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Allosteric modulators of the δ $GABA_A$ receptor subtype demonstrate a therapeutic effect in morphine-antinociceptive tolerance and withdrawal in mice

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Abstract

The present study evaluated the effects of compounds targeting extrasynaptic δ subunit-containing γ-aminobutyric acid type A receptors (δ*-$GABA_A$Rs) to interrogate the role of tonic inhibition in the development of antinociceptive tolerance caused by repeated morphine administration. We investigated the effect of subchronic or acute treatment with non-steroidal positive allosteric modulators (PAMs) of δ*-$GABA_A$Rs, such as 2–261, on the morphine-antinociceptive tolerance. Mice were treated twice daily with morphine for 9 days and antinociception was measured using the hot water tail immersion test. Co-treatment with 2–261 and morphine prevented morphine-antinociceptive tolerance and acute administration of 2–261 on day 9 was sufficient to reverse the tolerance. Other compounds with activity at δ*-$GABA_A$Rs also reversed morphine tolerance, whereas an enaminone that lacked activity at δ*-$GABA_A$Rs did not. Acute administration of 2–261 did not cause an additive or synergistic antinociceptive effect when combined with an acute submaximal dose of morphine. We then used Cre/LoxP recombination to generate $GABA_A$ δ-subunit knockout mice to corroborate the pharmacological results. Observations of male δ-knockout mice demonstrated that the δ*-$GABA_A$Rs was necessary for 2–261 modulation of both analgesic tolerance and somatic withdrawal symptoms produced by subchronic morphine. While female mice still benefited from the positive effects of 2–261, the δ-subunit was not necessary for these effects, highlighting a distinction of the different pathways that could have implications for some of the sex-related differences seen in human opioid-induced outcomes. Consequently, subtype-specific allosteric modulators of $GABA_A$Rs may warrant further investigation as pharmacological targets to manage tolerance and withdrawal from opioids.

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CRediT authorship contribution statement: K. Genaro, R. Yoshimura and K. Gee conceptualized and guided the project design. D. Hogenkamp performed the chemical synthesis of all compounds used in the study, and T. Johnstone, performed the electrophysiological data analysis. K. Genaro and R. Yoshimura performed the behavioral assays. B. Doan assisted in the behavioral assays, maintained the transgenic mouse colony and assisted in manuscript preparation. K. Genaro, R. Yoshimura and K. Gee wrote the manuscript.

Keywords

positive allosteric modulators; opioid; δ-subunit $GABA_A$ receptor; morphine-antinociceptive tolerance; opioid withdrawal symptoms

1. Introduction

The prevalence of opioid use in the United States stands in stark contrast to the potential danger it poses to the individuals who take them. From 2013–2016, about 1 in 15 Americans reported using a prescription opioid analgesic within the previous 30 days (Frenk et al., 2019). In 2017, opioids were involved in more than two-thirds of all overdose deaths in the United States (Scholl et al., 2019). Despite this significant liability, opioids have been commonly prescribed for the treatment of acute and severe pain because they are effective, inexpensive and there is a paucity of safer alternatives. The development of tolerance and physical dependence to repeated opioid exposure further complicate the use of opioids for chronic pain management (Montgomery, 2020).

Analgesic tolerance to long-term opioid use leads to the need to progressively increase the dose to achieve the same level of pain relief over time (Corder et al., 2018; Martyn et al., 2019). Even when chronic opioid treatment is discontinued, somatic and affective signs of withdrawal are typically observed (Rehni et al., 2013; Koob, 2020). While both tolerance to and withdrawal from opioids are likely due to neuroadaptive changes in the central nervous system at a molecular and cellular level, the mechanisms involved in each of these cases are still under intense investigation.

Preclinical research plays a key role in validating new drug targets with similar analgesic efficacy and minimized adverse effects. In this regard, γ-aminobutyric acid type A receptors ($GABA_A$Rs) are intriguing pharmacological targets for a variety of crucial processes in the nervous system. $GABA_A$Rs are ubiquitous and their activation is extremely nuanced depending on the precise localization of $GABA_A$R subunits and the individual subtype composition of receptors throughout the brain (Wisden et al., 1992; Fritschy and Mohler, 1995; Waldvogel et al., 2010; Sigel and Steinmann, 2012; Chua and Chebib, 2017). In addition to fast phasic inhibition, where $GABA_A$Rs are exposed to brief, saturating levels of GABA inside the synapse, there is also a tonic $GABA_A$R-mediated signaling in the extrasynaptic space (Brickley and Mody, 2012; Lu et al., 2020). This extrasynaptic inhibition typically involves δ-subunit-containing $GABA_A$Rs (δ*-$GABA_A$Rs) that mediate tonic inhibition and are sensitive to neurosteroids, the quintessential endogenous modulators of these extrasynaptic $GABA_A$Rs (Stell et al., 2003, Carver and Reddy, 2016). Mounting evidence indicates that hypofunction of GABAergic tone is a critical factor in chronic neuropathic pain (Gwak and Hulsebosch, 2011; Li et al., 2019). Molecular studies demonstrated decreased expression of components of the GABAergic system, including its receptors (Iura et al., 2016), GABA-synthesizing enzymes (Huang et al., 2016) and GABAergic interneurons (Meisner et al., 2010), resulting in impaired GABA synaptic inhibition that correlated well with behavioral reaction to nociceptive experiences. Similarly, we have observed that enhancing activity at δ*-$GABA_A$Rs reversed thermal and tactile

hypersensitivity in a spinal nerve ligation model (Johnstone et al., 2019; Luo et al., 2021). Subchronic treatment with neurosteroids prevented the development of morphine-antinociceptive tolerance and suppressed naloxone-precipitated withdrawal jumping in mice (Reddy and Kulkarni, 1997). Thus, there may be a relationship between these extrasynaptic δ*-$GABA_A$Rs and the aforementioned chronic opioid-related neuroadaptive changes.

