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Effect of Chronic Maternal Diazepam Treatment on the Development of Stress-Induced Antinociception in Young Rats

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MUHAMMAD, B. Y. AND I. KITCHEN. *Effect of chronic maternal diazepam treatment on the development of stress-induced antinociception in young rats.* PHARMACOL BIOCHEM BEHAV 47(4) 927–933, 1994.—The use and abuse of benzodiazepines is widespread and we have begun to address whether maternal exposure to these drugs influences the development of opioid systems. We have studied the effect of maternal diazepam treatment on stress-induced antinociception in the neonatal offspring. Diazepam (1 or 10 mg/kg) was administered twice daily to mothers from conception. Pre- and postweanling rat pups were assessed for opioid-mediated stress-induced antinociception by 3-min swimming and measuring nociception using the tail immersion test. In preweanling rats there was stress-induced antinociception in both vehicle- and diazepam-treated animals but in diazepam-treated groups (1 and 10 mg/kg) this was insensitive to reversal by the opioid antagonist naloxone, suggesting that nonopioid systems are operating this response. In postweanling rats a similar insensitivity to naloxone was observed in 1 mg/kg diazepam-treated groups; with 10 mg/kg diazepam there was no significant antinociception. The results suggest that maternal diazepam treatment interferes with the development of stress-mediated responses and that part of this toxicity is due to actions on opioid systems in the CNS.

Diazepam Ontogeny Stress-induced antinociception Opioid Maternal

A number of associations between the pharmacological effects of the benzodiazepines and opioids have been made at the clinical, physiological, and molecular level. For example, diazepam has been used clinically as an adjunct to analgesic drugs (16,25). In animal models, although benzodiazepines have been mostly shown to increase or have no effect on noxious stimuli alone (22,29,39,46), their predominant effect on opioid antinociception is inhibitory (10,29,34,36). Benzodiazepines have also been studied for their effects on stress-induced antinociception (SIA) in both the rat (39) and mouse (17,35), and antagonism of this adaptive response has been reported.

Analgesic responses to exogenously administered opioids appear as early as postnatal day 2 in the rat (32) and correlate well with the early ontogenesis of the μ- and κ-opioid receptors (19,43). SIA can be classified into both opioid and nonopioid forms [see (3)], and in the young there are marked differences in the devleopment of each of these systems. The nonopioid form of SIA does not appear until the postweanling period (at day 25) whilst opioid-mediated SIA is evident in preweanling animals and can be detected as early as postnatal day 10 (14,33). Further, in preweanling rats μ-opioid receptors operate this behaviour, but after weaning at day 21, a receptor transition occurs whereby δ-opioid receptors then control the response (14,20).

The therapeutic use of benzodiazepines for their anxiolytic and hypnotic actions is extremely widespread and indeed chronic abuse of these tranquillisers is prevalent in some populations throughout the world (30,41). We have begun to address the issue of how maternal exposure to benzodiazepines

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might influence the development of opioid systems and report here the effects of maternal diazepam treatment on the development of opioid-mediated SIA in their offspring.

METHOD

Animals and Diazepam Treatment

Wistar albino rats (University of Surrey strain) were used in all experiments and were maintained at 21 ± 1°C in a constant 12 L : 12 D cycle (lights on at 0700 h). Spratts expanded laboratory diet was fed ad lib. Two female rats were caged with one male rat and vaginal smears were taken daily and examined by phase-contrast light microscopy. Sperm-positive females were then housed singly and assigned to one of four treatment groups. Mothers were injected SC once daily at 1100 h with either vehicle (16% w/v ethanol and 64% w/v polyethylene glycol), diazepam 1 mg/kg, or diazepam 10 mg/kg using a dose volume of 0.1 ml/100 g. The area for SC injection was varied from day to day to minimise cutaneous tissue damage. Diazepam treatment of the mother was continued from conception throughout gestation and the postnatal period up to the day of experimental testing (postnatal day 10, 15, or 20) or weaning (day 21) for animals tested at day 25. Maternal and neonate body weight and fluid intake were measured thrice weekly throughout chronic diazepam treatment. At parturition the number of pups per litter was counted in each experimental group and litters were culled to eight pups. Litters within the same treatment group, born on the same day, were cross-fostered. Any litter with less than six pups or that subsequently fell to less than six pups due to neonatal mortality was removed from the study. Pups were weaned at day 21.

