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ORIGINAL ARTICLE

Chronic morphine and tramadol re-exposure induced an anti-anxiety effect in prepubertal rats exposed neonatally to the same drugs

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SUMMARY

Anxiety disorders are among the most common mental disorders. Drugs that are often administered to manage medical problems cause rebound anxiety. The use of morphine and tramadol has increased in recent decades. In the present study, the effects of morphine and tramadol exposure during the neonatal and prepubertal periods on anxiety-like behaviours in prepubertal rats were investigated. Male neonate rats were injected subcutaneously with saline, morphine or tramadol (3–21 mg/kg) on a daily basis from postnatal Day (P) 8 to P14. On P22, rats were divided into seven groups (saline/saline, saline/tramadol, saline/morphine, tramadol/saline, tramadol/tramadol, morphine/saline and morphine/morphine) and were injected with saline, tramadol or morphine for seven consecutive days. All rats were tested in an elevated plus maze (EPM) on P24 (acute effects), P27 (chronic effects) and P29. Locomotor activity was increased by the second and third exposure to the EPM. Re-exposure to chronic morphine and tramadol resulted in increased locomotor activity, whereas acute and chronic administration of these drugs induced no notable difference. Anxiety decreased markedly after re-exposure to tramadol and this anxiolytic-like behaviour was more dominant in EPM re-exposure in rats that had received higher doses of tramadol. Re-exposure to tramadol elicited a stronger anxiolytic-like behaviour than re-exposure to morphine. It can be concluded that repeated morphine and tramadol administration during the neonatal period followed by re-exposure to these drugs at an immature stage produces considerable anxiolytic-like behaviour. Exposure to chronic morphine and tramadol during the neonatal period may affect the developing brain, which may induce long-term changes in the opioid response.

Key words: anxiety, elevated plus maze, morphine, neonatal, prepubertal, tramadol.

INTRODUCTION

Anxiety disorders are among the most common mental disorders and up to 15% of all people suffer from an anxiety disorder during their lifetime.[1] Meanwhile, drugs that are administered to manage medical problems, including stimulants, sedatives, tranquillisers, monoamine oxidase inhibitors and antidepressants,[2] can cause rebound anxiety. Neonates, infants and children are often exposed to the pain caused by invasive procedures that are performed during intensive care and the postoperative period.[3] The use of analgesic drugs (e.g. morphine and tramadol) in neonatal intensive care units has increased in recent decades as a consequence of changes and advances in terms of the understanding, identification and treatment of pain in children.[4] Moreover, in many countries, tramadol is administered to children > 1 year of age.[5] Tramadol hydrochloride is a centrally acting analgesic that is prescribed for moderate to severe pain[6–8] and its analgesic effect is independent of changes in anxiety, nervous system and depression function.[9] Tolerance and dependence have not been described after the repeated administration of tramadol in humans[10–12] and this drug has not been associated with significant opioid side-effects, such as respiratory depression, constipation or sedation.[11,13] Tramadol and morphine bind μ-opioid receptors; however, the affinity of tramadol for μ-receptors is several thousand-fold weaker than that of morphine. The effects of tramadol are derived not only from its opioid action, but also from inhibition of noradrenaline and serotonin reuptake in the central nervous system.[14] Previous studies have shown that various neurotransmitters, such as serotonin, dopamine, GABA and glutamate, are involved in the behaviours triggered by morphine.[15,16] Serotonin, noradrenaline and GABA are considered the three main factors associated with anxiety, because anxiety is modulated by agonists and antagonists of these neurotransmitters.[17,18] The antinociceptive activity of morphine and tramadol, two main clinically useful antinociceptive drugs, has been investigated extensively, demonstrating that the analgesic potency of morphine is several fold higher than that of tramadol.[19] However, there remains a lack of knowledge as to the specific effects of morphine and tramadol after the long-term administration of these drugs to patients.[20] Moreover, the potential impact of exposure to morphine and tramadol during the immature period has not been well studied. Effects of morphine and tramadol administration during the immature period on anxiety-like behaviours is not

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clear; in addition, no studies have addressed the anxiety-like behaviours resulting from the neonatal administration of tramadol and morphine together. Therefore, the aim of the present study was to investigate and compare the effects of exposure to morphine and tramadol during the neonatal and prepubertal periods on anxiety-like behaviours in prepubertal rats.