A large body of evidence supports the existence of fundamental differences, including both pharmacokinetic and pharmacodynamic factors of opioid analgesia, between male and female responses and adaptations to pain and its treatment (Craft, 2003; Averitt et al., 2019). Numerous clinical and preclinical studies have found that steroid hormones, metabolic enzyme activities, and sexually dimorphic neural circuits in the brain interact to influence nociceptive sensitivity (Bartley and Fillingim, 2013; de Vries and Simerly, 2002). Sex-related differences in pain processing and analgesic sensitivity may also reflect differences in the endogenous GABA system. For example, a higher abundance of extrasynaptic δ*-$GABA_A$Rs transcripts in the ventral tegmental area leads to greater tonic inhibition in female mice compared to males (Darnieder et al., 2019). However, any role of extrasynaptic δ*-$GABA_A$Rs activity underlying sex-related differences in opioid sensitivity has yet to be revealed.

The present study examined the role of these extrasynaptic δ*-$GABA_A$Rs in preventing, reversing, and/or minimizing morphine-induced antinociceptive tolerance and somatic withdrawal symptoms. This study used compounds optimized for selectivity and potency at specific $GABA_A$R subtypes, including ganaxolone, etifoxine, and compounds developed in our laboratory with a distinct affinity for δ*-$GABA_A$Rs such as 2–261, 2–389 and 2–301 (Gee et al., 2010). With a genetic approach utilizing δ-subunit knockout (KO) mice, the role of δ*-$GABA_A$Rs on morphine-induced analgesic tolerance and somatic jumping behavior after naloxone-precipitated withdrawal in morphine tolerant mice was investigated. Additionally, we explored whether there were sex-related differences in the role of the δ-subunit in these behaviors.

2. Material and Methods

2.1. Oocyte electrophysiology

cDNA clones of human receptor subunits were synthesized by GENEWIZ (South Plainfield, NJ) and the subunit mRNA was prepared by TriLink Biotechnologies (San Diego, CA). Two-electrode voltage-clamp electrophysiology was performed on oocytes as previously described (Ng et al., 2007). Briefly, each compound was tested with a 30 s pre-treatment prior to co-application with a GABA EC_{10} (concentration of GABA that evokes 10% of the maximum response). Recorded currents in the presence of test compound were calculated as percent modulation relative to the control currents [($I_{modulated}/I_{control}$ * 100%) - 100%]. Concentration-response curves were fit to non-linear regression analysis on Prism 4.0 (GraphPad, San Diego, CA) to determine the maximal stimulation (E_{max}) and EC_{50} values.

2.2. Animals

Male CD-1 mice (Charles River, Wilmington, MA) weighing 35 to 40 g were used. They were housed under a 12:12-h light/dark cycle starting at 6:30 AM and tested according to the University of California, Irvine Institutional Animal Care and Use Committee (IACUC)-approved protocols. The animals had access to food and water *ad libitum* and were used after a minimum of 4 days of acclimatization under procedure room conditions.

2.3. Generation and validation of the $GABA_A$-δ subunit knockout mice

To understand what behaviors could be affected by reducing $GABA_A$-δ subunit function, we used a refined strategy for conditional gene inactivation that relies on the DNA recombinase Cre and its recognition *loxP* sites. Cre-*loxP* system is a widely used powerful technology for mammalian gene editing. This system has advantages which is very simple manipulation and do not require additional factors for efficient recombination (Nagy, 2000; Kim et al., 2018). In this study, floxed Gabrd mice (Lee and Maguire, 2014; JAX stock #023836), were crossed with CMV-Cre mice obtained from Jackson Laboratory (stock #006054) to generate mice deficient in the Gabrd gene. CMV-cre is a Cre-driver strain that express Cre recombination allowing deletion of *loxP*-flanked genes all tissues (Schwenk et al., 1995). Mouse genotypes from tail biopsies were determined using real-time PCR with specific probes designed for each gene: floxed, wild-type and Cre (Transnetyx, Cordova, TN). Mice of both sexes were used for experiments from 8–12 weeks of age (25–35 g).

Crossing Gabrd floxed$^{(+/+)}$ mice with CMV-cre$^{(+/+)}$ homozygous mice produced an F1 generation with the genotype Gabrd$^{+/-}$ CMV-cre^{+}. Subsequently, Gabrd$^{+/-}$ CMV-cre^{+} females and Gabrd$^{+/+}$ males were bred, leading to an F2 generation with a range of genotypes. In this manuscript we refer to the offspring which have an intact δ-subunit gene on both alleles as control mice (Gabrd$^{+/+}$ CMV-cre^{-} and Gabrd$^{+/-}$ CMV-cre^{-}). We refer to the offspring which have an intact δ-subunit gene on one allele as δ HET (Gabrd$^{+/-}$ CMV-cre^{+}). We refer to the offspring which have the δ-subunit gene deleted from both alleles as δ KO (Gabrd$^{-/-}$ CMV-cre^{+}).

2.4. Group sizes

The sample size “n” for each experimental condition represents independent observations, not replicates. For time-course studies and dose-response relationships, the data are presented as line graphs. Based on previous assessments of the reproducibility of morphine opioid tolerance (Martyn et al., 2019; Reddy and Kulkarni, 1997), a sample size of at least 6 was used in time-course studies assessing the effect of compounds targeting extrasynaptic $GABA_A$Rs on morphine-antinociceptive tolerance.

2.5. Compound administration

Morphine (Sigma Aldrich, St. Louis, MO) was diluted in saline and administered (dorsal neck region) subcutaneously (s.c.) twice a day in a volume of 10 mL/kg, at a final dose of 10 mg/kg. 2–261 and analogs were synthesized as previously described (Gee et al., 2010) and dissolved in 1.5% dimethyl sulfoxide (DMSO; Sigma Aldrich, St. Louis, MO), 1.5% solutol (Sigma Aldrich, St. Louis, MO), and 97% saline (0.9% NaCl). Ganaxolone (10 mg/kg) and loreclezole (30 mg/kg) were synthesized as previously described (Carter et al.,

1997; Wingrove et al., 1994; Hogenkamp et al., 2014), ganaxolone was dissolved in 35% beta-cyclodextrin and water, and loreclezole was dissolved in 5% dimethyl sulfoxide, 5% solutol and 90% saline. Etifoxine (Scynexis, Jersey City, NJ) at the dose of 50 mg/kg was dissolved in 5% dimethyl sulfoxide, 5% solutol and 90% saline. Gabazine and naloxone (Sigma Aldrich, St. Louis, MO) at the dose of 3 mg/kg and 1 mg/kg, respectively, were dissolved in saline (0.9% NaCl). The dose and incubation time for each compound were based on previous pharmacokinetic and behavioral experiments (Gee et al., 2010; Nuss et al., 2019). The volume for i.p. injections was 10 mL/kg.

