

RESPIRATORY μ -OPIOID AND BENZODIAZEPINE INTERACTIONS IN THE UNRESTRAINED RAT

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Summary—Interactions of μ -opioid receptors with the benzodiazepine system were studied by examining the modulatory effects of flumazenil (a benzodiazepine antagonist) and alprazolam (a benzodiazepine agonist) on the respiratory effects of the opioid peptide dermorphin. Dermorphin, 1–30 nmol administered i.c.v., to conscious, unrestrained rats decreased ventilation rate (VR) and minute volume (MV) dose-dependently. The ventilatory depression was antagonized by naloxone and by the benzodiazepine antagonist flumazenil. The benzodiazepine alprazolam potentiated the respiratory inhibition of a small (1 nmol) dose of dermorphin but antagonized that of a higher dose (3 nmol). The results suggest that the benzodiazepine/GABA receptor complex modulates respiratory depression induced by central μ -receptor stimulation in the rat.

Key words—dermorphin, opioid μ receptors, opioid–benzodiazepine interactions, respiration, flumazenil, benzodiazepine antagonist.

Dermorphin is a heptapeptide (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH₂) originally extracted from the frog skin (Montecucchi, De Castiglione, Piani, Gozzini and Erspamer, 1981). As a potent and selective opioid μ -receptor agonist (Rossi, De Castiglione and Perseo, 1985; Krumins, 1987; Lazarus, Guglietta, Wilson, Irons and De Castiglione, 1989) it depresses respiration (Feuerstein and Faden, 1983; Paakkari, Paakkari, Sirén and Feuerstein, 1990).

γ -Aminobutyric acid (GABA) modulates various opioid effects, as shown in studies describing the interactions of GABA or benzodiazepine agonists and antagonists with opioid-induced analgesia (Zonta, Zambotti, Vincentini, Tammiso and Mantegazza, 1981; Mantegazza, Parenti, Tammiso, Vita, Zambotti and Zonta, 1982; Andree, Kendall and Enna, 1983; Brady, Mansbach, Skurdal, Muldoon and Barrett, 1984; Zambotti, Zonta, Tammiso, Conci, Hafner, Ferrario, Zecca and Mantegazza, 1987). The intestinal effects of morphine (Fioramonti, Fargeas and Bueno, 1987) and the cataleptic effect of dermorphin (Paakkari and Feuerstein, 1988) were reversed by the selective benzodiazepine receptor antagonist flumazenil (Hunkeler, Möhler, Pieri, Polc, Bonetti, Cumin, Schaffner and Haefely, 1981). On the other hand, various effects of benzodiazepines—e.g. effects on conflict or feeding and drinking behavior (Lorens and Sainati, 1978; Duka, Cumin, Haefely and Herz, 1981;

Cooper, 1983) and on growth hormone secretion (Huupponen, Koulu, Pihlajamäki and Mäkinen, 1986)—have been attenuated by the opiate antagonist naloxone.

In the present study the selective benzodiazepine antagonist flumazenil or the benzodiazepine alprazolam were given prior to dermorphin to investigate whether opioid and benzodiazepine receptors also interact in mediating the respiratory effects of this μ -opioid agonist peptide. The experiments were conducted in conscious, freely moving rats to avoid the effects of anaesthesia and restraint on respiration.

METHODS

Conscious male Sprague–Dawley rats (250–330 g, Taconic Farms, Germantown, New York) were used. The animals received standard rat pellets and water *ad libitum* and were kept at 22°C and 12 hr light–dark cycle.

Procedures for drug administration

Rats were anesthetized with an intramuscular injection of ketamine (130 mg/kg) and acepromazine (1.3 mg/kg). A stainless steel guide cannula was stereotaxically inserted (DKI, California) through the skull into the right lateral brain ventricle (i.c.v.) and fixed with glue (Eastman 910 adhesive). Coordinates for the lateral ventricle were AP = –0.8 mm and L = 1.2 mm. After the operation the rats were allowed to recover 2–3 days. On the day of the

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experiment, a 7.5 mm long 30 g cannula was inserted into the ventricular space through the guide cannula. The injection cannula was connected via polyethylene tubing to a Hamilton microliter syringe and the drug solution was injected over a period of 20 sec in a volume of 10 μ l. The proper position of the i.c.v. cannula was ascertained at the end of the experiment by dissection of the brain after an injection of methylene blue (10 μ l).