RESULTS

Effects of repeated morphine and tramadol on entrance into open arms of the elevated plus maze

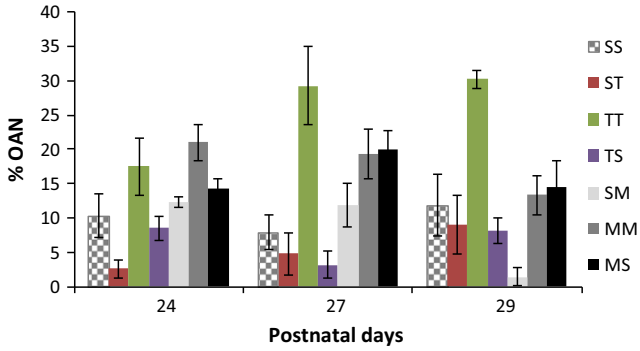
Table 1 summarizes data for open arm entry (OAE) in the elevated plus maze (EPM). Results of repeated-measures analysis indicated that effect of group was significant ($F_{(6,37)} = 17.39$; $P < 0.001$), whereas that of EPM re-exposure was not ($F_{(2,74)} = 0.414$; $P = 0.662$), but that of the EPM re-exposure × group interaction was ($F_{(12,74)} = 2.35$; $P = 0.013$). Because the effect of the EPM re-exposure × group interaction was significant, the data were analysed by the Bonferroni *post hoc* test. The multiple comparisons test indicated significant differences among groups, as indicated in Fig. 1.

Effects of repeated morphine and tramadol on time spent in the open arms of the EPM

Table 1 summarizes data for time spent in the open arms (OAT) of the EPM. Results of repeated-measures analysis indicated that effect of group was significant ($F_{(6,37)} = 27.58$; $P < 0.001$), whereas that of EPM re-exposure was not ($F_{(2,74)} = 1.31$; $P = 0.277$), but the interaction of EPM re-exposure × group was ($F_{(12,74)} = 5.81$, $P < 0.001$). Because the interaction of EPM re-exposure × group was significant, the data were analysed by the Bonferroni *post hoc* test. The multiple comparisons test indicated significant differences among groups, as indicated in Fig. 2.

Effects of repeated morphine and tramadol administration on locomotor activity

Table 1 summarizes data for locomotor activity. Repeated-measures analysis indicated that effect of group was significant ($F_{(6,37)} = 26.48$; $P < 0.001$), whereas that of EPM re-exposure was not ($F_{(2,74)} = 1.39$; $P = 0.277$), but the interaction of EPM re-exposure × group was ($F_{(12,74)} = 6.133$; $P < 0.001$). Because the effect of the EPM re-exposure × group interaction was significant, data were analysed by the Bonferroni *post hoc* test. The multiple comparisons test indicated significant differences among groups, as indicated in Fig. 3.



Group	P	Group	P	Group	P	Group	P	Group	P	Group	P
SS		ST		TT		TS		SM		MM	
ST	0.9	SS	0.9	SS	0.001	SS	0.9	SS	0.9	SS	0.06
TT	0.001	TT	0.001	ST	0.001	ST	0.9	ST	0.9	ST	0.01
TS	0.9	TS	0.9	TS	0.001	TT	0.001	TT	0.001	TT	0.07
SM	0.9	SM	0.9	SM	0.001	SM	0.9	TS	0.9	TS	0.01
MM	0.065	MM	0.001	MM	0.075	MM	0.001	MM	0.012	SM	0.01
MS	0.253	MS	0.001	MS	0.005	MS	0.005	MS	0.048	MS	0.9

Fig. 1 Effects of repeated morphine and tramadol administration on the percentage of open arm entries (%OAE). Data are expressed as the mean ± SD. The significance of differences between groups is indicated in the table (multiple comparisons for %OAE; Bonferroni test, group × day, group as between-subject factor and day as within-subject factors). SS, saline/saline; ST, saline/tramadol; SM, saline/morphine; TS, tramadol/saline; TT, tramadol/tramadol; MS, morphine/saline; MM, morphine/morphine.