Swim Stress Procedures and Nociceptive Testing

Swim SIA and its reversal by the opioid receptor antagonist, naloxone, was assessed in rat pups from vehicle- and diazepam-treated mothers at postnatal day 10, 15, 20, and 25. Separate groups of vehicle- and diazepam-treated animals were assessed at each age, and included both male and female neonates born in each litter. Experimental testing was carried out in a quiet, windowless, air-controlled laboratory between 1100 and 1600 h to minimise diurnal variation in nociceptive response. Litters were introduced into the laboratory 24 h prior to testing and pups were weighed individually and marked for administration of appropriate doses of drugs. Further, each litter was divided into four treatment groups (saline-injected unstressed, saline-injected swim stressed, naloxone-injected unstressed, naloxone-injected swim stressed) so that measures of nociception ($n \geq 6$) represents estimates from at least three litters carried out on at least three separate days. This protocol minimises interday and interlitter variation in nociceptive responses.

Nociceptive responses were recorded immediately before IP injection of 0.9% saline or naloxone (10 mg/kg) administration using the tail immersion test (15) at 50°C (15, 20, and 25 day) and 47.5°C (10 day) as previously described for use in neonates (9,21). In brief, rats were held upright in the hand, and the terminal 3 cm of the tail (in 10-day rats) and 5 cm (in older pups) was immersed in warm water. Nociceptive reaction times were determined by a hand-held stopwatch and

TABLE 1

EFFECT OF CHRONIC DIAZEPAM TREATMENT ON MATERNAL AND NEONATE WEIGHT IN THE PRE- AND POSTNATAL PERIODS

		Maternal Body Weight (g)			Mean Neonate Body Weight (g)	
Postnatal Day	Treatment Group	Starting Weight	Weight on Litter Day 1	Weight on Test Day	Weight on Litter Day 1	Weight on Test day
10	Control	242 ± 31	301 ± 12	332 ± 12	6.1 ± 0.26	20.4 ± 0.92
	Vehicle	260 ± 10	305 ± 15	354 ± 10	6.4 ± 0.06	25.3 ± 2.2
	Diazepam (1 mg/kg)	199 ± 31	269 ± 15	319 ± 13	6.4 ± 0.03	21.6 ± 0.96
	Diazepam (10 mg/kg)	264 ± 7	300 ± 6	330 ± 3	6.1 ± 0.3	21.9 ± 0.15
15	Control	229 ± 16	282 ± 7	330 ± 11	6.4 ± 0.07	34.8 ± 1.7
	Vehicle	240 ± 7	289 ± 6	338 ± 7	6.4 ± 0.23	35.5 ± 4.3
	Diazepam (1 mg/kg)	210 ± 18	269 ± 11	307 ± 7	6.4 ± 0.24	35.7 ± 1.5
	Diazepam (10 mg/kg)	249 ± 10	297 ± 11	336 ± 13	6.5 ± 0.20	40.0 ± 3.1
20	Control	206 ± 4	289 ± 4	309 ± 7	6.1 ± 0.26	42.7 ± 1.2
	Vehicle	266 ± 9*	316 ± 11	313 ± 31	6.0 ± 0.52	46.3 ± 5.0
	Diazepam (1 mg/kg)	217 ± 6	287 ± 14	330 ± 18	5.6 ± 0.32	40.5 ± 2.5
	Diazepam (10 mg/kg)	261 ± 31*	298 ± 17	349 ± 14	6.1 ± 0.24	44.8 ± 1.1
25	Control	240 ± 20	304 ± 20	332 ± 20	6.3 ± 0.19	62.6 ± 3.9
	Vehicle	284 ± 16	322 ± 5	364 ± 14	6.3 ± 0.18	68.2 ± 1.9
	Diazepam (1 mg/kg)	219 ± 3†	284 ± 1	329 ± 4	6.6 ± 0.03	67.3 ± 4.9
	Diazepam (10 mg/kg)	202 ± 8†	287 ± 13†	330 ± 14	6.7 ± 0.18	63.3 ± 3.2

Values (mean ± SEM) represent body weight at conception, after birth, and at the postnatal testing day for groups studied at 10, 15, 20, and 25 days of age. Values are the means of three to four mothers for each treatment group and values for neonate weight represents litter weight divided by pup number.