Recording of respiration

Ventilation rate (VR) and relative ventilation tidal volume (TV) were monitored by the Oxymax 85 system (Columbus Instruments, Columbus, Ohio). The recording system consists of 2 sealed plexi-glass test chambers and one reference chamber (internal dimensions: 112 \times 197 \times 297 mm, volume: 6.55 liter). Room air is pumped through each chamber at the constant rate of 2 l/min. Recording of the relative tidal volume and respiration rate is based on the minute pressure changes in the chamber caused by the subject's breathing. Tidal volume and calculated minute volume (MV) are expressed as arbitrary units.

The animals were allowed a 60 min of adaptation to the Oxymax plexiglass chamber prior to drug or vehicle administration. The respiratory data were gathered at 2 min intervals and averages of 5 consecutive recordings were calculated. The animals received the pretreatments (alprazolam, flumazenil or naloxone) 20 min prior to the i.c.v. administration of dermorphin. To minimize the interference of the animal handling on the respiratory results only the data prior to the pretreatment and those 30–50 min after the last drug injection were used. The base lines of the respiratory variables were calculated as averages of 5 consecutive measurements, i.e. 10 min period before the drug injection.

Drugs

Dermorphin (Sigma Chemical Company) and naloxone (kindly provided by DuPont Pharmaceuticals, Wilmington, Delaware) were dissolved in 0.9% NaCl. Flumazenil (gift from W. Haefely, Hoffmann-LaRoche Inc., Basel, Switzerland) was dissolved in 10% Emulphur 620 (GAF corp.)–ethanol mixture (1:1), warmed and sonicated immediately before i.p. administration. Alprazolam (Upjohn Co., Kalamazoo, Michigan) was dissolved in a solution of 10% ethanol, 40% propyleneglycol and 50% of 0.9% NaCl. Naloxone, flumazenil and alprazolam were administered i.p. in a volume of 0.3 ml 20 min before dermorphin injection when used as pretreatment.

Statistics

The data in text and figures are mean values \pm SE for 6–8 rats in each group. Some results are expressed as areas under the curves and the *x*-axes for the period of 30–50 min after drug injections (area scores). For calculation of area scores the trapezoidal method i.e. geometric approximation by means of

rectangles and triangles was used. The homogeneity of variances was tested with Bartlett's test. Groups with variances not significantly unequal were tested with one-way analysis of variance (ANOVA) followed by the multiple comparison test of Newman-Keul. Repeated observations within the same groups were analyzed with two-way ANOVA for repeated measures. For comparisons of repeated measurements within a group *t*-test for dependent means was used. For groups with significantly unequal variances the Kruskal-Wallis test was used. In case of a significant Kruskal-Wallis *P*-value the Dunn procedure was used for comparisons between several means.

For calculations the CSS: Statistical Software Package (StatSoft Inc., Tulsa, Oklahoma, release 3.0 C) for personal computers was used.

RESULTS

Effects of dermorphin i.c.v. on ventilation

Dermorphin 1, 3 and 30 nmol dose-dependently decreased ventilation rate (VR) to $74 \pm 14\%$ ($P < 0.10$), $54 \pm 9\%$ ($P < 0.01$) and $35 \pm 6\%$ [$P < 0.01$, dependent *t*-test, Figs 1(A), 2(A) and 3(A)] from the initial level, respectively. The corresponding effects on ventilation minute volume (MV) were $103 \pm 19\%$ ($P = 0.98$), $46 \pm 6\%$ and $41 \pm 7\%$ from the initial level, respectively [Figs 1(C), 2(C) and 3(C)]. The dermorphin-induced effects on ventilatory tidal volume (TV) were variable including increases at doses 1 or 30 nmol/kg (maxima: $131 \pm 14\%$ and $130 \pm 14\%$, both $P < 0.05$) and a negligible effect at 3 nmol/kg ($104 \pm 16\%$, $P = 0.40$, Fig. 4). Figures 1(B), (D), 2(B), (D), 3(B) and (D) show the corresponding area scores from samples 94–144 min post injection.