2.6. Nociceptive response test

According to Coderre and Rollman (1983), the warm-water tail immersion test was performed using a water bath with the temperature maintained at 55°C. Before injecting the mice, a baseline (control) latency was determined. Only mice with a control reaction time from 2 to 4 seconds were used. The average baseline latency for these experiments was 2.5 – 3.0 seconds. The test latency after morphine treatment was assessed at 30 minutes with a 10-second maximum cut-off time imposed to prevent tissue damage. Antinociception was quantified according to the method of Harris and Pierson (1964) as the percentage of maximum possible effect (%MPE), which was calculated as %MPE = [(test latency – control latency) / (10 – control latency)] × 100. Development of tolerance to the antinociceptive effect of morphine sulfate (10 mg/kg, twice daily) was measured in the tail-immersion test. Baseline tail-flick latency was measured before compound treatment and compared between treatment groups every other day (15 days).

In the co-treatment study, 2–261 (10 mg/kg) was dosed 30 minutes before each morphine injection and the tail-immersion test was performed 30 minutes after the morphine injection. In the acute treatment studies, effects of different compounds targeting δ*-$GABA_A$Rs were tested after developing morphine tolerance (on the 9^{th} day). The latency until withdrawal (rapid flick) was measured before compound treatment (baseline) and compared among treatment, 30 minutes after test compound and 30, 60, and 120 minutes following morphine injection groups, unless specified otherwise. Tail immersion tests were performed by an experienced experimenter blind to the treatment.

2.7. Naloxone-precipitated withdrawal

Withdrawal susceptibility was assessed by administration of the selective μ-opioid receptor antagonist naloxone. The mice were put on an escalating-dose administration paradigm for morphine (10–40 mg/kg, s.c., b.i.d.) for four consecutive days with an 8-h interval between doses given on the same day (day 1, 10 mg/kg; day 20 mg/kg; day 3, 30 mg/kg; and day 4, 40 mg/kg). On the morning of day 5, mice received a single morphine dose (40 mg/kg, s.c.) and then 90 minutes later received either vehicle or 2–261 (10 mg/kg, i.p.) in a counterbalanced fashion. 120 minutes after the morphine dose (30 minutes after vehicle or compound), the mice were then challenged with naloxone (1 mg/kg, s.c.) and placed in an acrylic chamber (10 cm in diameter, 30 cm high) where their jumping behavior was recorded for 30 minutes. The mice continued to receive 40 mg/kg morphine on the evening of day 5 and through day 6. On the morning of day 7, they were then put through the same procedure as on day 5, except now the mice were given whichever treatment they had not previously

received in a counterbalanced fashion. There were no significant differences between the day 5 and the day 7 results for any individual treatment group by one-way ANOVA with Bonferroni's multiple comparisons test. The videos were scored by a blinded observer who counted the number of jumps in the 30 minutes video.

2.8. Statistical analysis

Statistical differences were determined by two-way repeated measure ANOVA using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA). For assessment of behavioral experiments, all data are expressed as mean ± standard error. Two-way repeated-measures ANOVA with Tukey's or Bonferroni's multiple comparisons test were used to assess the effect of treatments, time and genotype. Selected *post hoc* statistical tests are specified in the results. In all cases, the threshold for significance was $p < 0.05$.

3. Results

3.1. Co-treatment study: 2–261 prevents the development of antinociceptive tolerance to subchronic morphine.

The effect of the δ*-$GABA_A$Rs positive allosteric modulator, 2–261 (10 mg/kg), on the development of antinociceptive tolerance following repeated morphine administration (10 mg/kg, twice daily) was examined in the tail-immersion assay. Figure 1A shows the timeline of the experiment and Figure 1B the antinociceptive response expressed as the percentage of the maximum possible effect (MPE) for 2–261 alone, morphine alone, and co-treatment with both 2–261 and morphine. Two-way repeated-measures ANOVA demonstrated a significant effect of time ($F_{7,224} = 25.42$; $p < 0.0001$), treatment ($F_{3,32} = 55.78$; $p = 0.0001$) and interaction of factors ($F_{21,224} = 5.9$; $p = 0.0001$). A Tukey post-test determined significant differences between treatment groups. Morphine initially produced maximum antinociception in both groups, but this antinociception was significantly reduced by the 9th day in vehicle + morphine mice while being preserved in 2–261 + morphine mice (Fig. 1B). 2–261 treatment alone produced a significant antinociceptive effect in saline-treated mice ($p < 0.05$), which was relatively consistent across all experimental sessions varying between 20 – 40% antinociception MPE, except on days 11 and 15 (17% antinociception MPE). There was no statistically significant reduction in the analgesic effect of 2–261 over time compared to day one. Morphine tolerance occurred in vehicle + morphine treated mice by day 7 compared to day 1 ($p < 0.05$). Antinociception was gradually reduced throughout the experimental sessions. Additionally, 2–261 + morphine treated mice showed significantly greater morphine analgesic sensitivity than saline + morphine treated mice from the 9th day of the treatment ($p < 0.05$). 2–261 + morphine treatment also slightly decreased nociceptive threshold on days 11 and 15 compared to day 1 of treatment (Fig. 1B). Absolute values of tail-flick latencies (seconds) for individual mice are shown in figure 1C.

3.2. Acute treatment study: 2–261 dose-dependently reverses morphine-induced antinociceptive tolerance.

We also examined the acute effect of a single injection of 2–261 on the 9th day, after antinociceptive tolerance was induced by morphine in the tail-immersion assay. Figure 2A shows the experimental timeline to investigate the dose-dependent effect of 2–261 on

morphine-induced antinociception in the tail immersion test. Two-way repeated-measures ANOVA demonstrated a significant effect of time ($F_{4,100} = 46.17$; $p < 0.0001$), treatment ($F_{3,25} = 44.3$; $p = 0.0001$) and interaction of factors ($F_{12,100} = 9.484$; $p = 0.0001$). A Tukey post-test determined significant differences between treatment groups. Morphine produced maximal antinociception on day 1, but there was significant tolerance to this antinociceptive effect by the 9th day of treatment ($p < 0.01$). A single injection of 2–261 (3 and 10 mg/kg), 30 min before the last morphine treatment on day 9, reversed the antinociceptive tolerance ($p < 0.001$). This response was not observed after the lowest dose of 2–261 (1 mg/kg) treatment ($p > 0.05$) at any time point (Fig. 2B). Absolute values of tail-flick latencies for individual mice are showed in figure 2C.

