As written in this article, a great number of genetic variations in the *OPRM1* gene have been analyzed for the opioid sensitivity, susceptibility to substance dependences and other disorders. Technologies for genetic analyses are developing remarkably in recent years and therefore genetic studies will be carried out more generally and inexpensively in the future. The pharmacogenetic information of the *OPRM1* gene including the associations with individual opioid sensitivity and susceptibility to substance dependences will be accumulated (see PharmGKB [205]), and these data will be absolutely essential for the establishment of personalized medicine for pain and drug abuse in the future.

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Executive summary

SNPs in the* OPRM1 *gene

- Over 700 polymorphisms have been identified from exon 1 to exon 4 of the *OPRM1* gene.
- Numerous studies have shown associations between these polymorphisms and opioid effects, substance dependence and susceptibility to other disorders, including epilepsy and schizophrenia.

Association of* OPRM1 *SNPs with opioid sensitivity

- The analgesic and side effects of opioid agonists may be lower in G-allele carriers of the A118G polymorphism (rs1799971) compared with AA patients.
- The effects of opioid antagonists for alcoholic treatment may be higher in G-allele carriers than in AA patients.

Association of* OPRM1 *SNPs with susceptibility to substance dependence & other disorders

- The G-allele of the A118G polymorphism may be a risk allele for alcoholism, opioid dependence, epilepsy and schizophrenia, but it may also be a protective allele for tobacco dependence, diabetes and obesity.
- The G-allele of the IVS1+A21573G polymorphism and C-allele of the IVS1-T17286C polymorphism may be risk alleles for alcohol and opioid dependence, respectively.
- By contrast, the G-allele of the TAA+A5359G polymorphism may be a protective allele for tobacco dependence.

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〔CINP2010 発表報告〕

Influence of GIRK Channel Inhibition on Alcohol Abstinence and Relapse Risk in Japanese Alcohol-Dependent Outpatients*

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GIRK channels are coupled to various G-protein-coupled receptors, including dopamine D_2 and opioid receptors, and play an important role in the inhibitory regulation of neuronal excitability (Kobayashi and Ikeda, 2006). Kobayashi et al (1999) reported that GIRK channels in the brain and heart are important targets for ethanol. Interestingly, cocaine self-administration is reportedly abolished in mice lacking the GIRK2 and GIRK3 channel subunits (Morgan et al, 2003). These findings suggest that GIRK channel inhibition may reduce the preference for drugs of abuse, including alcohol.

The present study examined the influences of GIRK inhibition on abstinence and relapse risk in Japanese alcohol-dependent outpatients. We hypothesized that patients who are treated with pharmacotherapeutics that inhibit GIRK exhibit improvements in abstinence and relapse risk compared with patients who are not treated with such medications. Additionally, we examined the influence of other medications, such as antidepressant, antipsychotic, anxiolytic, and anti-alcoholic, on abstinence and relapse risk.

METHODS

The participants of the present study were 68 alcohol-dependent outpatients, from whom we received written informed consent. The recruitment criteria were the following: at least 20 years old, history of alcohol abuse, diagnosis of alcohol-dependent based on the *Diagnostic and Statistical Manual of Mental Disorders*, 4th edition, outpatient at a Japanese mental hospital, and ability to understand Japanese. All participants belonged to the National Center of Neurology and Psychiatry Musashi Hospital. They twice answered a questionnaire that measured their alcohol abstinence and relapse risk. Participants who did not answer the follow-up questionnaire within the first 60 days after the first questionnaire were excluded. Data from the remaining 44 participants (32 males and 12 females; mean age, 50.27 years) were statistically analyzed. A correlation analysis was used for the examination of independence between GIRK inhibition treatment and the other treatments. A two-way mixed-design analysis of variance (ANOVA) was used to investigate whether GIRK inhibition increases abstinence and decreases relapse risk compared with the other treatments.

For medical treatment as the independent variable, information regarding the type of GIRK inhibition treatment, serotonin transporter blockade treatment (i.e., antidepressant treatment), dopamine D_2 receptor blockade treatment (i.e., antipsychotic treatment), anxiolytic treatment, and anti-alcoholic drug treatment were collected by the participants' psychiatrists. The medications with the ability of inhibiting GIRK were ifenprodil tartrate (Kobayashi et al, 2006a), paroxetine (Kobayashi et al, 2006b), and haloperidol (Kobayashi et al, 2000) in the present study. A total of 12 patients received GIRK inhibition treatment, and 32 patients received non-GIRK inhibition treatment. Additionally, paroxetine was categorized as both a GIRK inhibition treatment and antidepressant treatment. The type and dose of the medications regularly administered by the participants did not change until their follow-up rating.

Alcohol abstinence and relapse risk were the dependent variables. Alcohol abstinence was defined as "no consumption of any alcohol after the first rating" and measured by patients' self-reports or judgments by their psychiatrists. Relapse risk was measured using the Alcohol Relapse Risk Scale (ARRS; Ogai et al, 2009), which was a three-point Likert-type multidimensional scale, with 32 items and five subscales: (1) stimulus-induced vulnerability, (2) emotionality problems, (3) compulsivity for alcohol, (4) positive expectancy for alcohol, and (5) lack of negative expectancy for alcohol.

RESULTS AND DISCUSSION

No significant correlations were found between GIRK inhibition treatment and the other treatments, with the exception of the antidepressant treatment. A significant correlation was found between GIRK inhibition treatment and antidepressant treatment ($r=0.632$, $p<0.01$), possibly because paroxetine functions as both a GIRK inhibitor and serotonin transporter blocker. These results suggest that the GIRK inhibition treatment was independent of the other treatments, with the exception of the antidepressant treatment.

With regard to alcohol abstinence, a nearly significant effect of the GIRK inhibition treatment was observed ($F_{1,42}=2.96$, $p<0.10$), and a nearly significant difference was found in the first abstinence rating between groups ($t_{16}=-1.88$, $p<0.10$). Fig. 1 shows that the transition of the percentage of abstinence was different between the GIRK inhibition treatment and non-GIRK inhibition treatments. The GIRK inhibition treatment group tended to increase its percentage

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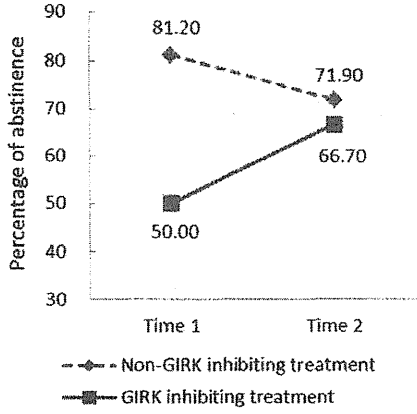

Fig. 1 Difference in the transition of the percentage of abstinence between GIRK inhibition treatment and non-GIRK inhibition treatment.

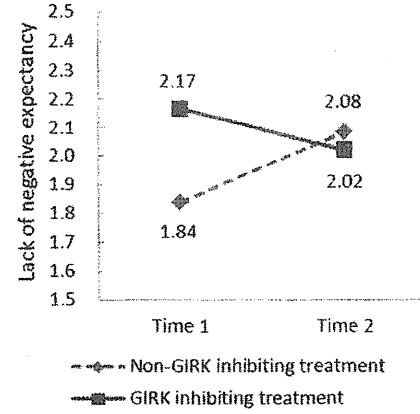

Fig. 2 Difference in the transition of the lack of negative expectancy between GIRK inhibition treatment and non-GIRK inhibition treatment.

of abstinence, whereas the non-GIRK inhibition treatment groups slightly decreased their percentages. Additionally, the antidepressant treatment, antipsychotic treatment, anxiolytic treatment, and anti-alcoholic drug treatment did not have any significant effects on alcohol abstinence. These results suggest that GIRK inhibition treatment promoted alcohol abstinence, and the other treatments did not have any effect on abstinence. These results are consistent with previous research, in which the antidepressant fluvoxamine, which does not inhibit GIRK but blocks the serotonin transporter, did not inhibit methamphetamine preference in mice, whereas the antidepressants fluoxetine and paroxetine, which exhibit both functions, inhibited preference (Takamatsu et al, 2006, 2011).