Interaction of naloxone and dermorphin 1 nmol

Pretreatment with naloxone (5 mg/kg, i.p.) antagonized the dermorphin-induced decrease in VR (Fig. 1). Likewise the concomitant increase in TV was attenuated by naloxone [Fig. 4(A)]. As could be expected, naloxone did not modify the negligible effect of dermorphin 1 nmol on MV (Fig. 1).

Interaction of alprazolam and dermorphin

Pretreatment with alprazolam (1 mg/kg, i.p.) did not alter the bradypneic effect of dermorphin 1 nmol while the dermorphin-induced increase in TV was completely abolished (Figs 1 and 4). Consequently, the combination alprazolam plus dermorphin resulted in a significant decrease in MV [Fig. 1(C) and (D)]. At dermorphin 3 nmol the alprazolam pretreatment (1 mg/kg, i.p.) slightly attenuated the dermorphin induced decrease in VR [Fig. 2(A) and (B)]. As the result of this and the concomitant slight increase of TV in the pretreatment group, the net effect alprazolam plus dermorphin combination on MV

was attenuated as compared to dermorphin alone [Fig. 2(C) and (D)].

Interaction of flumazenil and dermorphin

At dermorphin dose of 1 nmol the pretreatment with flumazenil (5 mg/kg, i.p.) clearly reverted the decrease in VR by dermorphin alone (about -80% from baseline) to an increase of about 110% from baseline [Fig. 1(A)]. Concomitantly the dermorphin-induced increase in TV was reverted to a slight negative shift [Fig. 4(A)] resulting in MV to a net effect close to the baseline and not significantly different from dermorphin alone [Fig. 1(C) and (D)].

Flumazenil antagonized the bradypneic effect of dermorphin 3 nmol [Fig. 2(A) and (B)] while neither dermorphin alone or flumazenil plus dermorphin significantly changed TV [Fig. 4(B)]. As a net effect flumazenil pretreatment antagonized the dermorphin induced decrease in MV. Dermorphin alone decreased MV by about 50% while after flumazenil pretreatment the decrease was 30%.

Flumazenil almost completely abolished the strong bradypneic effect of dermorphin 30 nmol [Fig. 3(A) and (B)] while increase in TV by dermorphin was slightly increased (Fig. 4). As the result MV was not

significantly altered in reference to the base line in the presence of the pretreatment in contrast to the strong decrease by dermorphin alone to about 40% of the initial level.

DISCUSSION

The strong respiratory depressant effect of dermorphin was abolished by flumazenil, a specific benzodiazepine antagonist which does not revert the effects of other central nervous depressants such as barbiturates, meprobamate, ethanol or valproate (Bonetti, Pieri, Cumin, Schaffner, Pieri, Gamzu, Muller and Haefely, 1982; Barrett, Brady and Witkin, 1985; Masur, Silva-Filho, de Souza and Pires, 1987). It has been reported previously that flumazenil antagonized the antinociceptive (Brady *et al.*, 1984) and intestinal (Fioramonti, Fargeas and Bueno, 1987) effects of centrally administered morphine or DAMGO, and it prevented also the cataleptic effect of dermorphin (Paakkari and Feuerstein, 1988). However, in contrast to our present results, flumazenil did not antagonize the respiratory depressant effect of i.v. injected morphine in rabbits (Bonetti *et al.*, 1982). The discrepancy may be due to the different species,

