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Full Paper

Role for μ-opioid receptor in antidepressant effects of δ-opioid receptor agonist KNT-127

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ABSTRACT

Previous pharmacological data have shown the possible existence of functional interactions between μ- (MOP), κ- (KOP), and δ-opioid receptors (DOP) in pain and mood disorders. We previously reported that MOP knockout (KO) mice exhibit a lower stress response compared with wildtype (WT) mice. Moreover, DOP agonists have been shown to exert antidepressant-like effects in numerous animal models. In the present study, the tail suspension test (TST) and forced swim test (FST) were used to examine the roles of MOP and DOP in behavioral despair. MOP-KO mice and WT mice were treated with KNT-127 (10 mg/kg), a selective DOP agonist. The results indicated a significant decrease in immobility time in the KNT-127 group compared with the saline group in all genotypes in both tests. In the saline groups, immobility time significantly decreased in MOP-KO mice compared with WT mice in both tests. In female MOP-KO mice, KNT-127 significantly decreased immobility time in the TST compared with WT mice. In male MOP-KO mice, however, no genotypic differences were found in the TST after either KNT-127 or saline treatment. Thus, at least in the FST and TST, the activation of DOP and absence of MOP had additive effects in reducing measures of behavioral despair, suggesting that effects on this behavior by DOP activation occur independently of MOP.

1. Introduction

Stress and trauma are important predisposing factors for mental illness symptoms, consequently affecting not only the lives of individual people but also imposing a great financial burden on society.[1] Epidemiological studies have shown that racial differences, gender, living environment, and a history of stressful and traumatic experiences play roles in the development of stress-induced psychiatric disorders, such as depression and anxiety, affecting the age of onset, treatment responsiveness, and disease progression.[2–5]

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There are three types of opioid receptors—μ (MOP), δ (DOP), and κ (KOP)—with different pharmacological properties but interacting physiological roles that are reflected by their analgesic and rewarding effects.[6] Opioid receptors are also well known to affect stress responses, and stressful experiences are known to affect responses to opioids.[7–9] We previously reported that MOP knockout (KO) mice exhibit a lower physical stress response compared with wildtype (WT) mice.[10,11] From the perspective of treating stress-induced psychiatric and physical diseases, pharmacological interventions via MOP may have therapeutic potential. However, the potential benefits of direct interventions with MOP agonists, such as morphine, are limited because opioids are highly addictive. Moreover, MOP stimulation is associated with the rapid development of tolerance.[12] Although alternative approaches for targeting MOP that do not produce tolerance have been investigated,[13] an alternative approach would potentially involve interactions between MOP and other opioid receptors.

Selective non-peptide DOP agonists, such as SNC80, have shown promise as analgesics, and many researchers have attempted to develop SNC80 derivatives with improved properties. However, studies of these drugs were halted at early clinical trial stages because of side effects and weak analgesic activity. DOP agonists have also been proposed as attractive candidates for some other psychiatric conditions because they do not have the same side effects as benzodiazepines or selective serotonin reuptake inhibitor antidepressants.[14] Additionally, research with rhesus monkeys showed that non-peptide DOP agonists do not produce respiratory-depressant or reinforcing effects,[15] which are major problems with other opioid agents. Although some studies have investigated analgesic roles of DOP, there are also reports of their emotional effects.[16,17] Numerous studies have been conducted from an antidepressant perspective. For example, DOP-KO mice exhibited an increase in anxiety-like behavior, in contrast to MOP-KO mice.[18] Indeed, the DOP agonists SNC80 and BW373U86 have antidepressant-like activity in the forced swim test (FST) in rodents.[19] Thus, the activation of DOP may be a novel approach to treat mood disorders that avoids problems that have plagued other drug classes.

Pharmacological studies have indicated the possible existence of interactions between MOP and DOP in specific neural pathways because analgesic and respiratory effects of DOP agonists are abolished in MOP-KO mice.[20] The relationship between MOP and DOP in stress responses and antidepressant-like effects is still unclear. Nagase et al.[21] recently synthesized a novel DOP agonist, KNT-127. KNT-127 has higher affinity for DOP ($K_i = 0.16$ nM) than TAN-67 and lower affinity for MOP ($K_i = 21.3$ nM) and KOP ($K_i = 153$ nM). KNT-127 also has greater selectivity for DOP than SN-28.[21] KNT-127 produces no convulsions or catalepsy in mice, in contrast to the conventional DOP agonist SNC80.[22] KNT-127 reduces the excitability of neurons in the prelimbic prefrontal cortex, a region that is associated with the control of emotional behavior, thereby reducing anxiety-like behavior.[23,24] MOP stimulation is associated with various undesirable side effects, including constipation, respiratory depression, tolerance, and physical dependence, but KNT-127 has low affinity for MOP, so these side effects should be minimized.