DISCUSSION

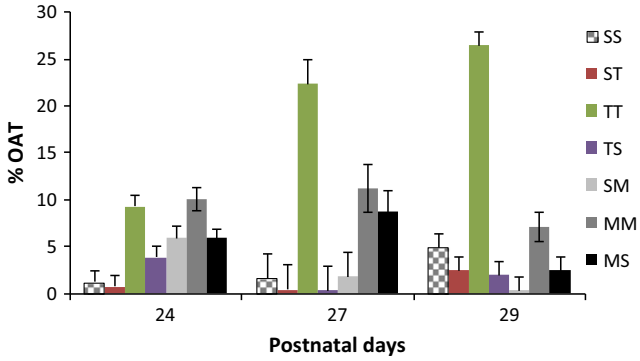
In the present study, neonatal rats received 3–21 mg/kg morphine, 3–21 mg/kg tramadol or saline daily from postnatal Day (P) P8 to P14. Rats were further divided into different subgroups and were given morphine, tramadol (3–21 mg/kg for both) or saline again from P22 to P28. All immature rats were subjected to an EPM test on P24, P27 and P29. The main finding of the present study was that neonatal tramadol and/or morphine exposure reduced anxiety in immature rats. Re-exposure to tramadol

Table 1 Descriptive analysis of anxiety behaviour in immature rats

	OAE%			OAT%			Locomotor activity (%)		
	P24	P27	P29	P24	P27	P29	P24	P27	P29
SS	10.3 ± 8.1	7.9 ± 6.4	11.8 ± 11.3	1.2 ± 0.9	1.6 ± 1.3	4.9 ± 3.5	5.8 ± 1.2	8 ± 2	9.8 ± 2.6
ST	2.5 ± 3.3	4.7 ± 7.4	9 ± 11	0.6 ± 0.7	0.4 ± 0.6	2.4 ± 2.8	14.3 ± 0.8	7.3 ± 1.7	9.8 ± 3.4
SM	12.3 ± 1.9	12 ± 8	1.4 ± 3.4	5.9 ± 2.1	1.8 ± 1.4	0.3 ± 0.7	8.3 ± 1.8	7.8 ± 2.1	8 ± 3
TS	8.5 ± 4.4	3 ± 5	8.2 ± 4.6	3.8 ± 2.5	0.3 ± 0.5	1.9 ± 1.2	9.2 ± 1.2	8.7 ± 0.8	10.2 ± 2.3
TT	17.5 ± 10.3	29.3 ± 14.1	30.2 ± 3.2	9.3 ± 6.4	22.3 ± 16.9	26.5 ± 8.6	10.5 ± 2.9	13 ± 3	16.2 ± 3.2
MS	14.2 ± 3.7	19.9 ± 7.3	14.4 ± 9.8	5.9 ± 0.8	8.7 ± 2.4	2.5 ± 1.7	8.6 ± 1.1	9 ± 2	5.9 ± 1.4
MM	21 ± 7	19.3 ± 8.9	13.4 ± 7.2	10.1 ± 3.4	11.2 ± 2.8	7.1 ± 3.2	12.8 ± 1.8	16.7 ± 4.1	14.8 ± 3.1

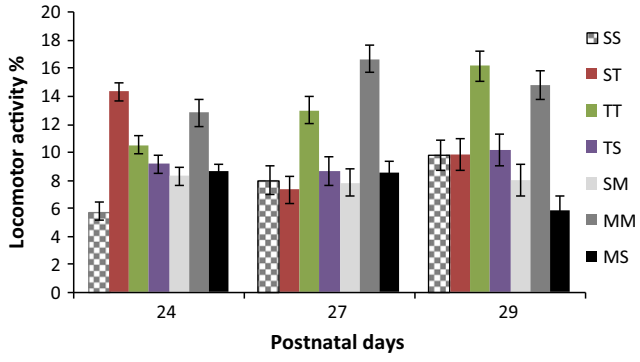
Data are the mean ± SD.

SS, saline/saline; ST, saline/tramadol; SM, saline/morphine; TS, tramadol/saline; TT, tramadol/tramadol; MS, morphine/saline; MM, morphine/morphine; OAE, number of open arm entries; OAT, time spent in the open arm; P, postnatal day.