*†Statistical comparisons within each series of treatments were made using one-way ANOVA and post hoc comparisons using Duncans New Multiple Range Test: *$p < 0.05$ vs. control untreated group; †$p < 0.05$ vs. vehcile-injected group.

TABLE 2

EFFECT OF CHRONIC DIAZEPAM TREATMENT ON FLUID CONSUMPTION IN THE PRE- AND POSTNATAL PERIODS

Postnatal Day	Treatment Group	Fluid Intake (ml)		
		Prenatally	Postnatally	Total
10	Control	648 ± 30	466 ± 10	1114 ± 38
	Vehicle	768 ± 35	597 ± 57	1365 ± 83*
	Diazepam (1 mg/kg)	862 ± 25*	688 ± 7*	1550 ± 25
	Diazepam (10 mg/kg)	890 ± 48*†	590 ± 80	1480 ± 124*
15	Control	768 ± 66	797 ± 87	1565 ± 147
	Vehicle	808 ± 24	995 ± 83	1803 ± 107
	Diazepam (1 mg/kg)	892 ± 75	1162 ± 224	2053 ± 298
	Diazepam (10 mg/kg)	1112 ± 182	1332 ± 140*	2443 ± 309*
20	Control	827 ± 24	1429 ± 109	2255 ± 132
	Vehicle	833 ± 64	1483 ± 168	2317 ± 224
	Diazepam (1 mg/kg)	847 ± 54	1192 ± 132	2038 ± 136
	Diazepam (10 mg/kg)	968 ± 34	1467 ± 45	2435 ± 50
25	Control	733 ± 64	1795 ± 72	2528 ± 102
	Vehicle	753 ± 18	2038 ± 52	2792 ± 62
	Diazepam (1 mg/kg)	967 ± 107*†	2138 ± 202	3105 ± 309*
	Diazepam (10 mg/kg)	1058 ± 34*†	1947 ± 61	3005 ± 74

Values (mean ± SEM) represent total fluid consumption and fluid consumption from conception to birth (prenatal) and from birth to the testing day (postnatal) for groups studied at 10, 15, 20, and 25 days of age. Values are the means of three to four litters for each treatment group. *†Statistical comparisons within each series of treatments were made using one-way ANOVA and post hoc comparisons using Duncans New Multiple Range Test: *$p < 0.05$ vs. control untreated group, †$p < 0.05$ vs. vehicle-injected group.

were taken as the point when the tip of the tail was withdrawn from the surface of the water. Ten minutes after saline or naloxone administration, animals were stressed by placing them individually in a plastic swim tank at 20°C for a period of 3 min as previously described for young rats (14). For 10-day-old pups a swimming flotation device was used (33). At the end of the swimming period, rat pups were removed from the water, dried, and returned to the home cage before subsequent nociceptive testing. Tail immersion responses were measured at 1, 5, 10, 15, and 30 min following swimming stress. Rat pups remained with their mothers at all times up to weaning, except during drug administration, swim stress, and nociceptive testing, to minimise effects of maternal deprivation.

Drugs and Statistical Procedures

Diazepam and naloxone were gifts from Roche and Dupont Pharmaceuticals, respectively. Diazepam was dissolved by first dispersing in polyethylene glycol (6.4 g) followed by the addition of ethanol (1.6 ml) and made up to 10 ml with water. The composition of this vehicle was 16% w/v ethanol/64% w/v polyethylene glycol. Naloxone was dissolved in 0.9% saline. Nociceptive treatment groups were compared using one-way ANOVA and post hoc comparisons at each time point made by Duncans New Multiple Range Test using the SUPERANOVA software package for the Macintosh.

RESULTS

Tables 1 and 2 show the effect of chronic diazepam treatment on maternal and neonate weight gain and fluid intake during gestation and the postnatal period up to the maximum age of testing (day 25). There were no significant effects of diazepam treatment upon maternal weight gain or upon litter weight in all groups over a chronic treatment period up to postnatal day 25. There were, however, significant effects on fluid consumption (Table 2). Diazepam treatment increased fluid intake by up to 67% compared to controls, an effect that was most marked in the pre- and early postnatal periods and in part was due to increased fluid consumption due to vehicle treatment. Significant differences (up to 40%) between diazepam- and vehicle-treated groups were only observed in the measures of fluid intake for the prenatal period (Table 2).