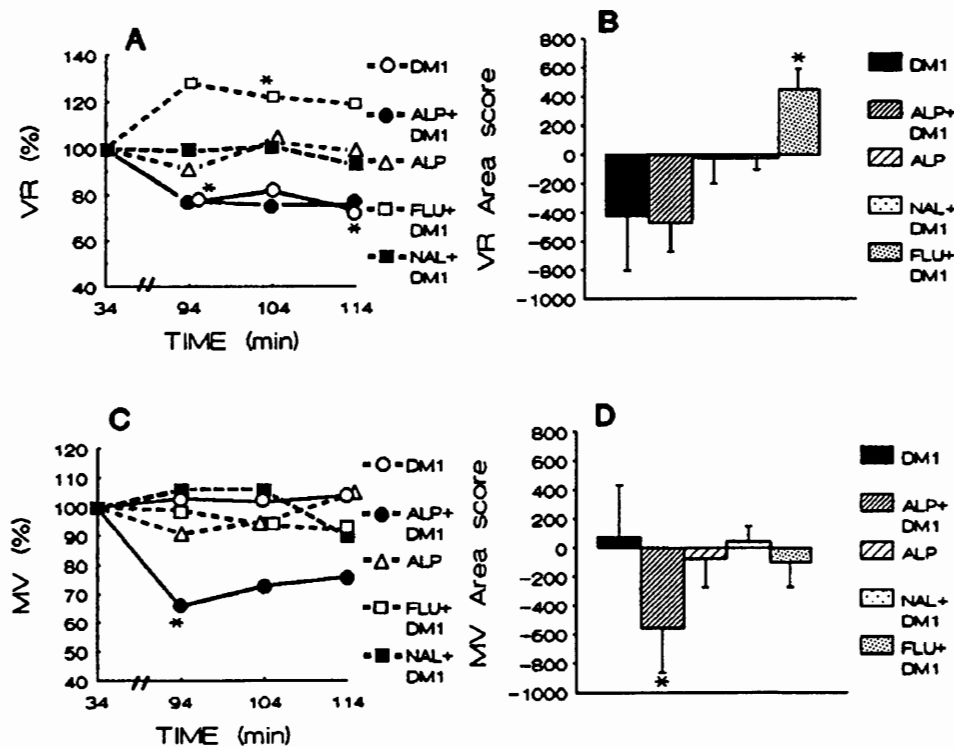


Fig. 1. Effect of dermorphin 1 nmol, i.c.v. (DM1) on ventilation rate (VR) and ventilation minute volume (MV). ALP = alprazolam, NAL = naloxone, FLU = flumazenil. (A) Time-course of the changes in VR after treatment. Two-way ANOVA for repeated measures: group $P < 0.001$, time $P = 0.24$, interaction $P < 0.05$. * $P < 0.05$ when compared to the base line, dependent t -test. (B) VR area scores between 94–114 min after treatment. One-way ANOVA between the groups: $P < 0.05$, * $P < 0.05$ DM1 vs FLU + DM1 (Dunnett's test). (C) Time-course of the changes in MV after treatment. Two-way ANOVA for repeated measures: group $P = 0.35$, time $P = 0.48$, interaction $P < 0.32$. * $P < 0.05$ when compared to the base line, dependent t -test. (D) MV area scores between 94–114 min after treatment. One-way ANOVA between the groups $P = 0.37$, * $P < 0.05$ DM1 vs all other treatments (Contrast analysis).