With regard to relapse risk, a significant interaction was found between GIRK inhibition treatment and the "lack of negative expectancy for alcohol drinking" subscale ($F_{1,40}=4.84, p<0.05$). Fig. 2 shows that the transition of the lack of negative expectancy was different between GIRK inhibition treatment and the non-GIRK inhibition treatments. The GIRK inhibition treatment group tended to decrease its lack of negative expectancy score, whereas the non-GIRK inhibition treatment groups appeared to increase their scores. Additionally, no significant difference was found in the first score of that subscale between groups ($t_{42}=1.53, n.s.$). These results suggest that the GIRK inhibition treatment group became more attentive to the negative influence of alcohol drinking, whereas the non-GIRK inhibition treatment group became gradually less attentive to that influence. No significant interactions were found with the other ARRS subscales.

The present study has some limitations. First, the medication schedule was not well controlled, and the patient data were analyzed retrospectively. Therefore, factors other than GIRK inhibition might have influenced the outcome as confounding variables. Second, the quality of each group, with the exception of the independent variable, could not be assured without using a random assignment procedure. Third, ifenprodil, paroxetine, and haloperidol were combined as a "GIRK inhibition treatment" category. Fourth, the sample size was relatively low. A sample of 12 GIRK inhibition treatment participants may have been too small to sufficiently support the ANOVA. More well controlled medical treatments and larger sample sizes may be necessary to confirm the present results.

In summary, the results of the present study indicated that GIRK inhibition treatment might improve alcohol abstinence and negative expectancy for alcohol, supporting the hypothesis that GIRK channel inhibition may reduce the preference for drugs of abuse, including alcohol.

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〔CINP2010 発表報告〕

Reduced Locomotor Sensitization Induced by Methamphetamine and Altered Gene Expression in ICER Overexpressing Mice*

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The inducible cyclic adenosine monophosphate (cAMP) early repressor (ICER) is the collective name for a group of proteins produced from the cAMP response element modulator (CREM)/ICER gene. Transcribed from the P2 internal promoter located in an intron of the CREM gene, ICER only contains two DNA binding domains (DBD I and DBD II) and lacks the activation and kinase-inducible domains (Molina et al, 1993). Consequently, ICER functions as an endogenous transcription repressor of several cAMP response element (CRE)-containing genes (Jaworski et al, 2003; Molina et al, 1993; Tinti et al, 1996). The P2 promoter of the ICER gene contains four CRE-like cAMP autoregulatory elements (CAREs). These CAREs are strongly inducible and recognized by a variety of CRE-binding proteins, including CREB. The phosphorylated CRE-binding protein (CREB) binds to CAREs in the P2 promoter and can induce transcription of the ICER gene. The increased ICER competes with CREB in binding with the CRE sequence, blocking transcription from CRE-containing promoters, including ICER's own promoter, and functioning as a potent endogenous CREB antagonist (Molina et al, 1993).

Alternative splicing of the ICER transcripts results in four ICER isoforms: ICER I, ICER Iγ, ICER II, and ICER IIγ. ICER I mRNA contains DBD I and DBD II, but DBD II is absent in the ICER I protein because a stop codon exists at the end of DBD I. The ICER II isoform contains only DBD II. ICER Iγ and ICER IIγ are characterized by a deficiency of exon γ from ICER I and ICER II, respectively (Mioduszewska et al, 2003).

ICER is expressed at low levels in the central nervous system, with the exception of neuroendocrine structures. However, a variety of physiological and non-physiological stimuli can dramatically upregulate ICER expression (for review, see Borlikova and Endo, 2009). Amphetamine injection increases ICER mRNA expression threefold in the striatum (Green et al, 2006), suggesting that ICER may participate in the mechanisms that underlie the effects of drugs of abuse.

Kojima et al (2008) generated two types of ICER mutant mice—ICER knockout mice and ICER-overexpressing mice—and showed that both ICER knockout mice and ICER-overexpressing mice displayed normal locomotor activity, sensory and motor functions, and emotional responses. However, long-term conditioned fear memory was attenuated in ICER-overexpressing mice and enhanced in ICER knockout mice, indicating the negative role of ICER in regulating long-term fear memory and epileptogenesis kindling. The present study investigated the role of ICER in methamphetamine (METH)-induced locomotor sensitization. We also screened gene expression profiles in METH-treated ICER I-overexpressing mice and their wildtype littermates using DNA microarrays purchased from Illumina.

ICER and METH-induced locomotor sensitization

Locomotor sensitization is characterized by the progressive enhancement of locomotor activity after repeated psychostimulant exposure (Pierce and Kalivas, 1997). The augmentation of this behavioral response can be maintained for several months after cessation of drug treatment (Robinson and Becker, 1986). This process closely resembles the course of relapse in METH-induced psychosis (Sato et al, 1983). In the present study, mice were first habituated to the apparatus for 180 min and then injected with METH (1 mg/kg, i.p.). Locomotor activity was then measured for 60 min after the injection. The procedure was repeated seven times, once every other day from Day 1 to Day 13. After a 7 day drug-free period, locomotor activity was measured again after METH injection (1 mg/kg, i.p.) on Day 20.

Methamphetamine-induced locomotor sensitization was significantly decreased in ICER I-overexpressing mice. Although METH-induced locomotor sensitization was not significantly altered in ICER knockout mice, they showed a minimal enhancement of METH-induced locomotor sensitization compared with wildtype mice. These data indicate the inhibitory role of ICER in METH-induced locomotor sensitization.

Altered gene expression in ICER I-overexpressing mice

To identify the downstream components of ICER and reveal a possible mechanism of the inhibitory role of ICER in METH-induced locomotor sensitization, we screened the gene expression profiles of ICER I-overexpressing mice and their wildtype littermates using DNA microarrays purchased from Illumina. Mice were decapitated, and the striatum

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(caudate putamen) was dissected. Total RNA was isolated using a Trizol reagent (Invitrogen Life Technology Japan, Tokyo, Japan). RNA amplification was conducted using the Illumina TotalPrep RNA amplification Kit (Applied Biosystems, Tokyo, Japan). The MouseRef-8 beadchip Kit (Illumina, Tokyo, Japan) was used for hybridization according to the manufacturer's instructions. Data were analyzed using the BeadStudio 3 Gene Expression Module (Illumina, Tokyo, Japan).

Of the 24,000 genes tested, 45 were downregulated and 37 were upregulated in ICER I-overexpressing mice compared with their wildtype littermates. Among the downregulated mRNAs, cocaine- and amphetamine-regulated transcript (CART) and prodynorphin (Pdyn) mRNA expression levels were reduced by approximately 50% in ICER I-overexpressing mice. The products of CART and Pdyn mRNAs are neurotransmitters expressed in brain regions associated with drug reward, including the nucleus accumbens and ventral tegmental area. CART knockout mice exhibited attenuated rewarding effects of drugs (Couceyro et al, 2005), and Pdyn knockout mice showed decreased cocaine-induced locomotor activity (Chefer and Shippenberg, 2006). ICER I-overexpressing mice, with decreased CART and Pdyn expression levels, consistently displayed attenuated METH-induced locomotor sensitization in the present study. Our results reveal the modulatory effects of the ICER/CART (Pdyn) pathway on METH-induced locomotor sensitization and provide an incentive for exploring the therapeutic potential of stimulating the ICER/CART (Pdyn) pathway in the treatment of drug abuse.