Recent studies indicate that MOP/DOP heteromers have a pharmacological profile that is distinct from either MOP or DOP alone.[25] The specific transport and signaling properties of MOP/DOP heteromers have been reported, suggesting the therapeutic potential of receptor heteromers to reduce opioid tolerance.[26] The lack of neuronal co-localization in the pre-Bötzinger complex suggests that MOP/DOP-specific targeting may provide analgesia without the side effect of respiratory depression.[27] There are also reports that the relationship between these two receptors is such that the analgesic effect of DOP agonism is abolished in the absence of MOP, confirming an interaction between MOP and DOP, at least in terms of analgesic effects. However, there are no reports on stress. Thus, targeting MOP and DOP appears to be an attractive strategy to reduce tolerance and withdrawal symptoms that are associated with opioid drugs.

The A118G polymorphism of the human MOP receptor (*OPRM1*) gene is being studied for its involvement in depression and stress. For example, the G allele of the rs1799971 single-nucleotide polymorphism results in an amino acid change (N40D) that down-regulates MOP expression and is associated with greater susceptibility to social rejection, a higher risk of developing major depressive episodes after adverse life events, and completed suicide.[28] This provides additional evidence of the importance of MOP in depression.

These findings led us to hypothesize that KNT-127 might be a potential novel treatment for mood disorders via actions at DOP that interact with MOP activity in a specific manner that limits undesirable effects of MOP agonism. In the present study, the ability of KNT-127 to affect depressive-like symptoms was examined, including whether DOP activation effectively suppresses depressive symptoms in MOP-KO mice.

2. Materials and methods

2.1. Animals

The original line of MOP-KO mice, described previously,[29] was used to produce a congenic mutant line by repeated backcrosses onto a C57BL/6J genetic background for 20 generations. After weaning at 4 weeks of age, the mice were housed in same-sex groups of two to four mice in translucent polyethylene cages (13 cm × 30 cm × 15 cm) with free access to food and water in a climate-controlled (22 °C ± 2 °C) vivarium on a reverse 12 h/12 h light/dark cycle with lights off at 8:00 p.m. The behavioral experiments were conducted during the light phase. All experiments were performed in accordance with the Regulations for Animal Experiments and Related Activities at Tohoku University (2007) and in conformity with all Japanese federal rules and guidelines under protocols that were approved by the Institutional Animal Care and Use Committee. All efforts were made to minimize the number of animals used and their pain and distress, within the limits of scientific necessity.

In the tail suspension test (TST), the present study used WT ($MOP^{+/+}$), heterozygous MOP-KO ($MOP^{+/-}$), and homozygous MOP-KO ($MOP^{-/-}$) 9-week-old male and female littermate mice. This test was conducted at the Tokyo Metropolitan Institute of Medical Science.

In the FST, 7- to 8-week-old male C57BL/6 N mice were purchased from CLEA Japan, Inc., and housed in standard polycarbonate mouse cages for at least 3 days before the experimental procedures. These animals were used as a control for comparisons to 8-week-old male MOP-KO mice. This test was conducted at Tohoku University.

2.2. Drugs

KNT-127 is a highly selective DOP agonist that was developed by Dr. Nagase's group at the University of Tsukuba.[21] KNT-127 was dissolved in 0.9% saline (Otsuka, Tokyo, Japan). For the behavioral tests, all mice were randomly assigned to the treatment groups, and 10 mg/kg KNT-127 or saline was administered intraperitoneally. Behavior was assessed by observers who were blind to genotype and treatment groups.

