blocking respiratory depression in male and female mice treated with BUF but not the fluorinated (4F-BUF) needs further investigation. The block of CRF_1 receptors enhances the reversal effect of naloxone in cardiac and respiratory responses. These findings corroborate the hypothesis on the role of stress factor in worsening the acute cardiorespiratory impairment triggered by the potent synthetic opioids and, in particular, the class of fentanyl analogues (Algera et al., 2019).

4.5 | Insights from ANT use in preclinical studies for opioid overdose

ANT, belonging to the pyrrolopyrimidine class, is the antagonist most closely related structurally to CP-154,526. It has slightly greater in vitro affinity for the CRF_1 receptor compared to CP-154,526 ($K_i = 0.8$ vs. 2.7 nM, respectively; Chorvat et al., 1999). It is a highly lipophilic compound (ClogP = 6.98; Hsin et al., 2002) that is readily able to cross the blood brain barrier (BBB) (Deak et al., 1999; Esposito et al., 2003). Previous pharmacokinetic studies on primates revealed that intravenous injections of ANT show a higher bioavailability with respect to oral administration (Habib et al., 2000). CRF_1 receptors are heterogeneously distributed throughout the rat brain, with the highest density found in the cortex, hypothalamus and pituitary (Seymour et al., 2003). CRF_1 receptors were also found in the periphery and in the testis and intestine in mice (Seymour et al., 2003). The metabolic profile of ANT is not yet established. Our results in vivo revealed that this compound is able to prevent motor and cardiorespiratory impairments induced by the potent fentanyl analogues. In contrast to previous studies that are mainly focusing on long-term effects of stress in the opioidergic system, this study examined the direct role of stress on opioid side effects, particularly respiratory depression. We have uncovered new mechanisms by which opioids may induce motor and cardiorespiratory alterations. We demonstrated that CRF_1 receptors are involved in motor and cardiorespiratory effects but not nociception. ANT blocked respiratory depression with BUF, and we suggested that this mechanism is played by off-target receptors of the adrenergic and cholinergic systems. CRF_1 antagonists should be considered more in preclinical studies of opioid toxicity and overdose.

5 | CONCLUSIONS

The present study revealed that BUF behaves similarly to fentanyl as a μ receptor agonist. In contrast to BUF, 4F-BUF acts as a partial agonist in vitro at μ opioid receptors and as a G protein-biased μ agonist. In vivo, the two compounds induced antinociception, modifications of motor activity and impaired cardiorespiratory functions in female and male mice, with a higher sensitivity of females to respiratory depression. In this study, we have uncovered a novel mechanism by which synthetic opioids might increase respiratory depression, shedding new light on the role of CRF_1 receptors in cardiorespiratory impairments caused by μ receptor agonists. This finding increases our understanding of μ receptor-mediated mechanisms and may have implications for antidotal therapies in case of overdose with synthetic opioids.

AUTHOR CONTRIBUTIONS

S. Bilel: Conceptualization (equal); data curation (equal); investigation (equal); methodology (equal); supervision (equal); writing—original draft (equal). **J. Azevedo Neto:** Data curation (equal); methodology (equal). **M. Tirri:** Data curation (equal); formal analysis (equal); methodology (equal). **G. Corli:** Data curation (equal); formal analysis (equal); methodology (equal). **M. Bassi:** Data curation (equal); formal analysis (equal); methodology (equal). **A. Fantinati:** Formal analysis (equal); methodology (equal). **G. Serpelloni:** Supervision (equal). **D. Malfacini:** Data curation (equal); formal analysis (equal); methodology (equal). **C. Trapella:** Formal analysis (equal); methodology (equal); supervision (equal). **G. Calo':** Conceptualization (equal); data curation (equal); investigation (equal); methodology (equal); supervision (equal). **M. Marti:** Conceptualization (equal); data curation (equal); investigation (equal); methodology (equal); supervision (equal); writing—original draft (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the supporting information of this article.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis and Animal Experimentation and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

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REFERENCES

Alexander, S. P. H., Christopoulos, A., Davenport, A. P., Kelly, E., Mathie, A. A., Peters, J. A., Veale, E. L., Armstrong, J. F., Faccenda, E., Harding, S. D., Davies, J. A., Abbracchio, M. P., Abraham, G., Agoulnik, A., Alexander, W., Al-Hosaini, K., Bäck, M., Baker, J. G., Barnes, N. M., … Ye, R. D. (2023). The Concise Guide to PHARMACOLOGY 2023/24: G protein-coupled receptors. *British Journal of Pharmacology*, *180*, S23–S144. https://doi.org/10.1111/bph.16177