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ARTICLE

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Active behaviours produced by antidepressants and opioids in the mouse tail suspension test

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Abstract

Most classical preclinical tests to predict antidepressant activity were initially developed to detect compounds that influenced noradrenergic and/or serotonergic activity, in accordance with the monoaminergic hypothesis of depression. However, central opioid systems are also known to influence the pathophysiology of depression. While the tail suspension test (TST) is very sensitive to several types of antidepressant, the traditional form of scoring the TST does not distinguish between different modes of action. The present study was designed to compare the behavioural effects of classical noradrenergic and/or serotonergic antidepressants in the TST with those of opioids. We developed a sampling technique to differentiate between behaviours in the TST, namely, curling, swinging and immobility. Antidepressants that inhibit noradrenaline and/or serotonin re-uptake (imipramine, venlafaxine, duloxetine, desipramine and citalopram) decreased the immobility of mice, increasing their swinging but with no effect on their curling behaviour. No differences were observed between antidepressants that act on noradrenergic or serotoninergic transmission. While opioid compounds also decreased the immobility of the mice [morphine, codeine, levorphanol, (−)-methadone, (±)-tramadol and (+)-tramadol], they selectively increased curling behaviour. Blocking opioid receptors with naloxone prevented the antidepressant-like effect of codeine, and μ-opioid receptor knockout decreased normal curling behaviour and blocked (±)-tramadol-induced curling, further demonstrating the reliability and validity of this approach. These results show that at least two behaviourally distinct processes occur in the TST, highlighting the antidepressant-like effects of opioids evident in this test. Furthermore, our data suggest that swinging and curling behaviours are mediated by enhanced monoamine and opioid neurotransmission, respectively.

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Key words: Antidepressants, behavioural despair, μ-opioid knockout, opioids, tail suspension test.

Introduction

There is substantial evidence implicating the opioid system in depression (Hegadoren *et al.* 2009), suggesting that compounds that enhance opioid neurotransmission may exert genuine antidepressant effects (Berrocoso & Mico, 2009*a*; Jutkiewicz, 2006; Tejedor-Real *et al.* 1998). Since the description of the 'opium cure' (Kraepelin, 1901), clinical reports have described the effectiveness of μ-opioid receptor (MOR) agonists in patients suffering depression, such as oxycodone, oxymorphone, tramadol and buprenorphine, especially in cases of refractory depression (Bodkin *et al.* 1995; Fanelli & Montgomery, 1998; Shapira *et al.* 2001; Spencer, 2000; Stoll & Rueter, 1999). We have studied the antidepressant-like effects of opioids and,

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in accordance with other studies (reviewed in (Berrocoso *et al.* 2009), we demonstrate the antidepressant-like activity of several opioids in animal paradigms of depression (Rojas-Corrales *et al.* 1998, 2004; Tejedor-Real *et al.* 1998). Indeed, we previously described a functional interaction between opioids and selective serotonin reuptake inhibitors (SSRIs) or noradrenaline (NA) reuptake inhibitors in the tail suspension test (TST: Berrocoso & Mico, 2009*a*), whereby both opioids and the more classic antidepressants decrease the time that the mice spend immobile (consistent with an antidepressant-like effect).

The mouse TST is a predictive behavioural test of antidepressant activity (Chermat *et al.* 1986; Steru *et al.* 1985, 1987) that is being increasingly used with the advent of transgenic mice. When mice are suspended by the tail they are subjected to short-term inescapable stress and they adopt an immobile posture. However, if antidepressant treatments are administered prior to the test, the mice will actively pursue escape-directed behaviours over longer periods of time. The increase in such activity (i.e. the decrease in immobility) in the TST is strongly correlated with antidepressant effects in humans (Cryan *et al.* 2005*a*). This clinical predictive value, together with the high degree of reliability in different laboratories, has led to the inclusion of the TST in almost all batteries used to screen new antidepressant drugs (Bravo *et al.* 2009), even though the TST in its traditional form cannot reliably detect specific modes of drug action. In our laboratory, previous studies with the TST suggested that while opioids decrease the time mice spend immobile they induce a pattern of activity that differs from that seen with classical monoaminergic antidepressants. To further investigate this effect, we have systematically analysed the behaviour of mice in the TST to compare the active behaviours induced by classical antidepressants and opioids.

Materials and method

Animals

Experiments were performed using male albino CD1 mice, male and female wild-type mice or heterozygous and homozygous MOR knockout (MOR-KO) littermates obtained by crossing heterozygous/heterozygous MOR-KO mice on a C57BL/6J genetic background (Sora *et al.* 2001). Animals were maintained under standard conditions: 12-h light/dark cycle (lights on 08:00 hours), *ad libitum* access to food and water and a constant temperature (21 ± 1 °C). Animals were housed in groups of 10 in standard polypropylene cages (1000 cm^2) and male and female mice shared the same room. All animal handling and procedures were performed in accordance with the European Communities directive 86/609-EEC and Spanish Law (RD 1201/2005) regulating animal research. The experimental protocols were approved by the Committee for Animal Experimentation of the University of Cádiz. All mice were experimentally naive, they weighed 25–30 g at the time of testing and they were only used once.

Drugs

The following drugs were used in this study: imipramine, desipramine, codeine and naloxone (Sigma-Aldrich-Quimica, UK); venlafaxine (Wyeth, USA); duloxetine (Eli Lilly, USA); citalopram, (±)-tramadol and (+)-tramadol (Grünenthal, Germany and Spain); morphine (Agencia Española de Medicamentos y Productos Sanitarios, Spain); (−)-methadone and levorphanol (RBI, USA). The selectivity of these compounds for opioid receptors (μ, δ and κ) and monoamine transporters (5-HT and NA) is summarized in Table 1.

All the drug solutions were prepared immediately before each trial and they were injected i.p., with the exception of naloxone, which was administered subcutaneously. All drugs were dissolved in physiological saline (NaCl 0.9%), with the exception of duloxetine, which was dissolved in distilled water, and the control animals received saline alone (NaCl 0.9%). All the solutions were injected in a volume of 10 ml/kg body weight 30 min prior to testing and the treatments were administered under blind conditions.

Tail suspension test

We used a modified form of the TST that was previously validated for NMRI mice (Steru *et al.* 1985). Accordingly, 30 min after injection the mice were individually suspended by the tail from an aluminium hook raised 20 cm above the floor using adhesive tape placed 2 cm from the tip of tail. The mice were positioned such that the base of their tail was aligned with the horizontal plane. Typically, mice demonstrated escape-oriented behaviour interspersed with successively longer bouts of immobility. Test sessions lasted for 6 min and they were videotaped and subsequently scored by a trained observer.

Behavioural scoring

The procedure used to analyse the test sessions was similar to that described previously for the forced

Table 1. Summary of the inhibitory effects of antidepressants and opioids on monoamine uptake and opioid receptor binding

Drugs	μ	δ	κ	NA	5-HT	Reference
Antidepressants						
Imipramine	3700	12 700	1800	6.6	21	Raffa *et al.* (1992)
Venlafaxine	–	–	–	1260	74	Beique *et al.* (1998)
Duloxetine	–	–	–	3	1.8	Beique *et al.* (1998)
Desipramine	–	–	–	0.31	129	Owens *et al.* (1997)
Citalopram	–	–	–	3042	0.75	Owens *et al.* (1997)
Opioids						
With no reuptake-inhibiting activity						
Morphine	0.34	92	570	IA	IA	Raffa *et al.* (1992)
Codeine	160	5130	5970	IA	IA	Raffa *et al.* (1992)
With reuptake-inhibiting activity						
Levorphanol	0.42	3.61	4.2	1220	86.3	Codd *et al.* (1995)
(−)-Methadone	0.945	371	1860	702	14.1	Codd *et al.* (1995)
(±)-Tramadol	2100	57 600	42 700	709	990	Raffa *et al.* (1992)
(+)-Tramadol	1300	62 400	54 000	2510	530	Raffa *et al.* (1993)

NA, Noradrenaline; IA, inactive at 10 μM.
The assays were performed on rat brain samples and the data represent K_i values ± S.E.M. (nM).