2.3. Tail suspension test

The TST was conducted as described previously,[10] with some modifications, to measure antidepressant-like effects of the DOP receptor agonist. Saline (i.p.) or KNT-127 (10 mg/kg, i.p.) was administered 30 min before the test (Fig. 1). The mice were suspended by their tail, which was taped to a metal hook in test chambers (20 cm × 20 cm × 25 cm) that were constructed of white plastic. Each hook was connected to a computerized strain sensor that was adjusted to detect mouse movements (Tail suspension System, Neuroscience, Inc., Osaka, Japan). Total immobility time was determined from the automatically detected immobility time by excluding the first minute of testing over the 9-min test. All behavioral tests were conducted between 10:00 a.m. and 4:00 p.m. The mice were returned to their home cages immediately after each test.

2.4. Forced swim test

The FST was conducted as described previously[10,30] to measure antidepressant-like effects of the DOP agonist. The FST procedure was based on methods that were described previously.[30] The mice were placed in a cylindrical Plexiglas tank (25 cm height × 15 cm diameter) that contained 14 cm deep water for 6 min. Saline (i.p.) or KNT-127 (10 mg/kg, i.p.) was administrated 30 min before the test (Fig. 1). The water temperature was maintained at approximately 25 °C. Immobility time (i.e., the total time spent immobile) was determined by a blind observer from digital recordings. All behavioral tests were conducted between 1:00 p.m. and 5:00 p.m. After the test, the mice were immediately removed from the container, dried with a towel until completely dry, and then returned to their home cages.

2.5. Data analysis

The data are expressed as the mean ± SEM. The analyses of pharmacological effects were performed using analysis of variance (ANOVA), with drug treatment and genotype as factors, followed by the Bonferroni-Dunn *post hoc* test to determine statistically significant differences between individual means. In the TST, three-way ANOVA was used to identify genotype × sex × treatment effects, followed by the Bonferroni *post hoc* test. The statistical analyses were performed using Prism 9 software (GraphPad, San Diego, CA, USA). Corrected values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Tail suspension test

Immobility time in the TST was analyzed in male and female $MOP^{+/+}$, $MOP^{+/-}$, and $MOP^{-/-}$ mice. The three-way ANOVA (genotype × sex × treatment) showed no overall sex differences in saline- and KNT-12T-treated male mice *vs*. saline- and KNT-127-treated female mice ($F_{5,161} = 1.54$, $p = 0.18$; Fig. 2A, B). There were significant effects of treatment ($F_{1,161} = 17.82$, $p < 0.001$) and genotype ($F_{5,161} = 6.51$, $p < 0.001$). Fig. 2A and B shows that the effects of treatment were sex-dependent ($F_{5,161} = 10.36$, $p < 0.001$), so each sex was analyzed separately in the subsequent analyses (see Fig. 3).

In male mice, as shown in Fig. 2A, KNT-127 clearly reduced immobility compared with saline-treated mice but appeared to do so independently of genotype. The two-way ANOVA (genotype × treatment) revealed a significant main effect of treatment ($F_{1,72} = 9.84$, $p = 0.002$) but not genotype ($F_{2,72} = 2.05$, $p = 0.14$) and no treatment × genotype interaction ($F_{2,72} = 0.08$, $p = 0.92$). The KNT-127-treated male groups exhibited a significant decrease in immobility time compared with the saline-treated male groups ($p = 0.01$ for $MOP^{+/+}$, $p = 0.03$ for $MOP^{+/-}$, $p = 0.03$ for $MOP^{-/-}$; Fig. 2A). The effects of KNT-127 were unaffected by MOP deficiency, with no effect of genotype.

In females, KNT-127 also reduced immobility but clearly had a greater effect in MOP-KO mice, particularly $MOP^{-/-}$ mice. The two-way ANOVA (genotype × treatment) revealed significant main effects of genotype ($F_{2,62} = 13.55$, $p < 0.001$) and treatment ($F_{1,62} = 27.61$, $p < 0.001$) and a significant genotype × treatment interaction ($F_{2,60} = 3.35$, $p = 0.04$). The female KNT-127-treated groups exhibited a strong decrease in immobility time compared with the saline-treated groups ($p < 0.001$ for $MOP^{+/+}$, $p < 0.001$ for $MOP^{+/-}$, $p < 0.001$ for $MOP^{-/-}$; Fig. 2B). Immobility time in saline-treated female $MOP^{+/-}$ and $MOP^{-/-}$ mice was modestly reduced compared with saline-treated $MOP^{+/+}$ mice, although this reduction was only significant in female $MOP^{+/-}$ mice ($p = 0.04$). Importantly, female mice that were treated with KNT-127 exhibited significant differences between genotypes ($p = 0.02$, $MOP^{+/+}$ *vs*.