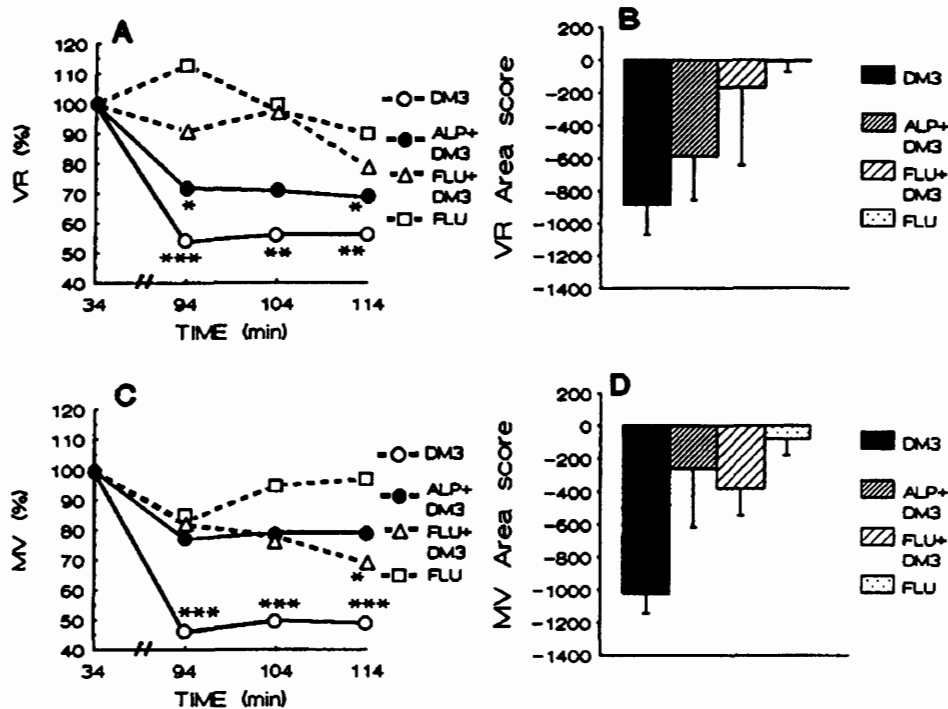


Fig. 2. Effect of dermorphin 3 nmol, i.c.v. (DM3) on ventilation rate (VR) and ventilation minute volume (MV). ALP = alprazolam, FLU = flumazenil. (A) Time-course of the changes in VR after treatment. Two-way ANOVA for repeated measures: group $P < 0.369$, time $P < 0.001$, interaction $P < 0.087$. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ when compared to the base line (dependent t -test). (B) VR area scores between 94–114 min after treatment. Kruskal–Wallis ANOVA $P = 0.098$, Median test $P < 0.05$. (C) Time-course of the changes in MV after treatment. Two-way ANOVA for repeated measures: group $P = 0.076$, time $P < 0.001$, interaction $P < 0.05$. * $P < 0.05$, *** $P < 0.001$ when compared to the base line (dependent t -test). (D) MV area scores between 94–114 min after treatment. Kruskal–Wallis ANOVA $P < 0.05$, Median test $P < 0.01$, $P < 0.05$ DM3 vs all other treatments (Contrast analysis).

or the use of different method in measuring the respiration. In the rabbit flumazenil–morphine study, the animals were mechanically immobilized and a mask with a pressure transducer was attached to the snout. In the present experiment the recordings were made from freely moving uninstrumented animals utilizing a fully automated, non-invasive system. Accordingly, the present results would reflect the opioid regulation of respiration in the absence of any major physical constraint. The assumption that changing the methodology may change the results is supported by the studies of Ward and Takemori (1983). In their study, β -funaltrexamine antagonized morphine-induced respiratory depression when the respiratory rate was recorded by body pletysmograph, whereas there was no antagonism when the rats were immobilized and the respiratory rate was counted by placing the snout of the mouse into the barrel of a syringe connected to a pressure transducer.

The interactions between benzodiazepine and opioid agonists in producing analgesia are complicated. Benzodiazepines have antagonized (Mantegazza *et al.*, 1982; Abott and Franklin, 1986) and enhanced (Moreau and Pieri, 1988; Bergman, Wynn, Peterson and Rudo, 1988) the antinociceptive effects of opi-

oids. Alprazolam had likewise dual effects on dermorphin-induced respiratory depression in the present study. It potentiated the decrease in respiratory minute volume induced by the lowest dose of dermorphin, but the effect of higher doses of dermorphin on all respiratory parameters was attenuated. The cataleptic effect of high doses of dermorphin (Paakkari and Feuerstein, 1988) may decrease the respiratory movements of the thorax and thus augment the respiratory depression. The well known muscle relaxing effect of benzodiazepines may partly explain the antagonistic effect of alprazolam.

Recently Rattan and coworkers (1991) reported an antinociceptive benzodiazepine–opioid interaction that is similar to our results. In the rat tail-flick test, midazolam (10 μg , i.t.) potentiated the antinociceptive effect of a low dose (10 μg , i.t.) of morphine, but antagonized the effect of higher doses (20 or 30 μg). In binding studies in rat spinal cord homogenates, the authors found that midazolam at a low dose potentiated the displacement of [^3H]naloxone by morphine whereas at higher doses midazolam inhibited the morphine-induced displacement of [^3H]naloxone. They suggest that in the presence of low concentrations of a benzodiazepine opioid receptors are

modulated in such a fashion that they have a higher affinity for morphine. This type of interaction could theoretically explain the dual effects of alprazolam in our studies. However, the binding studies were done in spinal receptors whereas we gave the opioids i.c.v. and alprazolam i.p.