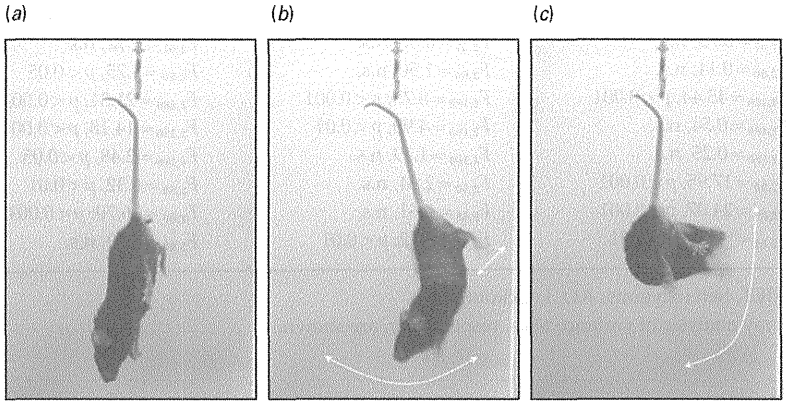

Fig. 1. Photographs illustrating the behaviours scored in the tail suspension test. (*a*) Immobility – the mouse hangs without engaging in any activity; (*b*) swinging – keeping its body straight, the mouse continuously moves its paws in a vertical position and/or moves its body from side to side; (*c*) curling – the mouse engages in active twisting movements.

swimming test (FST; Detke *et al.* 1995). A time-sampling technique was employed, whereby the predominant behaviour in each 5-s period of the 360 s test was recorded. The behaviours rated were: (1) immobility – a mouse was judged to be immobile when it hung by its tail without engaging in any active behaviour; (2) swinging – a mouse was judged to be swinging when it continuously moved its paws in the vertical position while keeping its body straight and/or it moved its body from side to side; (3) curling – a mouse was judged to be curling when it engaged in active twisting movements of the entire body (Fig. 1). The behavioural scoring was performed by a single experienced observer who was blind to the treatments. Several test sessions ($n = 30$ subjects) were then scored a second time by the observer to determine the test–retest reliability (i.e. two consecutive ratings by the same observer were compared). These sessions were then scored by a second blind observer to determine the inter-rater reliability.

Statistical analysis

The data were expressed as the mean + S.E.M. of the parameter measured and they were analysed by one-way analysis of variance (ANOVA) followed by the

Table 2. Effects of antidepressants and opioids on immobility and active behaviours in the tail suspension test

Drugs	Immobility	Swinging	Curling
Antidepressants			
(*a*) Imipramine	$F_{4,44}=3.34$, $p<0.05$	$F_{4,44}=3.55$, $p<0.05$	$F_{4,44}=0.69$, n.s.
(*b*) Venlafaxine	$F_{4,42}=7.82$, $p<0.001$	$F_{4,42}=3.30$, $p<0.05$	$F_{4,42}=0.13$, n.s.
(*c*) Duloxetine	$F_{6,63}=3.77$, $p<0.01$	$F_{6,63}=2.33$, $p<0.05$	$F_{6,63}=0.71$, n.s.
(*d*) Desipramine	$F_{3,32}=6.43$, $p<0.01$	$F_{3,32}=4.13$, $p<0.05$	$F_{3,32}=2.79$, n.s.
(*e*) Citalopram	$F_{5,51}=4.25$, $p<0.01$	$F_{5,51}=2.70$, $p<0.05$	$F_{5,51}=1.64$, n.s.
Opioids			
(*f*) Morphine	$F_{3,36}=5.36$, $p<0.01$	$F_{3,36}=12.02$, $p<0.001$	$F_{3,36}=26.78$, $p<0.001$
(*g*) Codeine	$F_{3,35}=3.13$, $p<0.05$	$F_{3,35}=3.70$, $p<0.05$	$F_{3,35}=9.35$, $p<0.001$
(*h*) Levorphanol	$F_{5,51}=3.14$, $p<0.05$	$F_{5,51}=7.15$, $p<0.001$	$F_{5,51}=13.52$, $p<0.001$
(*i*) (−)-Methadone	$F_{3,36}=3.44$, $p<0.05$	$F_{3,36}=3.61$, $p<0.05$	$F_{3,36}=8.61$, $p<0.001$
(*j*) (±)-Tramadol	$F_{3,36}=5.01$, $p<0.01$	$F_{3,36}=0.72$, n.s.	$F_{3,36}=5.85$, $p<0.01$
(*k*) (+)-Tramadol	$F_{3,33}=6.60$, $p<0.01$	$F_{3,33}=4.01$, $p<0.05$	$F_{3,33}=12.38$, $p<0.001$
Pharmacological opioid receptor blockade			
(*l*) Codeine	$F_{1,54}=7.93$, $p<0.01$	$F_{1,54}=2.19$, n.s.	$F_{1,54}=3.20$, n.s.
(*m*) Naloxone	$F_{2,54}=0.70$, n.s.	$F_{2,54}=12.23$, $p<0.001$	$F_{2,54}=16.26$, $p<0.001$
(*n*) Codeine × naloxone	$F_{2,54}=3.25$, $p<0.05$	$F_{2,54}=4.09$, $p<0.05$	$F_{2,54}=6.11$, $p<0.01$
MOR knockout			
(*o*) Gender	$F_{1,57}=0.32$, n.s.	$F_{1,57}=1.23$, n.s.	$F_{1,57}=0.02$, n.s.
(*p*) Genotype	$F_{2,57}=0.28$, n.s.	$F_{2,57}=1.23$, n.s.	$F_{2,57}=3.50$, $p<0.05$
(*q*) Gender × genotype	$F_{2,57}=0.73$, n.s.	$F_{2,57}=0.86$, n.s.	$F_{2,57}=0.12$, n.s.
(*r*) Genotype	$F_{2,60}=0.14$, n.s.	$F_{2,60}=1.50$, n.s.	$F_{2,60}=3.75$, $p<0.05$
(*s*) (±)-Tramadol	$F_{2,180}=45.44$, $p<0.001$	$F_{2,180}=8.78$, $p<0.001$	$F_{2,180}=21.31$, $p<0.001$
(*t*) Genotype	$F_{2,180}=0.54$, n.s.	$F_{2,180}=4.93$, $p<0.01$	$F_{2,180}=14.18$, $p<0.001$
(*u*) (±)-tramadol × genotype	$F_{4,180}=0.25$, n.s.	$F_{4,180}=1.19$, n.s.	$F_{4,180}=2.48$, $p<0.05$
(*v*) (±)-tramadol-WT	$F_{2,50}=17.95$, $p<0.001$	$F_{2,50}=2.84$, n.s.	$F_{2,50}=6.32$, $p<0.01$
(*w*) (±)-tramadol-HET	$F_{2,68}=24.07$, $p<0.001$	$F_{2,68}=0.61$, n.s.	$F_{2,68}=16.70$, $p<0.001$
(*x*) (±)-tramadol-KO	$F_{2,62}=9.77$, $p<0.001$	$F_{2,62}=6.12$, $p<0.01$	$F_{2,62}=1.59$, n.s.