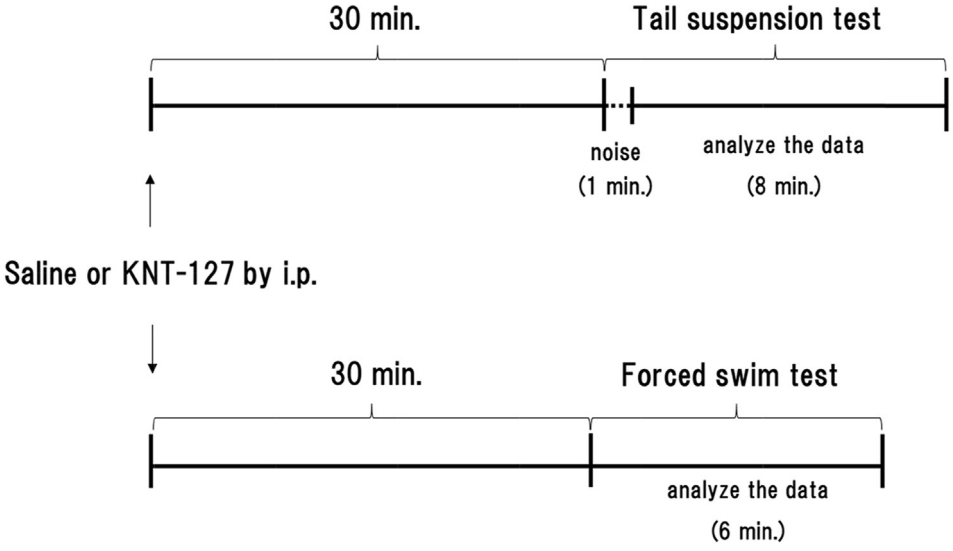

Fig. 1. Timeline of the experimental procedure. Saline (i.p) or KNT-127 (i.p) was administered 30 min before the TST or FST.