A small level of swim SIA (peak response latency 2.4–2.7 s vs. 1.2–1.6 s) was observed in vehicle- and both diazepam-treated groups in 10-day-old rat pups (Fig. 1). In 15-day-old animals swimming stress produced a more marked antinociception in vehicle- and diazepam-treated groups (Fig. 2). In rats treated with 10 mg/kg diazepam there was more variability in the nociceptive latencies and the antinociception did not reach statistical significance. Naloxone produced some attenuation of the swim SIA but this was not significant in any groups.

In 20-day-old preweanling rats a significant level of swim SIA, which was completely reversed by naloxone, was evident in vehicle-treated rats. In diazepam-treated animals (1 and 10mg/kg) there was stress-induced antinociception but it was insensitive to reversal by naloxone (Fig. 3). In postweanling rats marked swim SIA reversed by naloxone was observed in vehicle-treated rats, but the increase in response latencies observed in animals treated with 1 mg/kg diazepam was not affected by the opioid antagonist (Fig. 4). Further, in animals

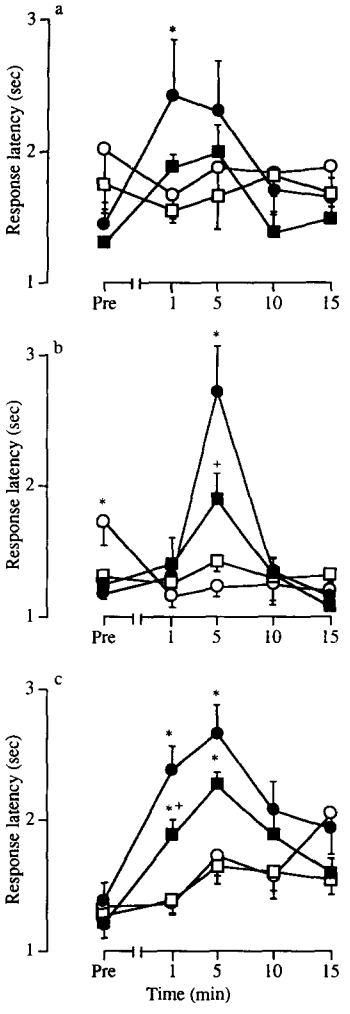

FIG. 1. Effect of chronic maternal (a) vehicle, (b) diazepam (1 mg/kg), and (c) diazepam (10 mg/kg) on tail immersion response latencies in 10-day-old rat pups. Saline treated, unstressed (○); saline treated, swim stressed (●); naloxone (10 mg/kg) treated, unstressed (□); naloxone (10 mg/kg) treated, swim stressed (■). Pre = pretest responses 10 min before swim stress. Responses at 30 min were equivalent to those at 15 min and are omitted for clarity of presentation. Values are the mean ± SEM of six to eight animals. $*p < 0.05$ vs. unstressed control; $+p < 0.05$ saline stressed vs. naloxone stressed.

treated with 10 mg/kg diazepam swim stress did not produce any significant antinociception.

DISCUSSION

Sedative and hypnotic drugs can produce maternal undernutrition as a side effect (13). Although some of the developmental exposure studies to diazepam have reported deficits in body weight gain (40,44), others at doses similar to those used in our study have shown no litter size or weight gain differences (6,12,24). The lack of overt nutritional effects of pre- and postnatal diazepam treatment in our study negates the possibility that effects on the behavioural responses result from developmental undernutrition. The lack of effect on body weight is perhaps surprising because it is well known that benzodiazepines cause hyperphagia and hyperdipsia and indeed that this effect is reversed by opioid antagonists [for reviews see (7,8)]. There was evidence of hyperdipsia in diazepam-treated animals, in part probably associated with the dipsogenic effects of alcohol used as a vehicle for the drug. The lack of body weight effects may reflect the overriding metabolic demands upon nursing mothers and accord with others who have failed to see effects of diazepam on body weight in pregnant rats (31). Also with respect to the neonates, the immaturity of benzodiazepine and opioid systems may be important; for example, effects of opioid antagonists on ingestive behaviour are not evident until 14 days (2).