3.3. Acute treatment study: 2–261 and related compounds on morphine-induced antinociceptive tolerance.

2–301, etifoxine and 2–261 have varying degrees of *in vitro* activity at δ*-$GABA_A$Rs (Supplementary Table S1). Their acute effects on morphine-induced antinociceptive tolerance were compared in mice. Figure 3 shows that compound activity at δ subunits is required to reverse morphine tolerance. Etifoxine and 2–261 reversed the morphine tolerance. Etifoxine has a dual mechanism of action to enhance GABAergic transmission, a direct effect on $GABA_A$Rs, and an indirect effect by enhancing neurosteroid synthesis (Rupprecht and Holsboer, 1999; Nuss et al., 2019). On the other hand, 2–301, a compound with similar activity at β subunit subtypes as 2–261, but with minimal activity at δ subunits (Johnstone et al., 2019), showed a significant effect *per se* 30 minutes after administration but did not show any significant effect against morphine tolerance induced reduction in tail-flick latency. The effects of 2–261 and related compounds on GABA EC10 – evoked currents in oocytes expressing representative human synaptic and extrasynaptic $GABA_A$Rs are summarized in the supplementary material (Supplementary Table S1).

Three other compounds with different activity at and selectivity for $GABA_A$R subtypes were also acutely administered in independent groups 30 minutes before the last morphine (10 mg/kg) injection on day 9 when tolerance was fully developed. All compounds tested have activity at δ*-$GABA_A$Rs, including g*anaxolone* (10 mg/kg), a synthetic analog of the endogenous neurosteroid allopregnanolone (Carter et al., 1997; Belelli et al., 2019); *loreclezole* (30 mg/kg), a PAM selective for $GABA_A$Rs containing β2 and β3 subunits (Holopainen et al., 2001); and 2–389, an enaminone with optimal potency/efficacy for the δ*-$GABA_A$Rs (Hogenkamp et al., 2019). All of these compounds reversed the morphine tolerance (Supplementary Figure S2). The effects of these compounds on morphine tolerance were blocked by pre-treatment with gabazine (3 mg/kg), a compound that acts as an antagonist at $GABA_A$Rs (Supplementary Figure S3).

3.4. 2–261 and etifoxine acutely modulate analgesic tolerance produced by subchronic morphine: effect of genetic manipulation.

We chose 2–261 and the clinically used drug, etifoxine, to further test the hypothesis that δ*-$GABA_A$Rs are necessary mediators for the reversal of morphine tolerance in males and females. We tested whether complete or partial deletion of δ*-$GABA_A$Rs affected the nociceptive response using a conditional–knockout strategy. The homozygous null mutant

mice $GABA_A$-δ subunit (δ KO), heterozygous mice (δ HET), and control mice exhibited similar tail-flick latency under baseline conditions (Supplementary Figure S4). The first injection of morphine (10 mg/kg) in all mice induced full analgesia. Morphine tolerance occurred in vehicle + morphine treated mice by day 11 compared to day 1 ($p < 0.001$).

As shown in Figure 4, etifoxine (50 mg/kg) or 2–261(10 mg/kg) had no significant effect *per se* on the nociceptive response when compared to the baseline response of the different genotypes; control, δ HET or δ KO, ($p > 0.05$). Control male mice pre-treated with 2–261 or etifoxine displayed a 2-fold increase in the percentage of antinociception compared to morphine effects in a baseline condition on day 11 (Supplementary Figure S4). Two-way repeated-measures ANOVA demonstrated a significant effect of time ($F_{2,48} = 80.23$, $p < 0.0001$), genotype ($F_{2,24} = 5.310$, $p = 0.0123$) and interaction of factors ($F_{4, 48} = 3.934$, $p = 0.0077$). A Tukey post-test determined significant differences in the genotype response to etifoxine between the control group and the δ-KO group at both 30 and 60 minutes ($p < 0.001$ and $p < 0.05$, respectively). There was not a significant difference between the control group and the δ-HET group at any time point. Two-way repeated-measures ANOVA demonstrated a significant effect of time ($F_{2,54} = 47.78$; $p < 0.0001$) and genotype ($F_{2,27} = 7.053$, $p = 0.0034$), but no interaction between the factors ($F_{4,54} = 1,065$; $p = 0.3826$). A Tukey post-test determined significant differences in the genotype response to 2–261 between the control group and the δ-KO group at both 30 and 60 minutes ($p < 0.01$ for both). There was a significant difference between the control group and the δ-HET group at 30 minutes ($p < 0.05$), but not at 60 minutes.

3.5. 2–261 modulates naloxone-precipitated withdrawal jumping behavior in males: effect of genetic manipulation.

Naloxone was used to precipitate physical withdrawal symptoms in mice that received twice-daily doses of morphine over five consecutive days (Figure 4C). Two-way repeated measures ANOVA demonstrated a significant effect of genotype ($F_{2,27} = 7.614$; $p = 0.0024$) and a borderline effect of treatment ($F_{1,27} = 3.867$; $p = 0.0596$). There was not a significant interaction between genotype and treatment ($F_{2,27} = 0.2109$; $p = 0.8112$). A Bonferroni post-test determined significant differences in the genotype response between the control group and the δ-HET group to either vehicle ($p < 0.01$) or 2–261 ($p < 0.001$). There was not a significant effect in the genotype response between the control group and the δ-KO group. A Bonferroni post-test determined a significant difference in the treatment response to 2–261 compared to vehicle for the control group ($p < 0.05$), but not for the δ-HET or the δ-KO groups (Figure 4D).

3.6. Sex-related differences: partial or global deletion of the δ-subunit $GABA_AR$ does not influence the effect of 2–261 on morphine-antinociceptive tolerance and naloxone-precipitated withdrawal jumping behavior in females.