MOR, μ-opioid receptor; WT, wild-type; HET, heterozygous; KO, knockout.
p values and F values from one- and two-way analysis of variance tests, respectively, are shown.

Dunnett's (for dose–response studies) or Tukey's test. For the mechanistic studies, the data were analysed using a two-way ANOVA followed by the Bonferrroni *post-hoc* test. The factors evaluated (between subjects) were codeine/(±)-tramadol treatment and naloxone treatment. A Pearson's correlation test was used to determine test–retest and inter-rater reliability and $p<0.05$ was considered statistically significant.

Results

In the TST employed here, the reliability of the scoring for each of the three behaviours contemplated was very high. Moreover, the test–retest reliabilities were: $r=0.89$ for immobility; $r=0.93$ for swinging; $r=0.88$ for curling. The concordance between raters was: $r=0.92$ for immobility; $r=0.86$ for swinging; $r=0.81$ for curling ($p<0.0001$).

Antidepressants

All the antidepressants administered here induced similar behavioural changes in the TST, reducing the immobility in conjunction with an increase in swinging, exerting no effect on curling behaviour [see Table 2(*a–e*) for one-way ANOVA data]. For example, the tricyclic antidepressant imipramine (2.5–20.0 mg/kg) induced a dose-dependent decrease in immobility while increasing swinging behaviour at both 10 and 20 mg/kg ($p<0.05$ in all cases), without affecting curling behaviour (Fig. 2*a*). The 5-HT and NA reuptake inhibitors venlafaxine (2.5–20.0 mg/kg) and duloxetine (1.25–40.0 mg/kg) induced similar behavioural changes, significantly decreasing immobility and increasing swinging behaviour in a dose–dependent manner. Moreover, like imipramine, neither venlafaxine nor duloxetine altered curling

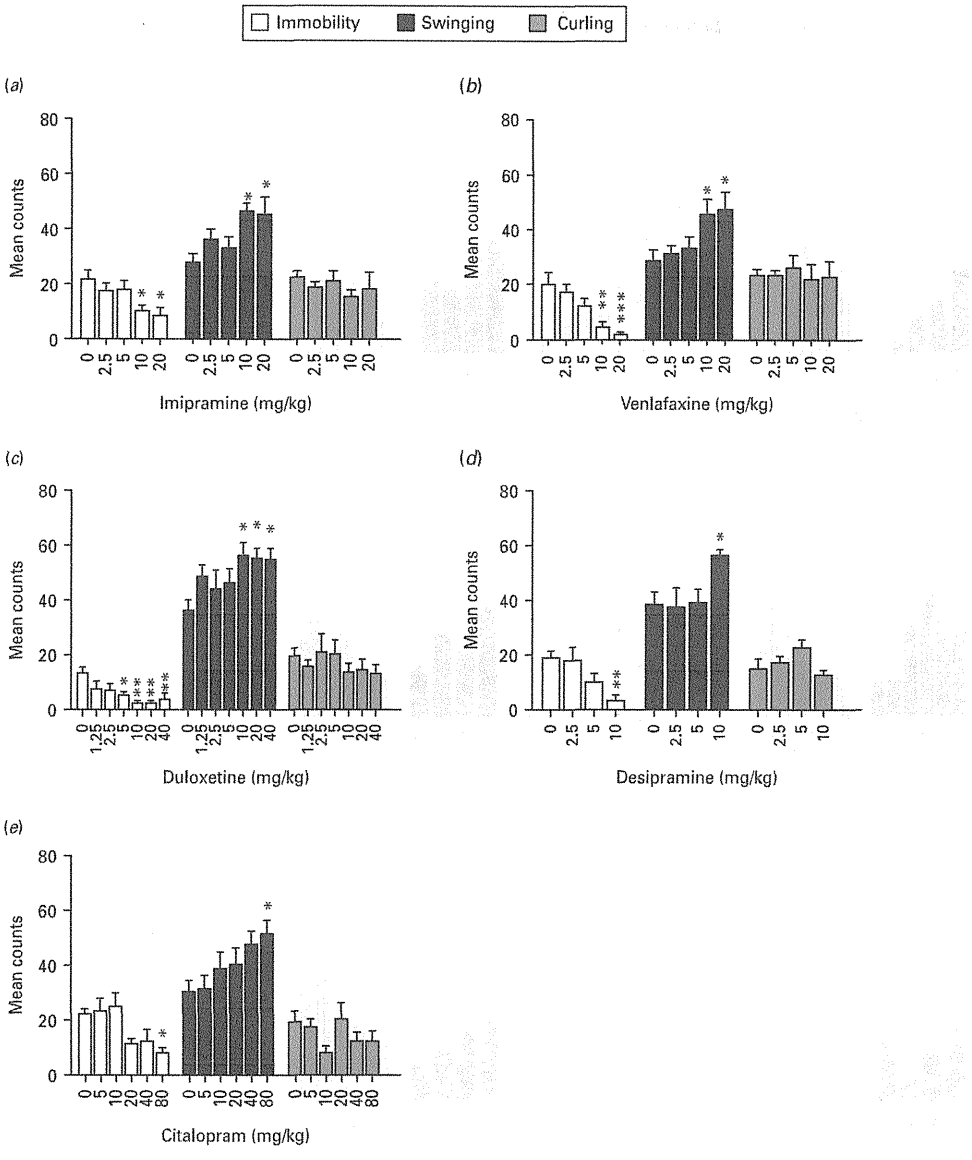

Fig. 2. Effects of (*a*) imipramine (2.5–20 mg/kg i.p.), (*b*) venlafaxine (2.5–20 mg/kg i.p.), (*c*) duloxetine (1.25–40 mg/kg i.p.), (*d*) desipramine (2.5–10 mg/kg i.p.) and (*e*) citalopram (5–80 mg/kg i.p.) on immobility and active behaviours in the tail suspension test. Drugs were administered 30 min before testing and the data represent the mean counts + S.E.M. from 8–11 animals per group. There were significant differences when compared to saline-treated mice (Dunnett's *post-hoc* test: * $p<0.05$; ** $p<0.01$; *** $p<0.001$).

behaviour (Fig. 2*b*,*c*). In a dose–response study to assess the antidepressant-like effects of the NA uptake inhibitor desipramine (2.5–10.0 mg/kg), 10 mg/kg desipramine reduced the time spent immobile ($p<0.01$) while increasing swinging ($p<0.05$), producing no effect on curling behaviour (Fig. 2*d*). Finally, the selective 5-HT uptake inhibitor citalopram (5–80 mg/kg) induced a behavioural pattern similar to that of the other antidepressants tested, decreasing immobility at 80 mg/kg ($p<0.05$) while increasing swinging behaviour ($p<0.05$), with no effect on curling behaviour (Fig. 2*e*).