Alexander, S. P. H., Fabbro, D., Kelly, E., Mathie, A. A., Peters, J. A., Veale, E. L., Armstrong, J. F., Faccenda, E., Harding, S. D., Davies, J. A., Annett, S., Boison, D., Burns, K. E., Dessauer, C., Gertsch, J., Helsby, N. A., Izzo, A. A., Ostrom, R., Papapetropoulos, A., … Wong, S. S. (2023). The concise guide to PHARMACOLOGY 2023/24: Enzymes. *British Journal of Pharmacology*, *180*, S289–S373. https://doi.org/10.1111/bph.16181

Algera, M. H., Kamp, J., van der Schrier, R., van Velzen, M., Niesters, M., Aarts, L., Dahan, A., & Olofsen, E. (2019). Opioid-induced respiratory depression in humans: A review of pharmacokinetic-pharmacodynamic modelling of reversal. *British Journal of Anaesthesia*, *122*(6), e168–e179. https://doi.org/10.1016/j.bja.2018.12.023

Amaducci, A., Aldy, K., Campleman, S. L., Li, S., Meyn, A., Abston, S., Culbreth, R. E., Krotulski, A., Logan, B., Wax, P., Brent, J., Manini, A. F., & Toxicology Investigators Consortium Fentalog Study Group. (2023). Naloxone Use in Novel Potent Opioid and Fentanyl Overdoses in Emergency Department Patients. *JAMA Network Open*, *6*(8), e2331264. https://doi.org/10.1001/jamanetworkopen.2023.31264

Bäckberg, M., Beck, O., Jönsson, K. H., & Helander, A. (2015). Opioid intoxications involving butyrfentanyl, 4-fluorobutyrfentanyl, and fentanyl from the Swedish STRIDA project. *Clinical Toxicology*, *53*(7), 609–617. https://doi.org/10.3109/15563650.2015.1054505

Barbosa-Leiker, C., Campbell, A. N. C., McHugh, R. K., Guille, C., & Greenfield, S. F. (2021). Opioid use disorder in women and the implications for treatment. *Psychiatric Research and Clinical Practice*, *3*(1), 3–11. https://doi.org/10.1176/appi.prcp.2019005

Bilel, S., Azevedo, N. J., Arfè, R., Tirri, M., Gregori, A., Serpelloni, G., De-Giorgio, F., Frisoni, P., Neri, M., Calò, G., & Marti, M. (2020). In vitro and in vivo pharmacological characterization of the synthetic opioid MT-45. *Neuropharmacology*, *171*, 108110. https://doi.org/10.1016/j.neuropharm.2020.108110

Bilel, S., Azevedo Neto, J., Arfè, R., Tirri, M., Gaudio, R. M., Fantinati, A., Bernardi, T., Boccuto, F., Marchetti, B., Corli, G., Serpelloni, G., De-Giorgio, F., Malfacini, D., Trapella, C., Calo', G., & Marti, M. (2022). In vitro and in vivo pharmaco-dynamic study of the novel fentanyl derivatives: Acrylfentanyl, ocfentanyl and furanylfentanyl. *Neuropharmacology*, *209*, 109020. https://doi.org/10.1016/j.neuropharm.2022.109020

Bilel, S., Murari, M., Pesavento, S., Arfè, R., Tirri, M., Torroni, L., Marti, M., Tagliaro, F., & Gottardo, R. (2023). Toxicity and behavioural effects of ocfentanil and 2-furanylfentanyl in zebrafish larvae and mice. *Neurotoxicology*, *95*, 83–93. https://doi.org/10.1016/j.neuro.2023.01.003

Broadbear, J. H., Winger, G., Rivier, J. E., Rice, K. C., & Woods, J. H. (2004). Corticotropin-releasing hormone antagonists, astressin B and antalarmin: Differing profiles of activity in rhesus monkeys. *Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology*, *29*(6), 1112–1121. https://doi.org/10.1038/sj.npp.1300410