The close relationship between opioid and benzodiazepine receptors is previously emphasized by the discovery of substituted 1,4-benzodiazepines which have no affinity for benzodiazepine binding sites but bind selectively on opiate receptors and act pharmacologically as opiates (Römer, Büscher, Hill, Maurer, Petcher, Zeugner, Benson, Finner, Milkowski and Thies, 1982). Furthermore, β -carbolines or harmalines had affinity for both benzodiazepine and opiate receptors (Airaksinen and Mikkonen, 1980). The pure benzodiazepine ligand β -carboline, 3-hydroxymethyl- β -carboline reversed the cerebral metabolic and hypnotic effects of flurazepam (Mendelson, Cain, Cook, Paul and Skolnick, 1983; Hoffmann, Feld, Larscheid, Cook, Albrecht and Miletich, 1985) as well as respiratory depression induced by the opiate fentanyl (Naughton, Hoffman, Larscheid, Cook, Albrecht and Miletich, 1985). Benzodiazepines also influence opiate receptor ontogeny: prenatal exposure

to diazepam decreased opiate binding sites in rat brain at 14 days of age (Watanabe, Shibuya, Salafsky and Hill, 1983).

On the basis of our *in vivo* results it is impossible to determine whether the opioid-benzodiazepine interactions observed are due to allosteric modulation of opioid binding by benzodiazepines, or whether the opioid and benzodiazepine/GABAergic pathways intervene physiologically. Interaction between these systems was implied by Waldrop, Eldridge and Millhorn (1983), who suggested that the depression of breathing that follows hindlimb muscle stimulation is mediated by endogenous opiates and GABA.

The respiratory depressant effect of barbiturates may, at least partly, be due to their enhancing effect on GABAergic transmission (Olsen 1981), since GABA and GABAergic drugs are known to produce ventilatory depression (Yamada, Hamosh and Gillis, 1981; Hedner, Hedner, Wessberg and Jonason 1984; Da Silva, Hartley, Hamosh, Quest and Gillis, 1987). A barbiturate-GABA-opioid interaction was reported by Suria, Nasreen and Saeed (1988), who found that in the rat thiopental-induced respiratory depression was associated with an increase of GABA