Opioids

All the opioids tested induced a similar behavioural pattern in the TST, decreasing immobility while increasing curling behaviour [see Table 2(*f*–*k*) for

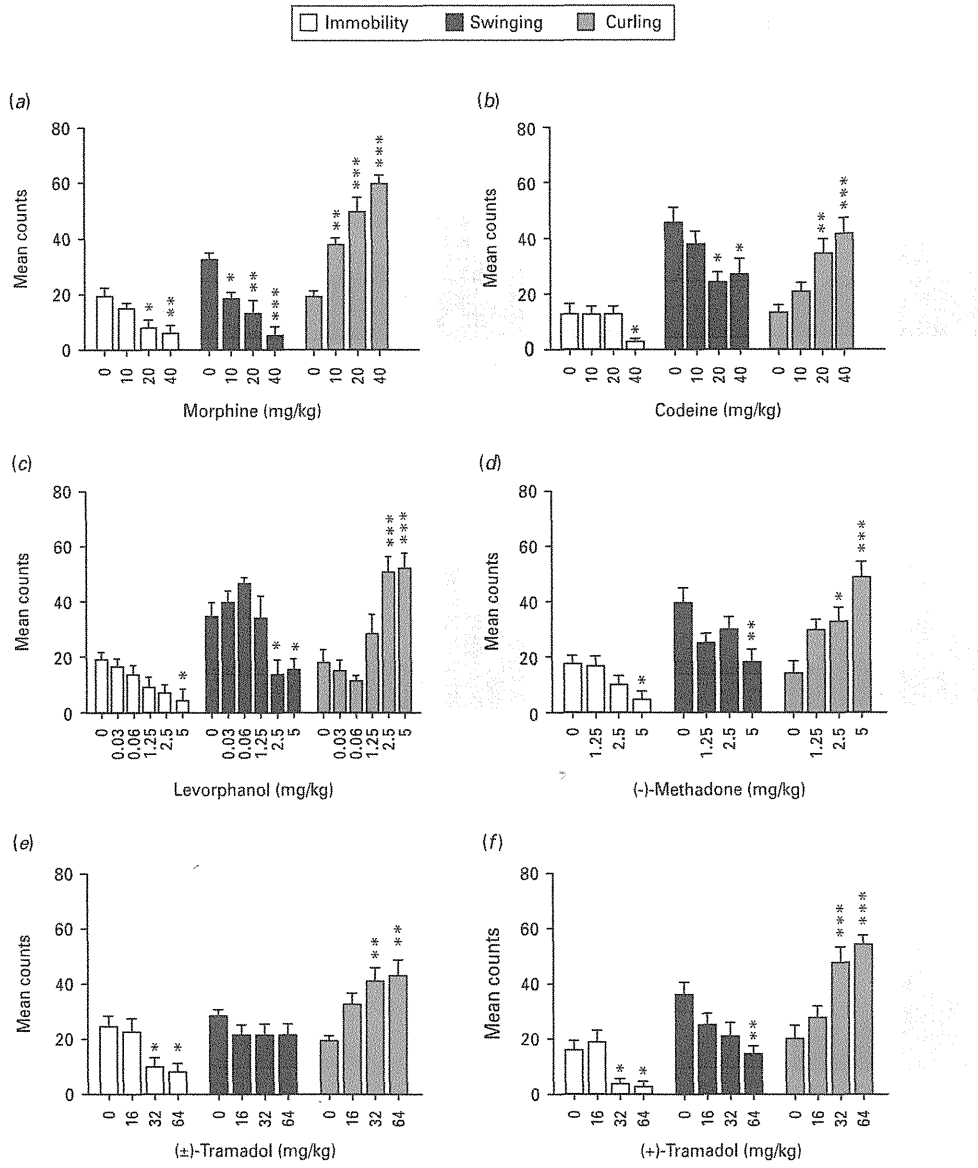

Fig. 3. Effects of (*a*) morphine (10–40 mg/kg i.p.), (*b*) codeine (10–40 mg/kg i.p.), (*c*) levorphanol (0.03–10 mg/kg i.p.), (*d*) (−)-methadone (1.25–5 mg/kg i.p.), (*e*) (±)-tramadol (16–64 mg/kg i.p.) and (*f*) (+)-tramadol (16–64 mg/kg i.p.) on immobility and active behaviour in the tail suspension test. Drugs were administered 30 min before testing and the data represent the mean counts + S.E.M. from 8–10 animals per group. There were significant differences when compared to saline-treated mice (one-way analysis of variance followed by Dunnett's *post-hoc* test: * $p<0.05$; ** $p<0.01$; *** $p<0.001$).

one-way ANOVA data]. Opioids that do not inhibit monoamine reuptake, such as morphine (10–40 mg/kg), attenuated immobility and swinging behaviour in a dose-dependent manner, while robustly increasing curling behaviour (Fig. 3*a*). Similar effects were observed for codeine (10–40 mg/kg), which as reported elsewhere (Berrocoso & Mico, 2009*a*), reduced immobility at 40 mg/kg ($p<0.05$), decreased swinging ($p<0.05$) and increased curling behaviour when administered at 20 or 40 mg/kg ($p<0.01$ and $p<0.001$, respectively; Fig. 3*b*). Opioids with monoamine uptake-inhibiting activity such as levorphanol (0.03–10.00 mg/kg) and (−)-methadone (1.25–5.00 mg/kg) induced a behavioural pattern similar to that seen for morphine and codeine, significantly reducing immobility ($p<0.05$ in both cases) and swinging behaviour and increasing curling behaviour (Fig. 3*c*,*d*). The behaviour elicited in response to (±)-tramadol

(16–64 mg/kg) differed slightly from that provoked by the other opioids (Fig. 3*e*) and, specifically, a significant reduction in immobility ($p<0.05$) and an increase in curling behaviour ($p<0.01$) at 32 and 64 mg/kg was not coupled with any effect on swinging behaviour. By contrast, the dextro enantiomer (+)-tramadol, which binds to the MOR and inhibits 5-HT reuptake more strongly than (±)-tramadol (Raffa *et al.* 1993), produced similar effects to the other opioids studied (Fig. 3*f*), decreasing immobility and increasing curling behaviour at 32 and 64 mg/kg and decreased swinging at 64 mg/kg ($p<0.01$; Fig. 3*f*).

Role of opioid receptors in antidepressant-like effects

Pharmacological blockage

To confirm the role of the opioid system in curling behaviour, an effective dose of codeine (40 mg/kg) was co-administered with the opioid receptor antagonist, naloxone [0.5–2.0 mg/kg; Fig. 4, Table 2(*l–n*)]. Two-way ANOVA revealed a significant effect of codeine on immobility ($p<0.01$), while swinging and curling behaviour remained unchanged. Two-way ANOVA also revealed significant effects of naloxone on swinging ($p<0.0001$) and curling behaviours ($p<0.001$). Furthermore, a significant interaction between factors was observed for all three behaviours: immobility ($p<0.05$); swinging ($p<0.05$); curling ($p<0.01$). Indeed, a Bonferroni's analysis revealed that naloxone significantly blocked the effects of codeine on immobility, swinging and curling ($p<0.01$ in all cases; Fig. 4).

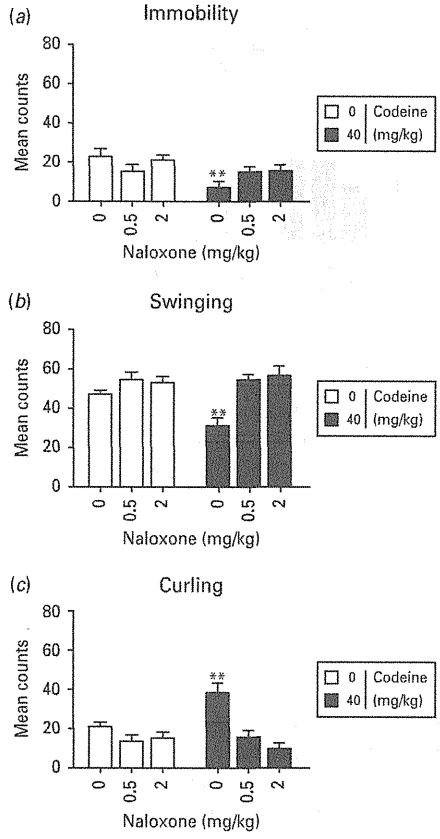

Fig. 4. Opioid receptor involvement in the effects of codeine on immobility, curling and swinging behaviour in the tail suspension test in mice. Codeine (40 mg/kg i.p.) and the opioid receptor antagonist naloxone (0.5–2 mg/kg s.c.) were administered 30 min before testing and the data represent the mean counts +S.E.M. from 10 animals per group. There were significant differences when compared to saline-treated mice (two-way analysis of variance followed by Bonferroni *post-hoc* test: $^{**}p<0.01$).