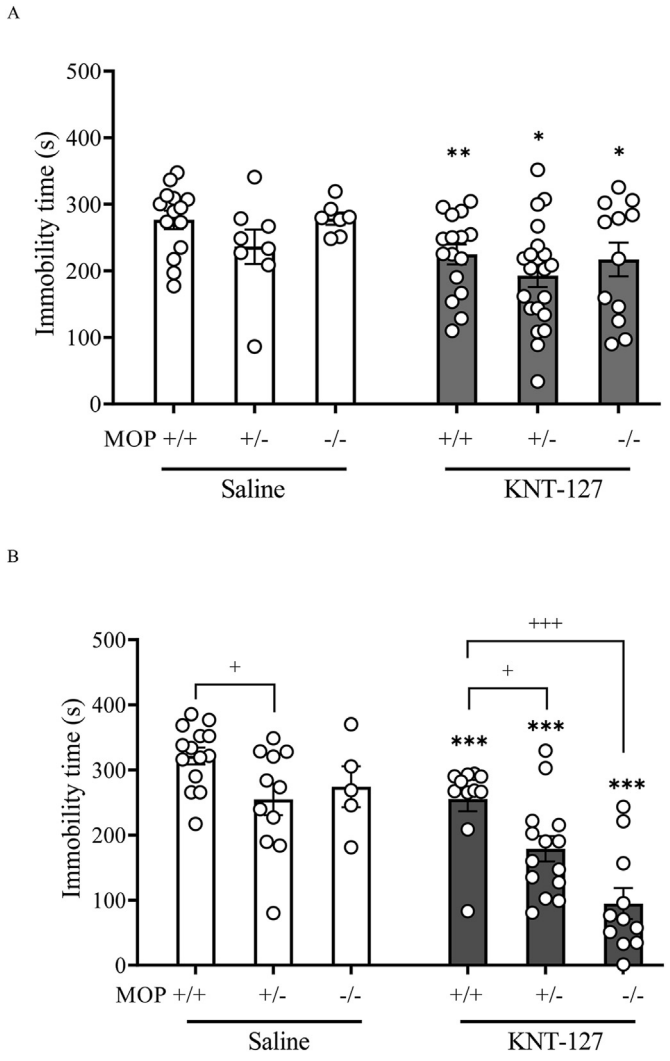

Fig. 2. (A) Immobility in male MOP$^{+/+}$, MOP$^{+/-}$, and MOP$^{-/-}$ mice in the TST. *$p < 0.05$, **$p < 0.01$, KNT-127 *vs.* saline group (two-way ANOVA followed by *post hoc* analysis). The data are expressed as the mean ± SEM. (B) Immobility in female MOP$^{+/+}$, MOP$^{+/-}$, and MOP$^{-/-}$ mice in the 8-min TST. ***$p < 0.001$, KNT-127 *vs.* saline group; $^{+}p < 0.05$, $^{+++}p < 0.001$, comparison between genotypes in saline or KNT-127 groups (two-way ANOVA followed by *post hoc* analysis). The data are expressed as the mean ± SEM.

MOP$^{+/-}$; $p < 0.001$, MOP$^{+/+}$ *vs.* MOP$^{-/-}$), showing that MOP $^{+/-}$ and MOP$^{-/-}$ mice were more sensitive to the immobility-reducing effects of KNT-127.

3.2. Forced swim test

Mice that were treated with 10 mg/kg KNT-127 exhibited a significant reduction of immobility time in both genotypes, but MOP-KO mice exhibited a reduction of immobility independent of these effects. The two-way ANOVA (genotype × treatment) revealed significant main effects of treatment ($F_{1,44} = 9.19$, $p < 0.01$) and genotype ($F_{1,44} = 15.56$, $p < 0.001$) but no treatment × genotype interaction ($F_{1,44} = 0.09$, $p = 0.76$). The *post hoc* tests showed that KNT-127-treated mice exhibited significantly lower immobility times compared with saline-treated mice ($p = 0.031$ for WT mice, $p = 0.041$ for MOP$^{-/-}$ mice). In the saline-treated group, immobility time was significantly reduced in MOP$^{-/-}$ mice compared with WT mice (*post hoc* test, $p = 0.013$). In the KNT-127-treated group, MOP$^{-/-}$ mice also exhibited a significant decrease in immobility time compared with WT mice (*post hoc* test, $p = 0.004$).

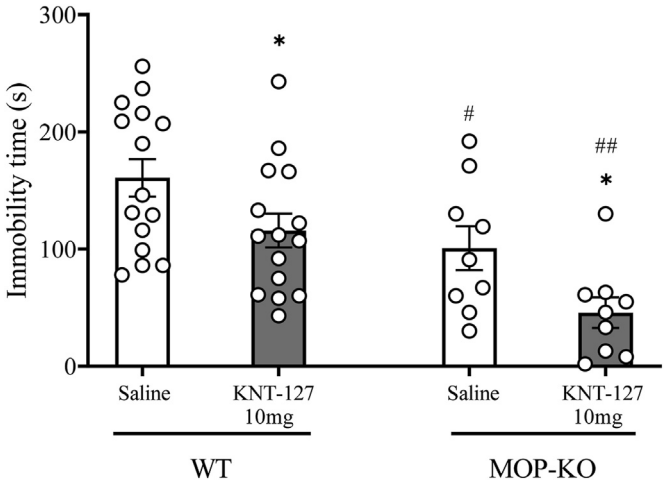

Fig. 3. Immobility time in WT and MOP-KO mice in the FST. *$p < 0.05$, KNT-127 *vs.* saline group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, WT *vs.* MOP-KO (two-way ANOVA followed by *post hoc* test). The data are expressed as the mean ± SEM.

4. Discussion

The primary symptoms of depression involve depressed mood, accompanied by cognitive changes that are characterized by a generally negative outlook, affecting both the interpretation of current events and expectations for the future. The primary approach to measuring core symptoms of depression has been to use classic animal models of behavioral despair, such as the TST and FST.[31] Additionally, both of these models involve hopelessness to some degree. Thus, they are assumed to represent the lack of motivated behavior that is frequently observed in depressed patients. We used these approaches to study potential antidepressant-like effects of KNT-127 and found evidence of mood-improving effects in mice in both the TST and FST. Both the FST and TST are stressful tests for rodents because of the forced nature and inescapability of the tests. One interpretation of the effect of MOP genotype could be that it conveys slight resistance to this aspect of these tests, which has been demonstrated in previous studies.[10,11] The effects on anhedonia, another aspect of the behavioral phenotype of depression, also need to be examined. The sucrose preference test is often used to confirm antidepressant drug effects and thought to specifically reflect anhedonia. The administration of an opioid receptor agonist increases sucrose intake, whereas the administration of an opioid receptor antagonist decreases sucrose intake.[32–34] Similarly, MOP-KO reduces sucrose consumption,[35,36] although this effect was also seen for standard food chow, so unclear is the degree to which these effects reflect lower motivation overall *vs.* effects on caloric intake.