Butelman, E. R., Huang, Y., Epstein, D. H., Shaham, Y., Goldstein, R. Z., Volkow, N. D., & Alia-Klein, N. (2023). Overdose mortality rates for opioids and stimulant drugs are substantially higher in men than in women: state-level analysis. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, *48*(11), 1639–1647. https://doi.org/10.1038/s41386-023-01601-8

Camarda, V., & Calo, G. (2013). Chimeric G proteins in fluorimetric calcium assays: Experience with opioid receptors. *Methods in Molecular Biology*, *937*, 293–Methods in Molecular Biology. https://doi.org/10.1007/978-1-62703-086-1_18

Chorvat, R. J., Corman, M. L., Breslin, M. J., Collins, J. T., Faraci, S., MacCoss, M., Dunaiskis, A. R., Fan, K. H., Schmidt, A. W., & Seymour, P. A. (1999). Cp-154,526: a potent and selective non-peptide antagonist of the corticotropin-releasing factor 1 (CRF1) receptor. *Journal of Medicinal Chemistry*, *42*(1), 135–138. https://doi.org/10.1021/jm980563h

Curtis, M. J., Alexander, S. P. H., Cirino, G., George, C. H., Kendall, D. A., Insel, P. A., Izzo, A. A., Ji, Y., Panettieri, R. A., Patel, H. H., Sobey, C. G., Stanford, S. C., Stanley, P., Stefanska, B., Stephens, G. J., Teixeira, M. M., Vergnolle, N., & Ahluwalia, A. (2022). Planning experiments: Updated guidance on experimental design and analysis and their reporting III. *British Journal of Pharmacology*, *179*(15), 3907–3913. https://doi.org/10.1111/bph.15868

Deak, T., Nguyen, K. T., Ehrlich, A. L., Watkins, L. R., Spencer, R. L., Maier, S. F., Licinio, J., Wong, M. L., Chrousos, G. P., Webster, E., & Gold, P. W. (1999). The impact of the nonpeptide corticotropin-releasing hormone antagonist antalarmin on behavioral and endocrine responses to stress. *Endocrinology*, *140*(1), 79–86. https://doi.org/10.1210/endo.140.1.6415

Di Chiara, G., & Imperato, A. (1988). Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proceedings of the National Academy of Sciences of the United States of America*, *85*(14), 5274–5278. https://doi.org/10.1073/pnas.85.14.5274

Di Francesco, G., Montesano, C., Vincenti, F., Bilel, S., Corli, G., Petrella, G., Cicero, D. O., Gregori, A., Marti, M., & Sergi, M. (2024). Tackling new psychoactive substances through metabolomics: UHPLC-HRMS study on natural and synthetic opioids in male and female murine models. *Scientific Reports*, *14*(1), 9432. https://doi.org/10.1038/s41598-024-60045-2

Drug Enforcement Administration, Department of Justice. (2016). Schedules of controlled substances: Temporary placement of butyryl fentanyl and beta-hydroxythiofentanyl into schedule I. Final order. *Federal Register*, *81*, 29492–29496.

Ducottet, C., Griebel, G., & Belzung, C. (2003). Effects of the selective non-peptide corticotropin-releasing factor receptor 1 antagonist antalarmin in the chronic mild stress model of depression in mice. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, *27*(4), 625–631. https://doi.org/10.1016/S0278-5846(03)00051-4

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

Esposito, P., Basu, S., Letourneau, R., Jacobson, S., & Theoharides, T. C. (2003). Corticotropin-releasing factor (CRF) can directly affect brain microvessel endothelial cells. *Brain Research*, *968*(2), 192–198. https://doi.org/10.1016/s0006-8993(03)02237-6

European Monitoring Centre for Drugs and Drug Addiction, 2015. *European drug report 2015: Trends and developments*. Publications Office of the European Union. https://www.emcdda.europa.eu/publications/edr/trends-developments/2015_en

European Monitoring Centre for Drugs and Drug Addiction, 2022. *European drug report 2022: Trends and developments*. Publications Office of the European Union. https://www.emcdda.europa.eu/publications/edr/trends-developments/2022_en

Fattore, L., Marti, M., Mostallino, R., & Castelli, M. P. (2020). Sex and gender differences in the effects of novel psychoactive substances. *Brain Sciences*, *10*(9), 606. https://doi.org/10.3390/brainsci10090606