MOR-KO study

To determine whether curling behaviour involves the activation of MORs, we evaluated the behaviour of C57BL/6J MOR-KO mice in the TST, both males and females. Initially, we investigated the effects of gender and genotype [Table 2(*o–q*)] and while a two-way ANOVA revealed no significant effect of gender and no gender × genotype interaction, a significant effect of genotype was evident [$p<0.05$, Table 2(*p*)]. As expected, a subsequent unpaired Student's *t* test revealed no significant difference in behaviour between male and female knockout mice (data not shown). Thus, the data from the male and female mice were pooled for the subsequent studies. While we were unable to detect any differences in the behaviour of heterozygous MOR-KO mice from their wild-type littermates, the homozygous knockout mice displayed significantly less curling behaviour compared to their wild-type littermates [$p<0.05$, Tukey's test; Fig. 5, Table 2(*r*)]. This result supports the hypothesis that curling behaviour involves MOR activation.

The opioid (±)-tramadol acts through both MOR and 5HT/NA transporters and, hence, we explored its effects on C57BL/6J MOR-KO mice over a range of active doses (32–64 mg/kg). Two-way ANOVA revealed a significant effect of (±)-tramadol on all the behaviour studied [Table 2(*s–u*); $p<0.001$] and a significant effect of genotype on swinging ($p<0.01$) and curling ($p<0.001$). Interestingly, the interaction of both factors ((±)-tramadol × genotype) was only significant for curling ($p<0.05$). To study the effect of (±)-tramadol on different behaviours, we performed a one-way ANOVA, followed by the Dunnett's test [Table 2(*v–x*)]. Results showed that (±)-tramadol

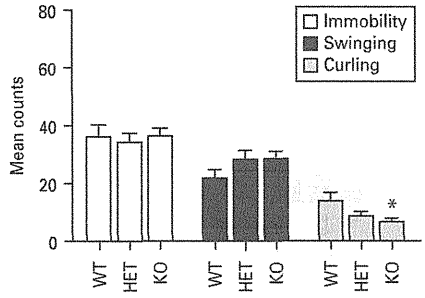

Fig. 5. Immobility, curling and swinging behaviour in wild-type (WT), heterozygous (HET) and homozygous μ-opioid receptor knockout (KO) mice in the tail suspension test. Data represent the mean counts +S.E.M. from 16–26 animals per group. There were significant differences when compared to saline-treated mice (one-way analysis of variance followed by Tukey's *post-hoc* test: * $p<0.05$). Data from male and female mice were pooled.

significantly decreased immobility to a similar degree in all three genotypes (Fig. 6), although the effect of (±)-tramadol on active behaviour varied according to the genotype. Thus, at both doses (±)-tramadol significantly increased swinging behaviour in knockout mice alone ($p<0.05$ and $p<0.01$, respectively), while curling behaviour was significantly increased at 64 mg/kg in wild-type and heterozygous mice ($p<0.01$ and $p<0.001$, respectively; Fig. 6). Note that the effect of (±)-tramadol was similar in CD1 and C57BL/6J wild type mice (Figs. 3*e*, 6).

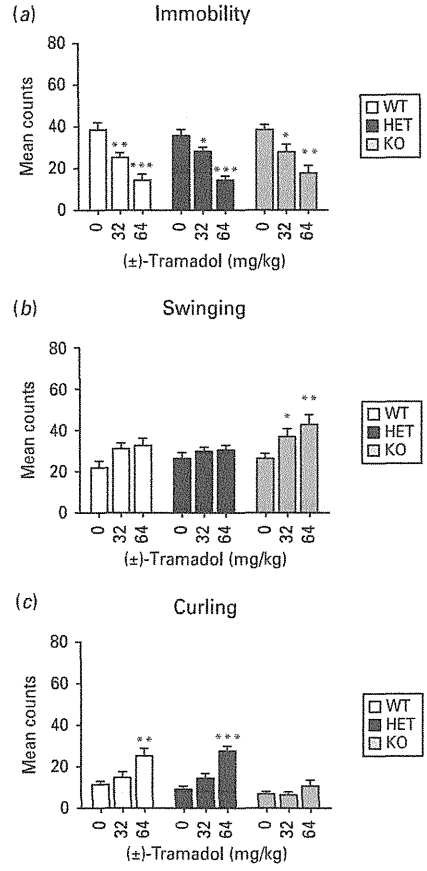

Fig. 6. Effects of (±)-tramadol (32–64 mg/kg i.p.) on immobility, curling and swinging behaviour in wild-type (WT), heterozygous (HET) and homozygous μ-opioid receptor knockout (KO) mice in the tail suspension test. (±)-Tramadol was administered 30 min before testing and the data represent the mean counts +S.E.M. from 15–24 animals per group. There were significant differences when compared to the corresponding saline-treated controls (one-way analysis of variance followed by Dunnett *post-hoc* test: * $p<0.05$; ** $p<0.01$; *** $p<0.001$). Data from male and female mice were pooled.

Discussion

The current study describes a modified means of scoring the TST, which differentiates between two active behaviours, swinging and curling. Using this approach, it was evident that antidepressants and opioids induce distinct active behaviours in the TST. While antidepressants increased swinging behaviour but had no effect on curling, opioids increased curling behaviour. Importantly, both antidepressants and opioids diminish the immobility of the mice, the traditional measure of antidepressant-like activity in the TST.

A modified form of the rat FST previously described a behavioural sampling technique that could distinguish between antidepressants with noradrenergic and serotonergic modes of action (Cryan *et al.* 2002). Hence, we employed a similar sampling technique to quantify and distinguish active behaviours induced by antidepressant treatment in the TST. While the traditional method of scoring the TST only measures the duration of immobility, we scored the frequency of the distinct behaviours in 5-s intervals throughout the test session. The results obtained using this approach do not differ from those obtained when each of the behaviours of interest are timed (Berrocoso & Mico, 2009*a*), yet they provided important information on the active behaviours that may be adopted when immobility is reduced. Significantly, our data are consistent with the strain differences previously observed in the TST (Cryan *et al.* 2005*a*; Liu & Gershenfeld, 2001) as CD1 mice were less immobile than C57BL/6J mice. Along similar lines, the immobility we observed in the dose–response experiments performed was in accordance

with the effects of antidepressants observed in other such screening tests. For example, the decrease in immobility following 10 and 20 mg/kg venlafaxine administration here is similar to its effect on mice in the FST (Berrocoso & Mico, 2009*b*). These findings validate the behavioural sampling technique and confirm that immobility is not to be confused with active behaviours.

The present study demonstrates that antidepressant compounds that differentially block the reuptake of NA and/or 5-HT induce a stereotypic behavioural profile that involves decreased immobility and increased swinging, with no effect on curling behaviour. These results implicate enhanced noradrenergic and/or serotenergic neurotransmission in swinging behaviour. Interestingly, and in agreement with previous data regarding the poor effect of SSRIs in animal models of depression (Berrocoso & Mico, 2009*a*; Lucki *et al.* 2001; Petit-Demouliere *et al.* 2005), a much higher dose of citalopram than that of other classes of antidepressants (noradenergic or dual) is required to significantly decrease immobility and increase swinging in CD1 mice. However, this behavioural sampling technique was unable to differentiate between antidepressants that selectively increase 5-HT or noradrenergic neurotransmission, unlike the modified version of the rat FST (Detke *et al.* 1995). It should be noted that, to date, all behavioural patterns in mouse tests of depression (including the FST) have been attributed to either serotoninergic or catecholaminergic signalling. The reasons for this restriction have not yet been elucidated but it suggests that differences in the coping strategies employed by mice and rats may be reflected in distinct active behaviours.