Previous studies reported that DOP activation has antidepressant-like effects.[37–40] In WT mice, the DOP agonist KNT-127 produced modest antidepressant-like effects in the FST and TST. Additionally, MOP deletion alone exerted only modest antidepressant-like effects, reflected by slight reductions of immobility in the FST and TST. The decrease in immobility time in MOP-KO mice compared with WT mice in both tests is consistent with previous studies.[10,18,41] The decrease in immobility time in MOP-KO mice compared with WT mice in both tests is consistent with previous reports.[10,18,41] KNT-127 exerts antidepressant-like and antinociceptive effects in mice.[30] The DOP agonists

SNC80[16,30] and TAN-67[42] also produce potent antidepressant-like and antinociceptive effects in animals. The present study investigated whether the effects of a DOP agonist depend on MOP receptors. Antidepressant-like effects were observed even in the absence of MOP. Although antidepressant-like effects of KNT-127 were unaffected by genotype in male MOP-KO mice, these effects were potentiated in female MOP-KO mice. The present study showed that the DOP-mediated effects of KNT-127 occur independently of MOP and that a combination of DOP agonism and MOP deletion has additive antidepressant-like effects. The present study found that the effects of KNT-127 were inhibited by a DOP antagonist. The mechanisms of this inhibition should be elucidated in future research, in addition to evaluating potential combinations of different types of MOP antagonists and DOP agonists. Notably, however, the antidepressant-like effects of KNT-127 were eliminated in DOP-KO mice in the FST.[40]

Several studies reported that KNT-127 had no effect on locomotor activity in C57BL/6J mice[40] or any apparent sedative effects in the open field test or elevated plus maze.[43] Baseline locomotor activity was unaffected by MOP-KO.[44] Therefore, the effects of KNT-127 at the doses that were used in the present study and MOP-KO on spontaneous locomotor activity did not influence immobility in the TST or FST, thus allowing us to evaluate effects on specifically depressive-like behavior. The present results showed that immobility time in the FST significantly decreased in mice that were treated with 10 mg/kg KNT-127 compared the saline-treated groups of WT mice. Similar results for this dose of KNT-127 were reported in ICR mice.[30] In the present study, similar results were found in the TST, in which a significant decrease in immobility time was observed in KNT-127-treated mice compared with saline treatment in $MOP^{+/+}$, $MOP^{+/-}$, and $MOP^{-/-}$ mice. These results provide evidence of antidepressant-like effects of DOP agonists, particularly KNT-127.

We hypothesized that MOP genotype would be an important moderating factor of the antidepressant-like effects of KNT-127. The effect of KNT-127 was observed in MOP-deficient mice, which was potentiated in female MOP-deficient mice. Sex differences in MOP-deficient mice have been observed previously.[45] Considering these findings and other previous studies, the efficacy of KNT-127 in MOP-KO mice in the FST and TST suggests that both DOP and MOP contribute to emotional outcomes in these tests of depressive-like behavior. However, the effects of KNT-127 clearly did not depend on the presence of MOP. These results indicate that unlike in the case of MOP and DOP in analgesia, the effects of DOP stimulation on emotional outcomes are potentiated by the elimination of MOP. Hence, there are different types of interactions between these receptors for different outcomes. Several studies reported that MOP and DOP are co-expressed in the same cells[12] and that MOP/DOP heteromers modulate signaling properties of individual receptors.[46] Martinez-Navarro et al. (2020)[47] reported that MOP activity in forebrain γ-aminobutyric acid-ergic neurons has antinociceptive effects in males, but females lack this protective mechanism and may be more vulnerable to this aspect of neuropathic pain sensitization. However, the effects of DOP on nociceptive sensitivity in nerve-damaged mice were opposite to the effects of MOP. Interactions between MOP and DOP have been identified with regard to analgesic effects, but no reports exist for affective responses such as those examined herein. The precise nature of this interaction remains uncertain, but it appears that DOP stimulation has antidepressant-like effects that are potentiated by the elimination of MOP. The present findings were insufficient to explain why MOP-KO mice that were treated with KNT-127 exhibited greater effects and why these effects were sex-dependent. It must also be considered that the nature of the interaction was not simply the result of the elimination of MOP and also resulted from some developmental changes that resulted from the life-long elimination of MOP.