Group		P	Group		P	Group		P	Group		P	Group		P	Group		P
SS	ST	0.9	ST	SS	0.9	TT	SS	0.01	TS	SS	0.9	SM	SS	0.9	MM	SS	0.007
	TT	0.01		TT	0.01		ST	0.01		ST	0.9		ST	0.9		ST	0.001
	TS	0.9		TS	0.9		TS	0.01		TT	0.01		TT	0.9		TT	0.001
	SM	0.9		SM	0.9		SM	0.01		SM	0.9		TS	0.9		TS	0.003
	MM	0.01		MM	0.01		MM	0.01		MM	0.003		MM	0.01		SM	0.009
	MS	0.9		MS	0.16		MS	0.01		MS	0.61		MS	0.9		MS	0.618

Fig. 2 Effects of repeated morphine and tramadol administration on the percentage of time spent in the open arm of the elevated plus maze (% OAT). Data are expressed as the mean ± SD. The significance of differences between groups is indicated in the table (multiple comparisons for %OAT; Bonferroni test, group × day, group as between-subject factor and day as within-subject factor). SS, saline/saline; ST, saline/tramadol; SM, saline/morphine; TS, tramadol/saline; TT, tramadol/tramadol; MS, morphine/saline; MM, morphine/morphine.



Group		P	Group		P	Group		P	Group		P	Group		P	Group		P
SS	ST	0.04	ST	SS	0.04	TT	SS	0.01	TS	SS	0.9	SM	SS	0.9	MM	SS	0.01
	TT	0.01		TT	0.03		ST	0.03		ST	0.9		ST	0.08		ST	0.01
	TS	0.9		TS	0.9		TS	0.01		TT	0.01		TT	0.01		TT	0.9
	SM	0.9		SM	0.08		SM	0.01		SM	0.9		TS	0.9		TS	0.01
	MM	0.01		MM	0.01		MM	0.9		MM	0.01		MM	0.01		SM	0.01
	MS	0.8		MS	0.01		MS	0.01		MS	0.6		MS	0.9		MS	0.01

Fig. 3 Effect of repeated morphine and tramadol administration on locomotor activity. Data are the mean ± SD. The significance of differences between groups is indicated in the table (multiple comparisons for percentage locomotor activity; Bonferroni test, group × day, group as between-subject factor and day as within-subject factor). SS, saline/saline; ST, saline/tramadol; SM, saline/morphine; TS, tramadol/saline; TT, tramadol/tramadol; MS, morphine/saline; MM, morphine/morphine.

elicited a stronger anxiolytic-like behaviour than re-exposure to morphine. Re-exposure to chronic morphine and tramadol increased locomotor activity, but there was no significant difference between the acute (P24) and chronic (P27) effects of these drugs.

The results of the present study showed that, in immature rats, tramadol and morphine had a similar effect on locomotor activity. In the saline/saline-treated group, locomotor activity increased in immature rats with each subsequent EPM test. Previous studies have reported differences in EPM behaviour when animals are exposed to the EPM on more than one occasion. For example, decreased activity on the open arms of the maze is typical on the second exposure to this task compared with the first.[21,22] Re-exposure to chronic morphine and tramadol increased locomotor activity, but there was no significant difference between the acute (P24) and chronic (P27) effects of these drugs. In this regard, some studies have reported that acute morphine has no notable effect on locomotor activity in adulthood.[23,24] It has also been reported that morphine re-injection increases locomotor activity[25] and that repeated administration of tramadol enhances D-amphetamine-induced locomotor hyperactivity.[26] Moreover, it has been reported that tramadol is effective in preventing anxiety in humans;[27] stress leads to changes in levels of central opioids[28] and opioids mediate the neuronal processes that are involved in moderating anxiety-related behaviours.[29] Intraperitoneal administration of morphine causes anxiolytic-like behaviours in rats[30] and Syrian hamsters.[29] The opioid antagonist naloxone increases anxiety-like behaviours in rats.[31] The present study showed that anxiety decreased after re-exposure to chronic tramadol in immature rats and that this anxiolytic-like behaviour was more prominent upon EPM re-exposure in rats on P27 than on P24. Re-exposure to chronic morphine slightly decreased anxiety, but the effect did not reach statistical significance. So, re-exposure to chronic tramadol induced a stronger anxiolytic-like behaviour compared with re-exposure to chronic morphine. In addition, anxiety-like behaviour in the tramadol groups was stable a day after the injection of the final dose (P29) in the third EPM test, but decreased in morphine groups. In agreement with the results of the present study, previous studies have reported that morphine causes dose- and time-dependent increases in the number of entries into and time spent in an open arm[22,32] and that the anxiolytic-like effect of morphine for acute, higher doses appears later than for lower doses.[22] It has also been suggested that low residual morphine levels remaining in the plasma 2–4 h after administration of 10 mg/kg morphine may be sufficient to elicit anxiolytic-like effects.[22] Moreover, repeated morphine exposure of rats and mice leads to a further increase in the duration and magnitude of anxiolytic-like effects.[22–24] Because the studies conducted on effects of neonatal administration of these drugs on anxiety are insufficient, the exact mechanism underlying the reduction in anxiety-like behaviours of immature rats following chronic re-exposure to tramadol and morphine should be clarified in future studies. Thus, changes in neurons and neurotransmitter levels were proposed in this paper. Studies have demonstrated that prenatal administration of morphine reduces neuronal packing density and the number of neurons in newborn rats.[33,34] Morphine may produce some of its effects by modulating the GABAergic system;[35] in addition, morphine-induced behavioural sensitization increases extracellular GABA concentrations.[36] Both GABA and serotonin are the main anxiety-associated neurotransmitters.[17,18] Several studies have suggested that, in rats with increased anxiety levels, brain concentrations of serotonin in serotonin projection areas are increased.[37,38] Microinjection of GABA into serotonergic projection areas such as the amygdala reduces the firing rate of serotonergic neurons.[39] Therefore, the reduction in anxiety-like behaviours following chronic morphine re-exposure observed in the present study could be due to increased GABA activity in the central nervous system. Moreover, it has been reported that tramadol inhibits noradrenaline and serotonin reuptake[14,40] and