A different outcome was observed in females receiving 2–261 in an acute treatment study of antinociceptive tolerance (Figure 5A). Two-way repeated-measures ANOVA demonstrated a significant effect of time ($F_{2,44} = 104.3$; $p < 0.0001$), but there was not a significant effect of genotype ($F_{2,22} = 1.329$; $p = 0.2851$), nor was there an interaction of factors ($F_{4,44} = 1.803$; $p = 0.1453$). A Tukey post-test determined that treatment with 2–261 caused

a significant reversal of tolerance at both the 30 and 60 minute timepoints compared to the 0 minute timepoint, and that this occurred across all genotypes ($p < 0.01$ for all). Treatment with 2–261 (10 mg/kg) before the nociceptive test had no significant effect on morphine-antinociceptive tolerance compared to the basal nociceptive response in females ($p > 0.05$).

Similarly, 2–261 treatment diminished naloxone-precipitated withdrawal jumping in the control group as well as in the δ HET and δ KO mice (Figure 5B). Two-way repeated-measures ANOVA demonstrated a significant effect of treatment ($F_{1,28} = 24.65$; $p < 0.0001$). In contrast to the males, there was not a significant effect of genotype ($F_{2,28} = 0.0584$; $p = 0.9434$). A Bonferroni post-test determined a significant difference in the treatment response of 2–261 compared to vehicle for the control group ($p < 0.05$), the δ-HET group ($p < 0.01$) and the δ-KO group ($p < 0.05$).

4. Discussion

The primary finding of these experiments is that acute administration of certain positive allosteric modulators of $GABA_A$Rs such as 2–261 can prevent or reverse subchronic morphine-induced antinociceptive tolerance. Although concomitant treatment with 2–261 can prevent morphine-antinociceptive tolerance, 2–261 at 10 mg/kg has a small but statistically significant antinociceptive effect when administered alone. It is unlikely that this effect of 2–261 is additive to the morphine tolerance effect on antinociception, because after 9 days of treatment, both 2–261 and morphine showed a range of 27–31% MPE of antinociception on their own, but in combination, the antinociceptive effect increases to 80% (Figure 1B). Importantly, a 3 mg/kg dose of 2–261 that has no effect on its own was also able to reverse morphine-antinociceptive tolerance when administered acutely on the final day (Figure 2B). We also tested whether there would be a possible antinociceptive potentiation following acute administration of 2–261 with morphine. Combinations of two different doses of 2–261 (3 or 10 mg/kg) with a submaximal dose of morphine (3 mg/kg) did not result in an additive or synergistic response in the tail flick model (Supplementary Figure S5). Thus, combining an allosteric modulator of δ*-$GABA_A$Rs with morphine yielded different results depending on the dosing regimen of morphine. Subchronic morphine (10 mg/kg) caused an antinociceptive tolerance that was reversed by acute administration of 2–261 at 3 or 10 mg/kg. However, acute submaximal morphine (3 mg/kg) does not appear to have an additive or synergistic effect when paired with 2–261 at 3 or 10 mg/kg. This suggests that the allosteric modulation of δ*-$GABA_A$Rs prevents and reverses morphine-antinociceptive tolerance, while not acutely potentiating morphine-induced antinociception.

It is worth mentioning that synergistic drug combinations potentially maximize the therapeutic effects while minimizing the adverse effects (Greco et al., 1995; Lehár et al., 2009; Foucquier and Guedj, 2015). However, it is challenging to quantify synergistic interactions accurately (Berthoud, 2013; Geary, 2013; Roell, 2017). Our findings support the development of drugs for selectivity and potency at specific δ*-$GABA_A$Rs as potential therapeutic strategies to prevent compensatory mechanisms and to improve the utility of opioids.

Using pharmacological probes such as 2–261 and 2–301, we demonstrated that acute allosteric modulatory activity at the extrasynaptic δ*-$GABA_A$Rs might be sufficient to reverse morphine-antinociceptive tolerance (Fig. 3). It should be noted that this does not entirely rule out a contribution from the synaptic $GABA_A$Rs and other pathways as we do not yet have a compound that exclusively activates δ*-$GABA_A$Rs. However, the effect of 2–261 or etifoxine is diminished with even a partial knockout of the δ*-$GABA_A$Rs in males (Fig. 4B). A similar effect was observed with neuroactive steroids having attenuated sensitivity in $GABA_A$R δ subunit knockout mice (Mihalek et al., 1999; Mihalek et al., 2001). Thus, activation of the δ*-$GABA_A$Rs by 2–261 and etifoxine is necessary to reverse morphine-antinociceptive tolerance in male mice. However, etifoxine is inactive at δ*-$GABA_A$Rs expressed in oocytes (Supplementary Table S1). This discrepancy is likely due to its dual mechanism of action to enhance GABAergic transmission. While etifoxine does directly modulate some $GABA_A$Rs, presumably via a high-affinity site on β subunit (Hamon et al. 2003), there is also an indirect pathway to δ*-$GABA_A$R modulation. This is because etifoxine also binds to the mitochondrial translocator protein (TSPO) and stimulates neurosteroidogenesis, the products of which then modulate δ*-$GABA_A$Rs (see Rupprecht and Holsboer, 1999; Nuss et al., 2019).

We saw a similar pattern of results with 2–261 when looking at somatic signs of morphine withdrawal (Figure 4D). 2–261 can acutely reduce naloxone-precipitated morphine withdrawal jumping and this effect is also diminished in the male δ-KO mice. This suggests that subchronic morphine treatment might have altered behavioral pathways that are amenable to treatment by δ*-$GABA_A$R modulators.

Opioid receptors are distributed at various sites located along nociceptive pathways and there is an overlap of the distribution for the three opioid receptors and $GABA_A$Rs (Svingos et al., 1997; Vaughan et al., 1997; Kalyuzhny et al., 2000; Erbs et al., 2015; Valentino and Volkow, 2018). Numerous key brain structures have been identified where the interaction of opioid and GABA mechanisms play an important role in the development of the pathological pain state, in particular for the reciprocal connections within the regions of the corticostriatal-limbic circuit (Chartoff and Connery, 2014; Haber, 2016; Koob and Le Moal, 2001). Opioid receptors can differentially modulate GABAergic neurons of the nucleus accumbens and periaqueductal gray, dopaminergic neurons of the ventral tegmental area, and glutamatergic neurons of the prefrontal cortex (Svingos et al., 1997; Madhavan et al., 2010; Li et al., 2016; Burns et al., 2019; Reeves et al., 2021). A growing body of evidence from preclinical and clinical studies has demonstrated relationships between $GABA_A$ and opioid receptor activation and these findings are broadening our understanding of how this interaction may contribute to pro- and anti-aversive effects, analgesia, reward, dependence, tolerance, somatic and affective signs of withdrawal, and relapse (Chartoff and Connery, 2014; Wise, 1989; Shen and Kalivas, 2013; Matsui et al., 2014; Fields and Margolis, 2015). In addition, neurosteroids were observed to have a modulatory role during the development of tolerance (Reddy and Kulkarni, 1997; Concas et al., 2006; Goodchild et al., 2009; Winter et al., 2003), and may alleviate at least some of the signs of morphine abstinence (Kulkarni and Reddy, 1995). Given the diversity and ubiquity of $GABA_A$Rs in the brain, it might be clinically valuable to pursue selective drugs that take advantage of opioid–$GABA_A$R interactions.