Fernandez, S. P., Broussot, L., Marti, F., Contesse, T., Mouska, X., Soiza-Reilly, M., Marie, H., Faure, P., & Barik, J. (2018). Mesopontine cholinergic inputs to midbrain dopamine neurons drive stress-induced depressive-like behaviors. *Nature Communications*, *9*(1), 4449. https://doi.org/10.1038/s41467-018-06809-7

Girardot, M. N., & Holloway, F. A. (1984). Intermittent cold water stress-analgesia in rats: Cross-tolerance to morphine. *Pharmacology, Biochemistry, and Behavior*, *20*(4), 631–633. https://doi.org/10.1016/0091-3057(84)90315-0

Glatfelter, G. C., Vandeputte, M. M., Chen, L., Walther, D., Tsai, M. M., Shi, L., Stove, C. P., & Baumann, M. H. (2023). Alkoxy chain length governs the potency of 2-benzylbenzimidazole 'nitazene' opioids associated with human overdose. *Psychopharmacology*, *240*(12), 2573–2584. https://doi.org/10.1007/s00213-023-06451-2

Guajardo, H. M., & Valentino, R. J. (2021). Sex differences in μ-opioid regulation of coerulear-cortical transmission. *Neuroscience Letters*, *746*, 135651. https://doi.org/10.1016/j.neulet.2021.135651

Habib, K. E., Weld, K. P., Rice, K. C., Pushkas, J., Champoux, M., Listwak, S., Webster, E. L., Atkinson, A. J., Schulkin, J., Contoreggi, C., Chrousos, G. P., McCann, S. M., Suomi, S. J., Higley, J. D., & Gold, P. W. (2000). Oral administration of a corticotropin-releasing hormone receptor antagonist significantly attenuates behavioral, neuroendocrine, and autonomic responses to stress in primates. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(11), 6079–6084. https://doi.org/10.1073/pnas.97.11.6079

Haile, C. N., Baker, M. D., Sanchez, S. A., Lopez Arteaga, C. A., Duddupudi, A. L., Cuny, G. D., Norton, E. B., Kosten, T. R., & Kosten, T. A. (2022). An immunconjugate vaccine alters distribution and reduces the antinociceptive, behavioral and physiological effects of fentanyl in male and female rats. *Pharmaceutics*, *14*(11), 2290. https://doi.org/10.3390/pharmaceutics14112290

Haouzi, P., & Tubbs, N. (2022). Effects of fentanyl overdose-induced muscle rigidity and dexmedetomidine on respiratory mechanics and pulmonary gas exchange in sedated rats. *Journal of Applied Physiology*, *132*(6), 1407–1422. https://doi.org/10.1152/japplphysiol.00819.2021

Heinrichs, S. C., Menzaghi, F., Schulteis, G., Koob, G. F., & Stinus, L. (1995). Suppression of corticotropin-releasing factor in the amygdala attenuates aversive consequences of morphine withdrawal. *Behavioural Pharmacology*, *6*(1), 74–80. https://doi.org/10.1097/00008877-199501000-00011

Hsin, L., Tian, X., Webster, E., Coop, A., Caldwell, T., Jacobson, A., Chrousos, G., Gold, P., Habib, K., Ayala, A., Eckelman, W., Contoreggi, C., & Rice, K. (2002). CRHR1 Receptor binding and lipophilicity of pyrrolopyrimidines, potential nonpeptide corticotropin-releasing hormone type 1 receptor antagonists. *Bioorganic and Medicinal Chemistry*, *10*(1), 175–183. https://doi.org/10.1016/s0968-0896(01)00261-9

Kaplovitch, E., Gomes, T., Camacho, X., Dhalla, I. A., Mamdani, M. M., & Juurlink, D. N. (2015). Sex differences in dose escalation and overdose death during chronic opioid therapy: A population-based cohort study. *PLoS ONE*, *10*(8), e0134550. https://doi.org/10.1371/journal.pone.0134550

Kenakin, T. (2004). *A pharmacology primer*. Elsevier Academic Press.