may increase anxiety-like behaviours via one of these mechanisms. In contrast, chronic tramadol re-exposure resulted in marked anxiolytic-like behaviours. Because there are not sufficient studies about the long-term effects of tramadol on anxiety, the mechanism underlying this effect is unclear and requires further investigation.

In conclusion, the results of the present study suggest that repeated morphine and tramadol administration during the neonatal period followed by re-exposure to these agents at an immature stage produced considerable anxiolytic-like behaviour, which was more pronounced for tramadol than morphine. It may be concluded that repeated morphine and tramadol administration during the neonatal period may affect the developing brain, which may induce long-term changes in the opioid response. This, in turn, produces strong anxiolytic-like behaviour after chronic re-exposure to these agents at the immature stage of life. However, future studies should be performed to determine how these changes occur in immature rats and whether these effects are specific to drug exposure during the immature period or not.

METHODS

Animals

Twelve-week-old male and female Wistar rats (200–250 g) were obtained from Urmia University of Medical Sciences (Urmia, Iran). Rats were housed in same-sex groups with four animals per cage under a 12 h light–dark cycle (lights on from 0700 to 1900 h) at 22 ± 2°C with free access to food and water. All experimental protocols and procedures complied with the guidelines of Medical Ethics Committee, Ministry of Health, Iran. In addition, the Regional Medical Ethics Committee of West Azerbaijan Province, Iran, approved this study. All female rats were mated at 13 weeks of age with a sexually experienced male of the same genotype. Four pregnant females were housed per cage for the entire gestation period.

Morphine and tramadol administration

After parturition, pups from different litters were mixed and divided evenly among dams. Male neonatal rats were identified by loop observation of the genital area. Then, male rats (n = 43) were randomly divided into three groups: saline (S; n = 17), morphine (M; n = 14) and tramadol (T; n = 12). Pups in the morphine and tramadol groups were injected with morphine or tramadol (5 mL/kg, s.c.) on the back, above the tail, at doses of 3, 6, 9, 12, 15, 18 and 21 mg/kg on P8, P9, P10, P11, P12, P13 and P14, respectively.[41,42] Similar doses of morphine and tramadol were used to simplify the comparison of the effects of these drugs on anxiety. Pups in the saline group were injected with an equal volume of saline solution (5 mL/kg). We started injections on P8 in the present study because it has been reported that rats at this age have a similar level of neurological development to that of a newborn human[43] and are physiologically immature.[3] Each pup was injected with saline, morphine or tramadol once a day from P8 for seven consecutive days. On P21, rats were weaned and then separated from the dams. Their sex was checked again to confirm that the pups under investigation were all male. The study was continued from P22 until P29 to compare the effect of exposure to morphine and tramadol on anxiety-like behaviours at two different stages (weaning and weaned) in immature rats. Thus, rats in the three groups were subdivided on P22 into saline/saline (SS; n = 6), saline/tramadol (ST; n = 5), saline/morphine (SM; n = 6), tramadol/saline (TS; n = 5), tramadol/tramadol (TT; n = 7), morphine/saline (MS; n = 8) and morphine/morphine (MM; n = 6) groups, which were injected with saline, tramadol or morphine (at the same doses as used on P8–P14) once a day for 7 consecutive days. All morphine, tramadol and saline injections were performed at the same time each day (1100 and 1200 h). Morphine sulphate (Temad, Tehran, Iran) was dissolved in 0.9% saline and prepared fresh prior to use. Tramadol hydrochloride (Atlantis Life Sciences, Mumbai, India) was in liquid form.