A secondary finding of these experiments is that while the δ-subunit appears to be necessary for the 2–261 effects in males, it is not necessary for females. Importantly, 2–261 had a therapeutic effect in control mice independent of sex in both the antinociceptive and withdrawal paradigms. While the 2–261 effects were diminished in male δ-KO mice, the female δ-KO mice retained the antinociceptive effect of morphine (Figure 5A). It should be noted that this may be obscured in the withdrawal jumping paradigm due to baseline differences across genotypes in males that were not seen in females (Figure 5B). These data suggest that 2–261 may also be exerting an effect through a δ-independent pathway in the female mice that is not available in the males. It could be that 2–261 modulates a synaptic $GABA_A$ pathway that is sufficient to reverse the effects of subchronic morphine in female mice, but not in male mice. This could be due to a differential in the compensatory response to the δ-subunit deletion in female mice.

One important contrast to note is that these therapeutic results observed by δ*-$GABA_A$Rs modulation on morphine-related adverse effects were identified with a model of phasic pain. For comparison, neuropathic pain is more resistant to opioid analgesia, but opioids continue to develop tolerance in neuropathic pain patients. It is not clear whether positive allosteric modulators of δ*-$GABA_A$Rs would prevent or reverse tolerance in a neuropathic pain model. Pain modulation may represent a distinct strategy for pain therapy and the present study indicates a possible target to prevent, reverse or minimize opioid side effects. It remains to be confirmed that tonic inhibition elicited through δ*-$GABA_A$Rs could modulate opioid side effects in different pain paradigms. This study demonstrates that certain positive allosteric modulators can acutely reverse morphine-antinociceptive tolerance and reduce a somatic sign of morphine withdrawal in both male and female control mice. Extrasynaptic δ*-$GABA_A$Rs are necessary for these effects in males but not in females. Further investigation of the mechanisms that underlie these phenomena will allow us to better understand the neural adaptations to chronic opioid use and develop better treatments to combat opioid addiction.

5. Conclusions

Our findings provide pharmacological, genetic, physiological, and behavioral evidence that GABAergic and opioid systems are closely linked and that activity at extrasynaptic δ*-$GABA_A$Rs might conditionally modulate tolerance and withdrawal in rodents. Furthermore, therapeutic strategies for mitigating pain and opioid side effects should include considerations about potential mechanistic differences between males and females, given the evidence for sexual dimorphism. Other physiological conditions, including neurotransmitter release, the function of ion channels, synaptic connectivity, neural circuitry or the interaction of any of these factors, can influence the pharmacological effects of allosteric modulation of extrasynaptic $GABA_A$Rs. These findings support that modulation of GABAergic neurotransmission via δ*-$GABA_A$Rs may present as a therapeutic strategy to improve the utility of opioids. Thus, our results provide a rationale for additional investigation and further evaluation of the mechanism of δ*-$GABA_A$Rs PAMs as an innovative and potentially safer approach to reverse and prevent opioid side effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Concomitant modulation of δ-$GABA_A$Rs prevents the development of morphine-antinociceptive tolerance
- δ-$GABA_A$R positive allosteric modulators reverse morphine-antinociceptive tolerance
- Acute 2–261 does not potentiate antinociceptive effects of an acute submaximal dose of morphine
- 2–261 reduces signs of morphine withdrawal in both male and female control mice
- δ-$GABA_A$Rs are necessary for the therapeutic effect in males, but not in females

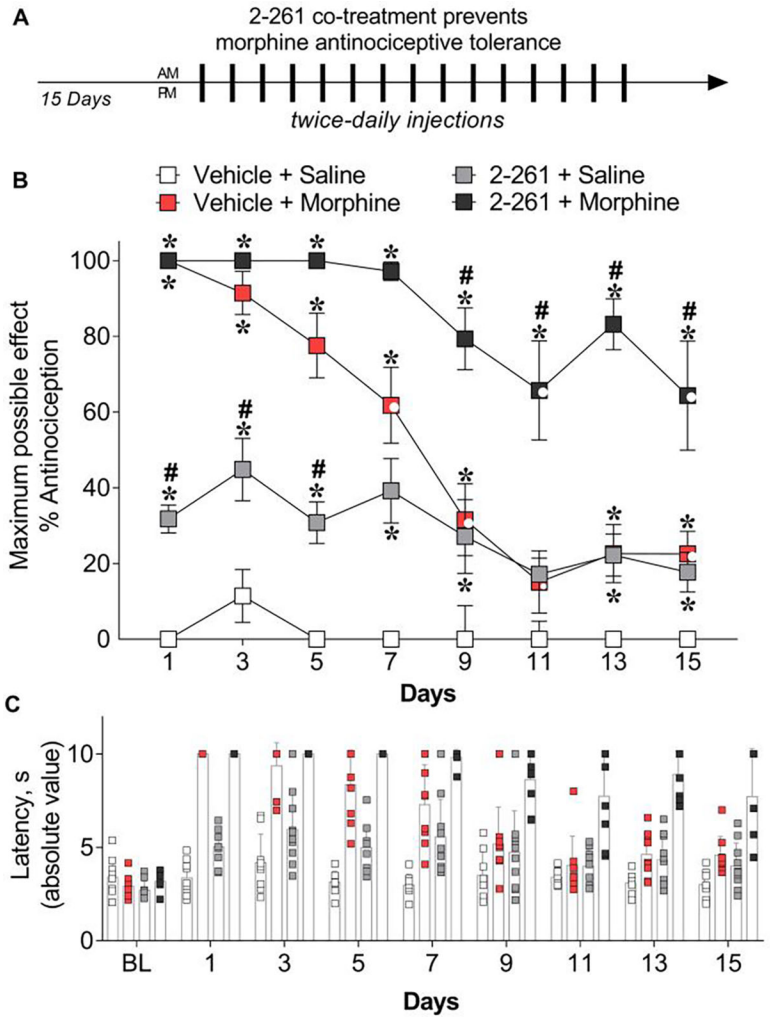

Fig. 1. 2–261 co-treatment prevents morphine-antinociceptive tolerance.