Krantz, M. J., Palmer, R. B., & Haigney, M. C. P. (2021). Cardiovascular complications of opioid use: *JACC* state-of-the-art review. *Journal of the American College of Cardiology*, *77*(2), 205–223. https://doi.org/10.1016/j.jacc.2020.11.002

Lilley, E., Stanford, S. C., Kendall, D. E., Alexander, S. P., Cirino, G., Docherty, J. R., George, C. H., Insel, P. A., Izzo, A. A., Ji, Y., Panettieri, R. A., Sobey, C. G., Stefanska, B., Stephens, G., Teixeira, M., & Ahluwalia, A. (2020). ARRIVE 2.0 and the British Journal of Pharmacology: Updated guidance for 2020. *British Journal of Pharmacology*, *177*(16), 3611–3616. https://doi.org/10.1111/bph.15178

Little, K. M., & Kosten, T. A. (2023). Focus on fentanyl in females: Sex and gender differences in the physiological and behavioral effects of fentanyl. *Frontiers in Neuroendocrinology*, *71*, 101096. https://doi.org/10.1016/j.yfrne.2023.101096

Lui, P. W., Lee, T. Y., & Chan, S. H. (1990). Involvement of coerulospinal noradrenergic pathway in fentanyl-induced muscular rigidity in rats. *Neuroscience Letters*, *108*(1–2), 183–188. https://doi.org/10.1016/0304-3940(90)90728-r

Malcolm, N. J., Palkovic, B., Sprague, D. J., Calkins, M. M., Lanham, J. K., Halberstadt, A. L., Stucke, A. G., & McCorvy, J. D. (2023). Mu-opioid receptor selective superagonists produce prolonged respiratory depression. *iScience*, *26*(7), 107121. https://doi.org/10.1016/j.isci.2023.107121

Malfacini, D., Ambrosio, C., Gro', M. C., Sbraccia, M., Trapella, C., Guerrini, R., Bonora, M., Pinton, P., Costa, T., & Calo', G. (2015). Pharmacological profile of nociceptin/orphanin FQ receptors interacting with G-proteins and β-arrestins 2. *PLoS ONE*, *10*(8), e0132865. https://doi.org/10.1371/journal.pone.0132865

Marchette, R. C. N., Carlson, E. R., Frye, E. V., Hastings, L. E., Vendruscolo, J. C. M., Mejias-Torres, G., Lewis, S. J., Hampson, A., Volkow, N. D., Vendruscolo, L. F., & Koob, G. F. (2023). Heroin- and fentanyl-induced respiratory depression in a rat plethysmography model: Potency, tolerance, and sex differences. *The Journal of Pharmacology and Experimental Therapeutics*, *385*(2), 117–134. https://doi.org/10.1124/jpet.122.001476

Matsui, A., Jarvie, B. C., Robinson, B. G., Hentges, S. T., & Williams, J. T. (2014). Separate GABA afferents to dopamine neurons mediate acute action of opioids, development of tolerance, and expression of withdrawal. *Neuron*, *82*(6), 1346–1356. https://doi.org/10.1016/j.neuron.2014.04.030

Molinari, P., Vezzi, V., Sbraccia, M., Grò, C., Riitano, D., Ambrosio, C., Casella, I., & Costa, T. (2010). Morphine-like opiates selectively antagonize receptor-arrestin interactions. *The Journal of Biological Chemistry*, *285*(17), 12522–12535. https://doi.org/10.1074/jbc.M109.059410

Papaleo, F., Kitchener, P., & Contarino, A. (2007). Disruption of the CRF/-CRF1 receptor stress system exacerbates the somatic signs of opiate withdrawal. *Neuron*, *53*(4), 577–589. https://doi.org/10.1016/j.neuron.2007.01.022

Peckham, E. M., & Traynor, J. R. (2006). Comparison of the antinociceptive response to morphine and morphine-like compounds in male and female Sprague-Dawley rats. *The Journal of Pharmacology and Experimental Therapeutics*, *316*(3), 1195–1201. https://doi.org/10.1124/jpet.105.094276

Percie du Sert, N., Hurst, V., Ahluwalia, A., Alam, S., Avey, M. T., Baker, M., Browne, W. J., Clark, A., Cuthill, I. C., Dirnagl, U., Emerson, M., Garner, P., Holgate, S. T., Howells, D. W., Karp, N. A., Lazic, S. E., Lidster, K., MacCallum, C. J., Macleod, M., … Würbel, H. (2020). The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. *PLoS Biology*, *18*(7), e3000410. https://doi.org/10.1371/journal.pbio.3000410