In common with our previous findings in naive animals (33), vehicle-treated rats exhibit a small level of SIA as early as postnatal day 10. Further, in pre- and postweanling rats (20 and 25 days) the reversal of SIA by naloxone accords with opioid receptor mediation of this behaviour (14,20). However,

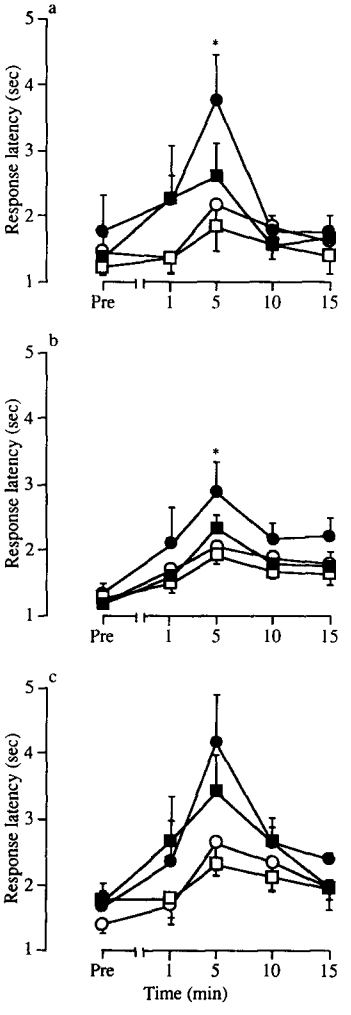

FIG. 2. Effect of chronic maternal (a) vehicle, (b) diazepam (1 mg/kg), and (c) diazepam (10 mg/kg) on tail immersion response latencies in 15-day-old rat pups. Saline treated, unstressed (○); saline treated, swim stressed (●); naloxone (10 mg/kg) treated, unstressed (□); naloxone (10 mg/kg) treated, swim stressed (■). Pre = pretest responses 10 min before swim stress. Responses at 30 min were equivalent to those at 15 min and are omitted for clarity of presentation. Values are the mean ± SEM of six to eight animals. $*p < 0.05$ vs. unstressed control.

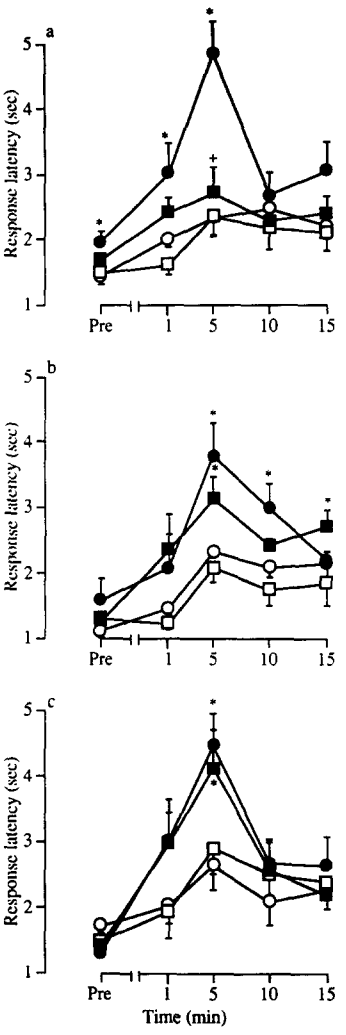

FIG. 3. Effect of chronic maternal (a) vehicle, (b) diazepam (1 mg/kg), and (c) diazepam (10 mg/kg) on tail immersion response latencies in 20-day-old rat pups. Saline treated, unstressed (○); saline treated, swim stressed (●); naloxone (10 mg/kg) treated, unstressed (□); naloxone (10 mg/kg) treated, swim stressed (■). Pre = pretest responses 10 min before swim stress. Responses at 30 min were equivalent to those at 15 min and are omitted for clarity of presentation. Values are the mean ± SEM of six to eight animals. $*p < 0.05$ vs. unstressed control; $+p < 0.05$ saline stressed vs. naloxone stressed.

in diazepam-treated animals at 20 days of age, although SIA is present its lack of reversal by naloxone suggests that nonopioid receptor mechanisms mediate this effect. This may suggest a developmental toxicity of diazepam upon opioid-mediated analgesic systems as they develop between the second and third postnatal weeks. It is an effect that persists through to the postweanling period, and indeed in the animals treated with 10 mg/kg diazepam there is a blunting of the SIA response, showing disruption of stress-mediated behaviour even when diazepam is no longer being administered. Whether the effect of chronic maternal diazepam is an action upon opioid receptor development is not possible to say at this stage, although diazepam and its major metabolite do not have affinity for μ- or κ-opioid receptors (37). However, pre- and postnatal diazepam treatment has been shown to cause a small decrease in κ-opioid receptors (45) as well as a small downregulation of benzodiazepine receptors themselves (27,38), although it should be stressed that others have failed to observe changes in benzodiazepine sites after diazepam treatment (23,31). Whether an interaction of diazepam with opioid systems is manifested in the CNS or the spinal cord is not possible to determine from the current evidence, but it should be stressed that the tail immersion response used to assess SIA is recognised to be a spinally mediated reflex.