Anxiety assignment

The EPM test is a rodent model for measuring anxiety[44] and the anxiolytic-like effects of drugs and other experimental agents.[44–46] The method used in the present study was basically the same as that described by Pellow and File.[47] It has been demonstrated that exposure to a novel environment immediately before testing in the EPM increases motor activity in the EPM and leads to a greater likelihood of entering the open arms of the maze.[32,48] So, in the present study, rats were placed in the room used for experiments at least 1 h before testing. All rats were randomly tested. This experimental model of anxiety was unconditioned and animals did not need any training or learning before the test.[49] Testing was conducted in a quiet behavioural testing room. On P24 and P27, each rat was tested individually 30 min after morphine, tramadol or saline injection. Finally, on P29, the test was repeated without any injection for all the groups. Recent reports have suggested some differences in EPM behaviour when animals are exposed to the EPM on more than one occasion. For example, decreased activity on the open arms of the maze is typical on the second exposure to this task compared with the first.[21,22] Therefore, the EPM test was repeated three times in the present study, not to investigate the effects of age, but to clarify whether there were any differences in the EPM behaviour of different experimental groups. Finally, because the behaviour of rats in terms of the EPM can be influenced by circadian rhythms and/or the light cycle,[50,51] all the rats were tested at the same time (from 1100 to 1300 h).

The mouse EPM was used because of the similar size of the immature rats in the present study with adult mice. The maze was made of Plexiglas and consisted of a central square (5 × 5 cm), two closed arms with Plexiglas walls (5 × 30 × 15 cm) and two open arms without walls (5 × 35 cm). A 0.25 cm high edge was placed around the open arms to prevent the rats from falling off the maze. The maze was elevated 50 cm above the floor. The apparatus was located in a soundproof room illuminated by a 100 W red light bulb. For the EPM test, rats were placed at the junction of the four arms of the maze, facing an open arm and the entry into and time spent in each arm and the total number of entries into both arms was recorded simultaneously on videotape and by an observer for 5 min. After each test, the EPM was cleaned before testing a new rat to avoid any odourant confounder during the test.

The OAT% was used to represent the time spent by a rat in the open arm and was calculated as:

$$\mathrm{OAT\%} = \mathrm{OAT}/\mathrm{OAT} + \mathrm{CAT} \times 100$$

where OAT is the time spent in the open arm and CAT is the time spent in the closed arm time. Similarly, OAE% was used to represent the number of entries into the open arm and was calculated as:

$$\mathrm{OAE\%} = \mathrm{OAE}/\mathrm{OAE} + \mathrm{CAE} \times 100$$

where OAE is the number of entries into the open arm and CAE is the number of entries into the closed arm. The number of total arm entries is used as a measure of spontaneous locomotor activity;[52] thus, in the present study, the total number of entries into the open and closed arms was considered an index of locomotor activity.

Statistical analyses

Results are expressed as the mean ± SD. Data were analysed using repeated-measures analysis of variance (ANOVA) with the general linear models procedure of SPSS version 16.0 (SPSS, Chicago, IL, USA) using experimental groups (treatments) as between-subject factors and time (P24, P27 and P29) as within-subject factors. Therefore, a two-way ANOVA for one repeated-measures analysis was performed, followed by a multiple comparison test (Bonferroni test) when indicated. Anxiety behaviours at different time points (P24, P27 and P29) were treated as repeated measures for each animal. Differences were considered significant if two-tailed $P < 0.05$.

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DISCLOSURE

The authors declare no conflicts of interest.

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