Pintér, D., Balangó, B., Simon, B., Palotai, M., Csabafi, K., Dobó, É., Ibos, K. E., & Bagosi, Z. (2021). The effects of CRF and the urocortins on the hippocampal acetylcholine release in rats. *Neuropeptides*, *88*, 102147. https://doi.org/10.1016/j.npep.2021.102147

Ramos-Gonzalez, N., Groom, S., Sutcliffe, K. J., Bancroft, S., Bailey, C. P., Sessions, R. B., Henderson, G., & Kelly, E. (2023). Carfentanil is a β-arrestin-biased agonist at the μ opioid receptor. *British Journal of Pharmacology*, *180*(18), 2341–2360. https://doi.org/10.1111/bph.16084

Reyes, B. A., Glaser, J. D., & Van Bockstaele, E. J. (2007). Ultrastructural evidence for co-localization of corticotropin-releasing factor receptor and μ-opioid receptor in the rat nucleus locus coeruleus. *Neuroscience Letters*, *413*(3), 216–221. https://doi.org/10.1016/j.neulet.2006.11.069

Richeval, C., Baillieux, M., Pawlak, G., Phanithavong, M., Wiart, J.-f., Humbert, L., Batisse, A., Lamoureux, C., Pfau, G., Nefau, T., Allorge, D., & Gaulier, J.-M. (2019). Benzoylfentanyl and parafluorobutyrfentanyl: Some analytical and metabolism data. *Toxicologie Analytique et Clinique*, *31*, 258–267. https://doi.org/10.1016/j.toxac.2019.01.004

Rodgers, R. J., & Randall, J. I. (1985). Social conflict analgesia: Studies on naloxone antagonism and morphine cross-tolerance in male DBA/2 mice. *Pharmacology, Biochemistry, and Behavior*, *23*(5), 883–887. https://doi.org/10.1016/0091-3057(85)90087-5

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.

Sauvage, M., & Steckler, T. (2001). Detection of corticotropin-releasing hormone receptor 1 immunoreactivity in cholinergic, dopaminergic and noradrenergic neurons of the murine basal forebrain and brainstem nuclei—Potential implication for arousal and attention. *Neuroscience*, *104*(3), 643–652. https://doi.org/10.1016/s0306-4522(01)00137-3

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 mu 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

Seymour, P. A., Schmidt, A. W., & Schulz, D. W. (2003). The pharmacology of CP-154,526, a non-peptide antagonist of the CRH1 receptor: A review. *CNS Drug Reviews*, *9*(1), 57–96. https://doi.org/10.1111/j.1527-3458.2003.tb00244.x

Shaham, Y., Erb, S., Leung, S., Buczek, Y., & Stewart, J. (1998). CP-154,526, a selective, non-peptide antagonist of the corticotropin-releasing factor1 receptor attenuates stress-induced relapse to drug seeking in cocaine- and heroin-trained rats. *Psychopharmacology*, *137*(2), 184–190. https://doi.org/10.1007/s002130050608

Sharp, J. L., Pearson, T., & Smith, M. A. (2022). Sex differences in opioid receptor mediated effects: Role of androgens. *Neuroscience and Biobehavioral Reviews*, *134*, 104522. https://doi.org/10.1016/j.neubiorev.2022.104522

Staeheli, S. N., Baumgartner, M. R., Gauthier, S., Gascho, D., Jarmer, J., Kraemer, T., & Steuer, A. E. (2016). Time-dependent postmortem redistribution of butyrfentanyl and its metabolites in blood and alternative matrices in a case of butyrfentanyl intoxication. *Forensic Science International*, *266*, 170–177. https://doi.org/10.1016/j.forsciint.2016.05.034

Steidl, S., Wasserman, D. I., Blaha, C. D., & Yeomans, J. S. (2017). Opioid-induced rewards, locomotion, and dopamine activation: A proposed model for control by mesopontine and rostromedial tegmental neurons. *Neuroscience and Biobehavioral Reviews*, *83*, 72–82. https://doi.org/10.1016/j.neubiorev.2017.09.022

Stinus, L., Cador, M., Zorrilla, E. P., & Koob, G. F. (2005). Buprenorphine and a CRF1 antagonist block the acquisition of opiate withdrawal-induced conditioned place aversion in rats. *Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology*, *30*(1), 90–98. https://doi.org/10.1038/sj.npp.1300487