(A) Timeline of the experimental protocol. (B) Subchronic morphine exposure resulted in antinociceptive tolerance to morphine (10 mg/kg; s.c.) that was prevented by co-treatment with 2–261(10 mg/kg; i.p.) in the tail-immersion assay. Results are expressed as the percentage of maximum possible effect (%MPE ± SEM). A “white dot” inside the square symbol ($p < 0.01$) indicates difference of %MPE compared to day 1 with the same treatment group; * $p < 0.01$ different from vehicle + saline; # $p < 0.01$ different from vehicle + morphine, N = 8–10/group. (C) Absolute values of tail-flick latencies for individual mice, 10-second maximum cut-off time.

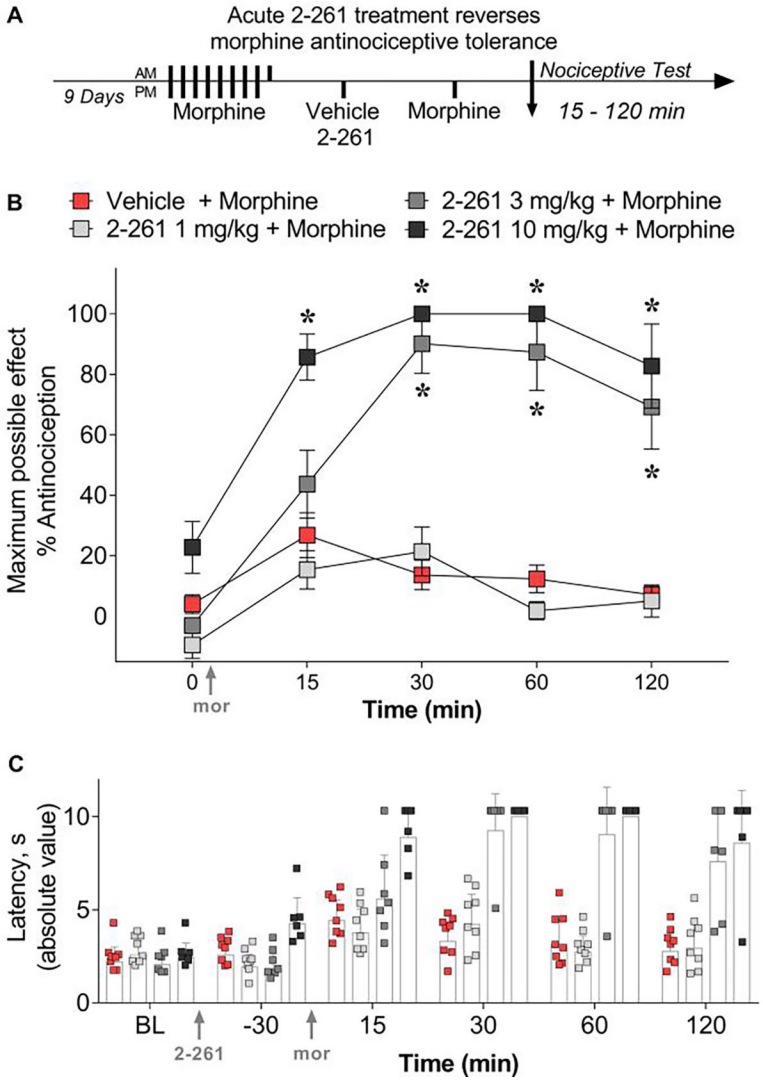

Fig. 2. Acute 2–261 treatment reverses morphine-antinociceptive tolerance.

(A) Timeline of the experimental protocol. (B) Subchronic morphine exposure resulted in antinociceptive tolerance to morphine (10 mg/kg, twice daily; s.c.) that was reversed by acute 2–261 (3 and 10 mg/kg; i.p.) pre-treatment (30 minutes before the final morphine injection in the tail-immersion assay on day 9. Results are expressed as the %MPE ± SEM in response to morphine. * $p < 0.01$ compared to vehicle + morphine group, N = 6–8/group. (C) Individual absolute values of tail-flick latencies, 10-second maximum cut-off time. The arrows at the x-axis indicate the final morphine (mor) injection and 2–261 injection.

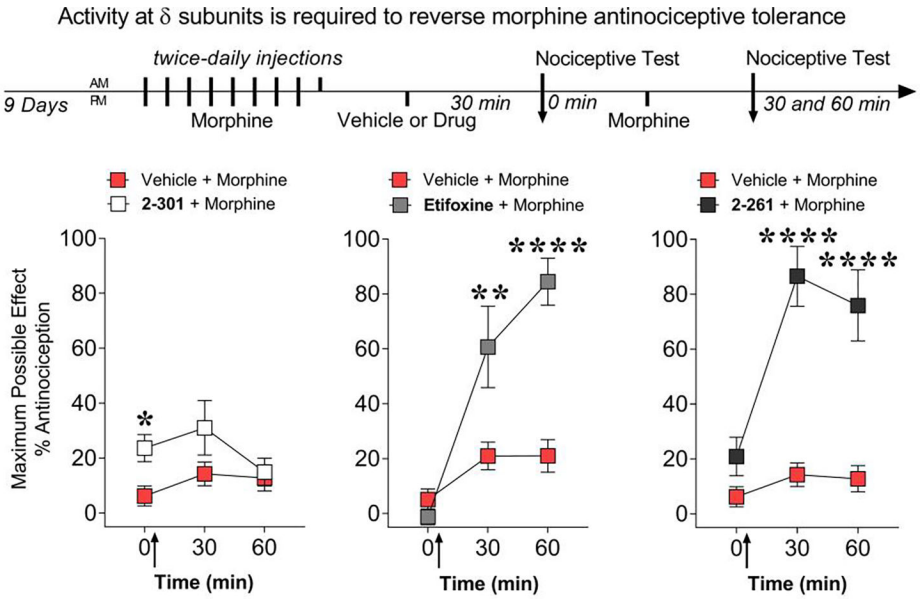

Fig. 3. 2–261 and related compounds on morphine-antinociceptive tolerance.