Disruption of cholinergic, GABAergic, 5-hydroxytryptamine, and noradrenergic systems (11,18,42) after prenatal diazepam has been shown, and these have been implicated as "downstream neurotransmitter" in the mediation of SIA [see (4)]; thus, the toxic effect of diazepam does not necessarily have to reflect a direct action on opioid systems.

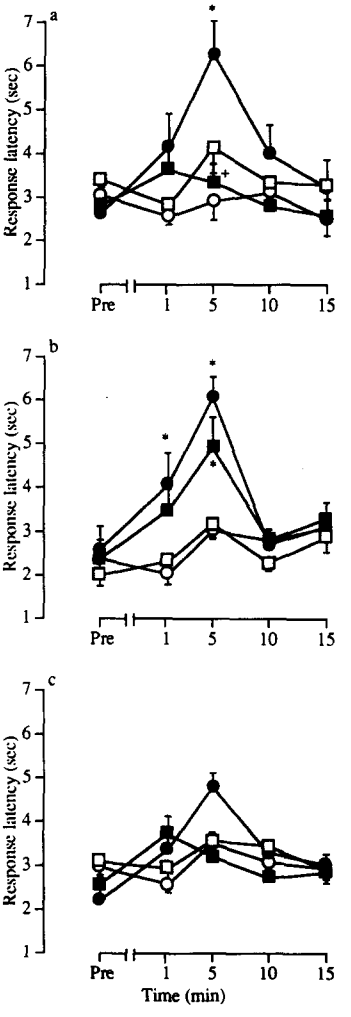

FIG. 4. Effect of chronic maternal (a) vehicle, (b) diazepam (1 mg/kg), and (c) diazepam (10 mg/kg) on tail immersion response latencies in 25-day-old rat pups. Saline treated, unstressed (○); saline treated, swim stressed (●); naloxone (10 mg/kg) treated, unstressed (□); naloxone (10 mg/kg) treated, swim stressed (■). Pre = pretest responses 10 min before swim stress. Responses at 30 min were equivalent to those at 15 min and are omitted for clarity of presentation. Values are the mean ± SEM of six to eight animals. $*p < 0.05$ vs. unstressed control; $+p < 0.05$ saline stressed vs. naloxone stressed.

Specific acute interactions of benzodiazepines upon opioid-mediated SIA have been shown in the adult. Benzodiazepine agonists inhibit and benzodiazepine antagonists potentiate foot shock-induced analgesia (39) and both benzodiazepine ligands inhibit antagonism of SIA by naloxone. This observation is consistent with our chronic ontogenetic study that shows diazepam treatment blocks naloxone reversal of SIA, and in the adult Rovati et al. (39) have shown diazepam reverses stress-induced downregulation of opioid binding sites labelled with [^{3}H]naloxone. It should be noted, however, that the nature of the stressor is important in determining the effect of the benzodiazepines, as cold water swim stress analgesia is potentiated by diazepam (26) and tail shock analgesia can be unaffected or reduced by diazepam, dependent on the duration of shock (28). Further, Alleva et al. (1) showed prenatal oxazepam exposure in mice had no effect on morphine analgesia in neonates but delayed morphine hyperactivity, thus suggesting selective effects of benzodiazepines on opioid-mediated behaviours. We can now add that stress-induced analgesic responses are modified by benzodiazepine treatment and this differential effect on analgesic mechanisms accords with Bodnar et al. (5), who showed chronic pretreatment of adult rats with chlordiazepoxide attenuated cold water swim antinociception without affecting morphine analgesia.

In conclusion, maternal exposure to diazepam modifies opioid-mediated SIA, further confirming the interrelationship between opioid and benzodiazepine systems and showing that maternal diazepam exposure causes opioid system dysfunction.

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