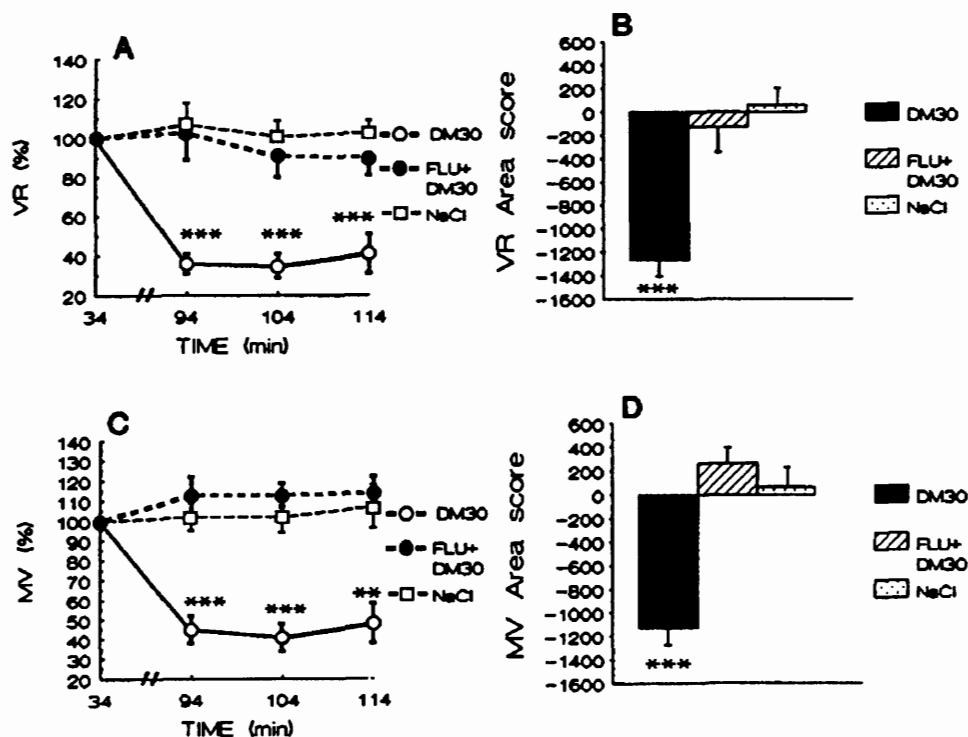


Fig. 3. Effect of dermorphin 30 nmol, i.c.v. (DM30) on ventilation rate (VR) and ventilation minute volume (MV). FLU = flumazenil. (A) Time-course of the changes in VR after treatment. Two-way ANOVA for repeated measures: group $P < 0.001$, time $P < 0.001$, interaction $P < 0.001$. *** $P < 0.001$ when compared to the base line, dependent t -test. (B) VR area scores between 94–114 min after treatment. ANOVA $P < 0.001$, *** $P < 0.001$ DM30 vs FLU + DM30 or NaCl, Newman-Keul test. (C) Time-course of the changes in MV after treatment. Two-way ANOVA for repeated measures: group $P = 0.001$, time $P < 0.01$, interaction $P < 0.001$. ** $P < 0.01$ and *** $P < 0.001$ when compared to the base line, dependent t -test. (D) MV area scores between 94–114 min after treatment. ANOVA $P < 0.001$, *** $P < 0.001$ DM30 vs FLU + DM30 or NaCl.

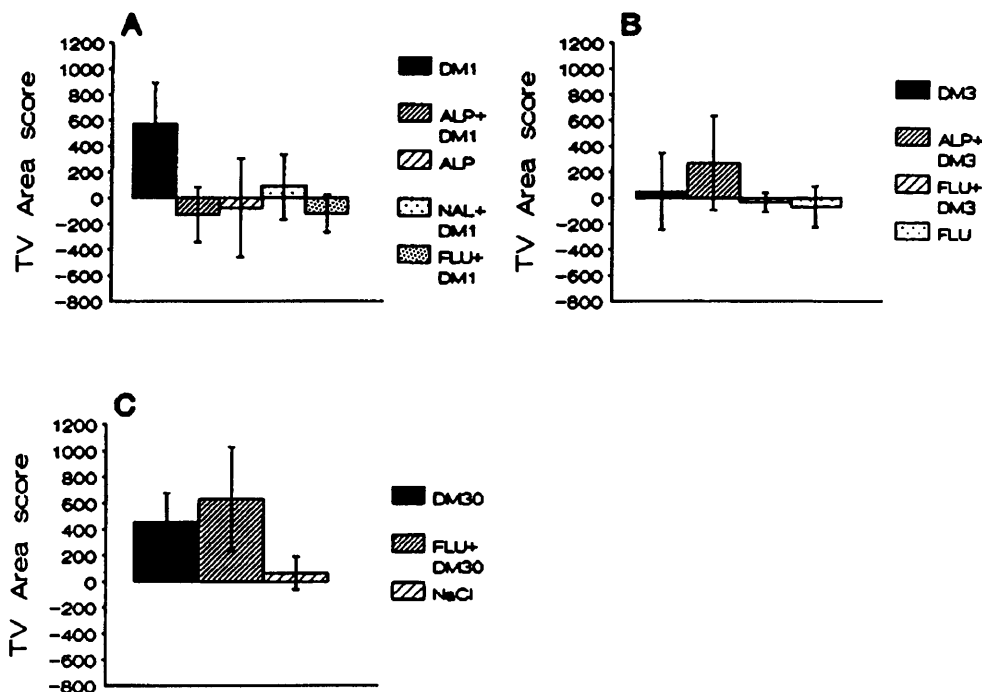


Fig. 4. Effect of dermorphin 1, 3 or 30 nmol, i.c.v. (DM1, DM3 or DM30) on ventilation tidal volume (TV) expressed as area scores between 94–114 min after treatment. ALP = alprazolam, FLU = flumazenil, NAL = naloxone. (A) DM1 $P = 0.33$. (B) DM3 Kruskal–Wallis ANOVA $P = 0.76$. (C) DM30 ANOVA $P = 0.27$.

in brain stem, and naloxone 2.5 mg/kg reversed both the respiratory paralysis and the increase of GABA levels. The strong antagonistic effect of flumazenil on dermorphin-induced respiratory depression suggests that benzodiazepine/GABAergic receptors and/or pathways take part also in opioid induced respiratory depression, analogous to interactions implied in analgesic, gastrointestinal and cataleptic actions of opioids.

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