(A) Timeline of the experimental protocol. (B) Comparison of the effects of 2–301 (10 mg/kg), etifoxine (50 mg/kg), and 2–261(10 mg/kg) in mice submitted to subchronic morphine treatment. Results are expressed as the percentage of maximum possible effect (%MPE ± SEM) in response to morphine. * $p < 0.05$; ** $p < 0.01$ **** $p < 0.0001$ compared to vehicle + morphine (10 mg/kg) treatment at the same time point. The arrow at the x-axis indicates the final morphine injection. N=8–10/group.

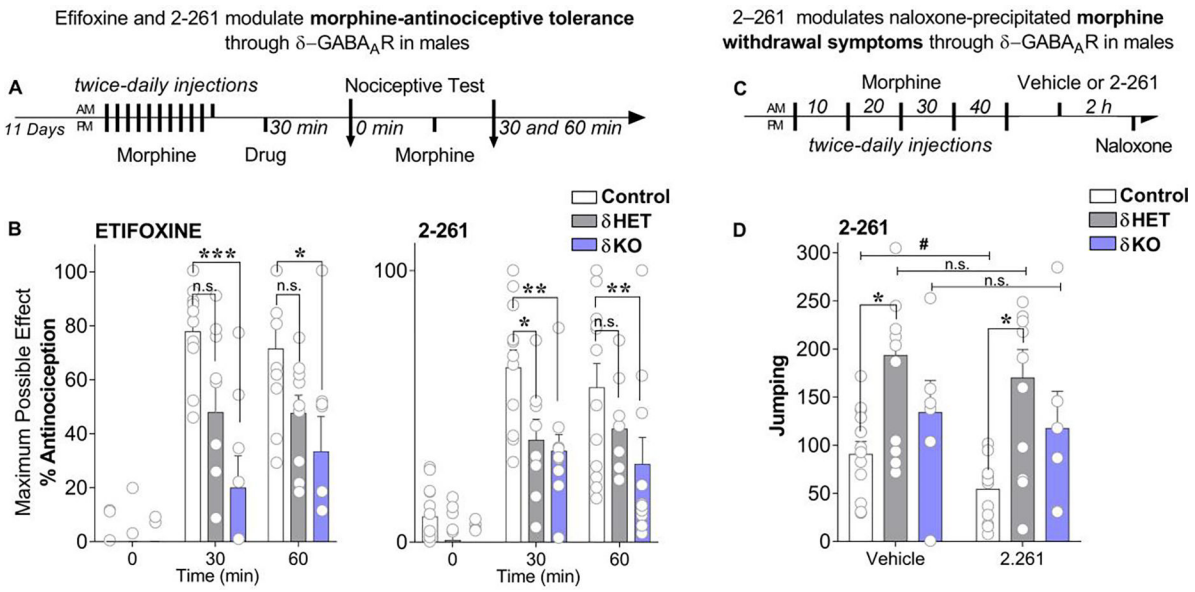

Fig. 4. δ-containing GABA$_A$Rs are necessary for etifoxine and 2–261 to reverse morphine-antinociceptive tolerance in males.

(A) Timeline of the experimental protocol. (B) Comparison of the effects of Etifoxine (50 mg/kg) and 2–261 (10 mg/kg) in null GABA$_A$ δ-subunit mutant mice (δ KO), heterozygous mice (δ HET) and control mice submitted to chronic morphine treatment. Results are expressed as the percentage of maximum possible effect (%MPE ± SEM) in response to drug treatment (time 0); 30 and 60 min after last morphine (10 mg/kg) injection. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; no significant (n.s.) compared between genotypes. N=8–12/group. (C) Timeline of the experimental protocol testing the involvement of 2–261 in morphine dependence. (D) 2–261 diminished naloxone-precipitated withdrawal jumping in control male mice ($p < 0.05$), but not in the δ HET or δ KO mice.

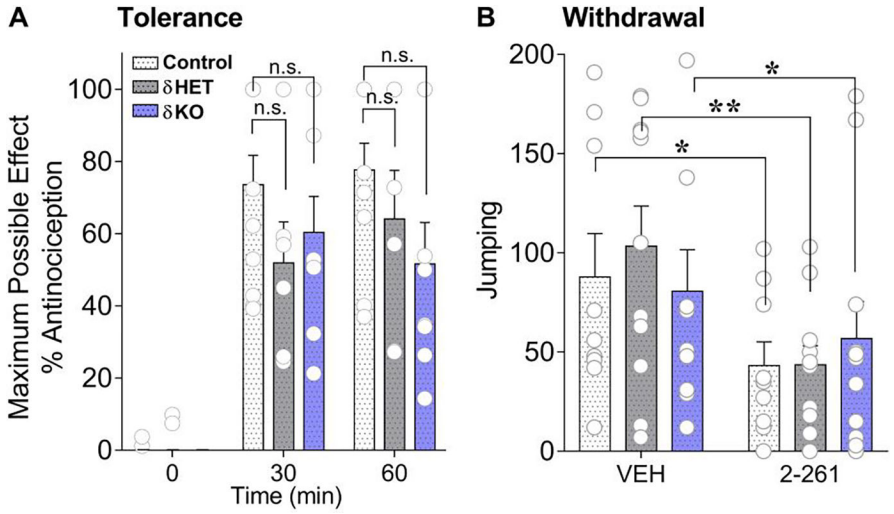

Fig. 5. δ-containing $GABA_A$Rs are not necessary for 2–261 to reverse antinociceptive tolerance and naloxone-precipitated withdrawal jumping behavior in females.

Comparison of the effects of 2–261 (10 mg/kg) in null $GABA_A$ δ-subunit mutant mice (δ KO), heterozygous mice (δ HET) and control mice submitted to chronic morphine treatment. (A) Tolerance: Results are expressed as the percentage of maximum possible effect (%MPE ± SEM) in response to 2–261 treatment (time 0) and after last morphine (10 mg/kg) injection (30 and 60 min). No significant (n.s.) difference was found between genotypes. N=8–12/group. (B) Withdrawal: Acute administration of 2–261 significantly reduced naloxone-precipitated withdrawal jumping for females in all groups ($p < 0.05$).