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

Towers, E. B., Setaro, B., & Lynch, W. J. (2022). Sex- and dose-dependent differences in the development of an addiction-like phenotype following extended-access fentanyl self-administration. *Frontiers in Pharmacology*, *13*, 841873. https://doi.org/10.3389/fphar.2022.841873

Tsai, M. M., Chen, L., Baumann, M. H., Canals, M., Javitch, J. A., Lane, J. R., & Shi, L. (2023). The in vitro functional profiles of fentanyl and nitazene analogs at the μ-opioid receptor—High efficacy is dangerous regardless of signaling bias. bioRxiv, 2023.11.10.566672. https://doi.org/10.1101/2023.11.10.566672

United Nations Office on Drugs and Crime. (2023). World drug report: Executive summary.

Vachon, L., Costa, T., & Herz, A. (1987). Opioid receptor desensitization in NG 108-15 cells: Differential effects of a full and a partial agonist on the opioid-dependent GTPase. *Biochemical Pharmacology*, *36*(18), 2889–2897. https://doi.org/10.1016/0006-2952(87)90199-7

Vandeputte, M. M., Van Uytfanghe, K., Layle, N. K., St Germaine, D. M., Iula, D. M., & Stove, C. P. (2021). Synthesis, chemical characterization, and μ-opioid receptor activity assessment of the emerging group of "nitazene" 2-benzylbenzimidazole synthetic opioids. *ACS Chemical Neuroscience*, *12*(7), 1241–1251. https://doi.org/10.1021/acschemneuro.1c00064

VanHouten, J. P., Rudd, R. A., Ballesteros, M. F., & Karin, A. (2019). Mack: Drug overdose deaths among women aged 30–64 years—United States, 1999–2017. *MMWR. Morbidity and Mortality Weekly Report*, *68*(1), 1–5. https://doi.org/10.15585/mmwr.mm6801a1

Varshneya, N. B., Hassanien, S. H., Holt, M. C., Stevens, D. L., Layle, N. K., Bassman, J. R., Iula, D. M., & Beardsley, P. M. (2022). Respiratory depressant effects of fentanyl analogs are opioid receptor-mediated. *Biochemical Pharmacology*, *195*, 114805. https://doi.org/10.1016/j.bcp.2021.114805

Varshneya, N. B., Hassanien, S. H., Holt, M. C., Stevens, D. L., Layle, N. K., Bassman, J. R., Iula, D. M., & Beardsley, P. M. (2023). Fentanyl analog structure-activity relationships demonstrate determinants of diverging potencies for antinociception and respiratory depression. *Pharmacology, Biochemistry, and Behavior*, *226*, 173572. https://doi.org/10.1016/j.pbb.2023.173572

Varshneya, N. B., Walentiny, D. M., Moisa, L. T., Walker, T. D., Akinfiresoye, L. R., & Beardsley, P. M. (2019). Opioid-like antinociceptive and locomotor effects of emerging fentanyl-related substances. *Neuropharmacology*, *151*, 171–179. https://doi.org/10.1016/j.neuropharm.2019.03.023

World Health Organization. (2017). 4-fluorobutyrylfentanyl (4F-butyrylfentanyl) critical review report. Expert Committee on Drug Dependence, 39th Meeting. Geneva, Switzerland. https://www.who.int/medicines/access/controlled-substances/4F-Butyrylfentanyl.pdf.

Zhang, X., Zhang, Y., Asgar, J., Niu, K. Y., Lee, J., Lee, K. S., Schneider, M., & Ro, J. Y. (2014). Sex differences in μ-opioid receptor expression in trigeminal ganglia under a myositis condition in rats. *European Journal of Pain*, *18*(2), 151–161. https://doi.org/10.1002/j.1532-2149.2013.00352.x

Zhao, J., Elgeti, M., O'Brien, E. S., Sár, C. P., Ei Daibani, A., Heng, J., Sun, X., White, E., Che, T., Hubbell, W. L., Kobilka, B. K., & Chen, C. (2024). Ligand efficacy modulates conformational dynamics of the μ-opioid receptor. *Nature*, *629*(8011), 474–480. https://doi.org/10.1038/s41586-024-07295-2

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