In the present study, KNT-127 was effective in MOP-KO mice, suggesting its efficacy for the treatment of stress-induced depression, although this will require further examinations of longer-term stress-inducing depressive outcomes. MOP agonists have various adverse effects,[48–50] and a pathway that affects opioid function that does not involve MOP is important for the development of antidepressants that act through these systems. Compared with MOP agonists,[51–53] molecules that act on DOP generally have fewer side effects.[14] There have been many studies of antidepressant-like effects of SNC80 in mice that lack MOP,[40,52] but possible sex differences were not examined. We hypothesized that the antidepressant-like effects of KNT-127 are mediated not only by DOP but also by MOP. Not entirely clear is why KNT-127 has a superior profile of effects compared with other DOP agonists. The calculated K_i values for SNC80 were ~500-fold greater for DOP *vs*. MOP and 250-fold greater for DOP *vs*. KOP, indicating that this compound has a significant degree of DOP selectivity.[53] This selectivity is actually slightly greater than KNT-127,[21] although KNT-127 has greater selectivity for DOP than TAN-67 or SN-28,[21] which might account for its superior profile compared with those drugs. Collectively, there are many reports that DOP agonists have antidepressant-like effects. TAN-67[54] exerted antidepressant-like effects in the FST, similar to the tricyclic antidepressant imipramine. However, SNC80 and some of its derivatives have been reported to induce spasticity in mice, rats, and monkeys. The DOP-selective peptide agonist DPDPE also exerted antidepressant-like effects in animal models but at doses that caused seizures. Unclear are from where these effects derive and why KNT-127 is different. In contrast, KNT-127 shows systemic antinociceptive, antidepressant, and anxiolytic activity without causing seizures.[30,55] Thus, KNT-127 has fewer side effects than other DOP agonists. Unclear are what mediates these differences between DOP agonists, but it appears that opioid system interventions with DOP as the main target may be an alternative strategy for the treatment of depression. Several questions still remain unanswered and require further investigation, including whether such effects of DOP agonists are more effective in specific models of depression or specific genetic backgrounds (e.g., MOP deficiency) and whether there are notable sex differences.

Sex differences in emotional responses that are relevant to the development of depression and antidepressant-like effects clearly exist, and such differences are clearly seen in MOP-KO mice.[10,45] Extensive research has reported sex differences in rates of depression in humans, but few behavioral studies of antidepressant-like effects have been conducted in female mice. The TST and FST are widely used as predictors of antidepressant-like effects using male mice. There are no previous studies that used male and female mice in the TST with KNT-127; thus, no comparisons can be made between the present findings and previous work. Nonetheless, the present study showed a decrease in immobility time with KNT-127 treatment in females and males, an effect that depended on genotype. This might suggest that MOP status might be a sex-specific biomarker for antidepressant-like effects of KNT-127. In males, KNT-127-treated mice exhibited a significant reduction of immobility, with no significant difference that was attributable to MOP deficiency. Several reports of sex differences in stress responses may be relevant to these findings.[2,45,56,57] MOP and DOP are G_i-type G-protein-coupled receptors[58] that can be expressed in the same neurons or in different parts of a circuit. Previous research indicated sex differences in stress sensitivity between male and female MOP-KO mice. The application of new approaches to label receptors[59] would be useful for elucidating mechanisms that are relevant to the present

findings, perhaps how sex differences in MOP/DOP interactions might underlie the sex differences that were observed herein.

5. Conclusions

In conclusion, we found that KNT-127 produced antidepressant-like effects, and these effects depended on sex and MOP genotype. MOP and DOP independently play important roles in modulating depression-like responses in standard tests of behavioral despair. Thus, at least in the TST and FST, DOP activation and low MOP expression interact to reduce depressive-like behavior. This may involve alterations of the response to the inherently stressful nature of these tests, based on previous observations in MOP-KO mice. The nature of the interaction and whether it involves MOP/DOP interactions in the same or different cells remain to be determined in future studies.

Declaration of competing interest

The authors declare no conflict of interest.

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