One of the main findings in this study is the clear demonstration of the antidepressant-like effect exerted by opioids, as witnessed by a decrease in immobility. These findings are consistent with previous studies using the TST or other behavioural tests to screen for depression or antidepressant effects using similar doses of these opioids (Fichna *et al.* 2007; Rojas-Corrales *et al.* 2002; Tejedor-Real *et al.* 1993, 1998). In the learned helplessness paradigm, morphine exerts an antidepressant-like effect and is antagonized by naloxone, indicating that it is indeed mediated by the opioids (Besson *et al.* 1996; Tejedor-Real *et al.* 1995). Antidepressant effects of other MOR agonists, such as (−)-methadone and levorphanol, have also been reported in the rat learned helplessness test (Rojas-Corrales *et al.* 2002). Thus, our findings validate the use of the TST as a test to screen for antidepressant-like activity of compounds that modulate opioid neurotransmission. Furthermore, the characteristic reduction in immobility indicative of antidepressant-like activity was accompanied by a consistent increase in curling behaviour with all the opioids evaluated. The pharmacological blockade of this behaviour by naloxone suggests that the antidepressant-like effect of opioid drugs is mediated by the opioid system and not other neurotransmission systems. Furthermore, the increase in curling behaviour seems to be accompanied by a decrease in swinging. However, it is important to note that the observed dose-dependent reduction in swinging may be a consequence of the increase in curling, i.e. if the animals spend a substantial amount of the time curling then the time left for other behaviours is reduced. While opioids caused a 3- to 6-fold decrease in immobility at the highest doses, swinging was only reduced ~2-fold (with the exception of morphine), indicating that many opioids induce a shift towards relatively more swinging in the 'non-curling' periods. This would suggest a contribution (although less relevant) of the monoaminergic system in the antidepressant-like effect of opioids.

It could be argued that curling behaviour reflects an opioid-induced increase in spontaneous motor activity or, alternatively, the induction of Straub tail, an S-shaped dorsiflexion of the mouse tail, produced by contraction of the sacro-coccygeal dorsalis muscles. Such effects would suggest that curling behaviour is not an escape-oriented behaviour but, rather, that it can be considered as a false positive score in the TST. However, we previously showed that codeine (40 mg/kg) did not modify spontaneous motor activity or coordination (Berrocoso & Mico, 2009*a*). In addition, spontaneous locomotion is not modified in the genetic knockout of the MOR (Ide *et al.* 2010), ruling out any possible increase in spontaneous motor activity. While Straub tail may be produced by high doses of opiates (Bilbey *et al.* 1960; Narita *et al.* 1993), genetic blockade of MORs significantly and specifically decreases curling behaviour without modifying immobility, indicating that Straub tail and curling are modulated by different mechanisms. Therefore, it seems unlikely that these events are confounded in the analysis of active behaviours in the TST.

While many studies have described antidepressant-like effects of δ (Tejedor-Real *et al.* 1998; Torregrossa *et al.* 2005, 2006) and κ (Mague *et al.* 2003; Pliakas *et al.* 2001; Shirayama *et al.* 2004) receptor antagonists, the specific roles of these opioid receptors on opioid-induced behaviour was not evaluated here. Hence, further studies with opioids that specifically act through these receptors will be necessary to determine their individual contributions. Indeed, since δ-opioid receptors are thought to be critical for the analgesic

activity of tricyclic antidepressants (Benbouzid *et al.* 2008), it would be interesting to evaluate their possible contribution to the effect observed in the TST. Finally, in the light of experiences with the FST (Cryan *et al.* 2005*b*), it would also be very interesting to test other effective or potential antidepressants that do not directly target monoamines (such as ketamine, AMPA potentiators or CRF1 antagonists) in order to determine how they affect the active behaviours described above.

We evaluated our modified scoring method using (±)-tramadol, a compound that has both opioid and monoaminergic effects. This compound is a weak agonist of the MOR and, like many antidepressant drugs, it inhibits the reuptake of 5-HT and NA. When compared to its parental compound, (+)-tramadol binds more potently to MOR and it inhibits the reuptake of 5-HT (Raffa *et al.* 1993; see Table 1). Indeed, (±)-tramadol decreases the immobility of CD1 mice and is suggestive of an antidepressant-like action (Berrocoso *et al.* 2006; Rojas-Corrales *et al.* 2002, 2004; Yalcin *et al.* 2007), further validating our sampling method. While (±)-tramadol significantly increased curling behaviour (consistent with an increase in MOR activity) it had no effect on swinging behaviour, despite its monoaminergic mode of action. Interestingly, (+)-tramadol, which possesses greater affinity for MOR, acted like a typical opioid, decreasing immobility and swinging behaviour and increasing curling. These data are consistent with the stronger affinity of (±)-tramadol and (+)-tramadol for MOR than for 5-HT/NA transporters (Table 1).

The administration of (±)-tramadol to MOR-KO mice provided further evidence of its specific mode of action. Thus, (±)-tramadol showed similar antidepressant-like effects in all three genotypes (wild-type, heterozygous and knockout), as indicated by the decrease in immobility. However, the increase in curling behaviour in wild-type mice suggests that (±)-tramadol acts through the opioid system, although the increase in swinging induced by (±)-tramadol in MOR-KO mice suggests that it enhances monoaminergic neurotransmission. Heterozygous mice displayed a profile similar to that of wild-type mice, indicative of a predominant opioid-mediated effect. It is also noteworthy that (±)-tramadol does not apparently affect swinging in wild-type mice, despite its well-known influence on monoamine transporters. The decrease in immobility but not swinging indicates a shift towards more swinging in the 'non-curling' periods. However, (±)-tramadol-induced swinging is more pronounced in MOR-KO mice, indicating that curling induced by MOR activation might mask the effects on swinging. These findings demonstrate the utility of this approach to explore the mode of action of monoaminergic/opioidergic compounds. Furthermore, we show that (±)-tramadol has both a central monoaminergic and opioidergic activity and that it can elicit antidepressant-like effects, even when one of these signalling mechanisms is blocked. This finding may be particularly relevant in pathological conditions in which both receptor profiles are modified.

Depression displays remarkable inter-individual variation in terms of symptoms and drug response. For example, opioid therapy has been successful in treating some refractory cases of depression such as when using the MOR agonists, oxycodone and oxymorphone, the partial agonist, buprenorphine, and the atypical opioid (±)-tramadol (Bodkin *et al.* 1995; Fanelli & Montgomery, 1998; Shapira *et al.* 2001; Spencer, 2000; Stoll & Rueter, 1999). Accordingly, alterations to the opioid system may underlie the neuroendocrine abnormalities observed in some groups of patients with this illness (Kennedy *et al.* 2006). Indeed, significant alterations in MOR activation have been observed in several brain areas (e.g. rostral region of the anterior cingulate) in patients with a major depressive disorder who did not respond to SSRI treatment. Interestingly, these alterations were correlated with corticotropin and cortisol plasma levels. Thus, although it remains to be confirmed, it is possible that the behaviour's affect in the TST could serve to predict different symptom clusters or therapeutic effects.

In summary, using a novel approach to scoring the TST, two specific active behaviours can be characterized that enable serotonergic/noradrenergic antidepressants to be distinguished from opioid compounds: swinging and curling. While traditional antidepressants that inhibit serotonin and/or NA reuptake decrease immobility and increase swinging behaviour, opioids, having decreased immobility, increase curling behaviour. Analysing these active behaviours may be useful to evaluate the mode of action of opioids and of opioids that also display monoaminergic properties, providing an important means of analysing the antidepressant effects of opioid compounds.

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