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INVESTIGATIONS ON DYNORPHIN STRUCTURALLY-RELATED OPIOID PEPTIDES. I. IMPACT ON NOCICEPTIVE TRANSMISSION. II. REGULATION OF PITUITARY CONTENT

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Summary. – *In the present work the effects have been investigated of a series of pro-dynorphin derived peptides, intrathecally administered to rats: dynorphin 1-17 (Dyn A), dynorphin 1-32 (Dyn 1-32), dynorphin B (Dyn B). In addition, investigations have been carried out on pituitary dynorphinergic system, by pharmacological and surgical manipulations, in order to obtain information on mechanism(s) that regulate its storage and possible biological functions. Studies at spinal level have shown an influence of these peptides on rat response to aversive stimuli and on motor function of the animal. Both alterations induced by dynorphin-related peptides are mediated by an opioid receptor of the k-type since they are antagonized by a pre-treatment with a k-antagonist (MR 1452) and, moreover, a cross-tolerance to these effects is shown with the relatively pure k-agonist, ethylketocyclazocine. However, different mechanism(s), possibly non opioid in nature, may contribute to the prolonged depression of motor function, since the k-receptor blocker when administered* after *the peptide, only normalized the vocalization threshold. Concerning investigations on pituitary dynorphinergic system, a reduction of the neurohypophyseal pool of ir-dyn was observed in rats radiofrequency lesioned in the supraoptic, paraventricular, anterior hypothalamic nucleus as well as in the medial basal hypothalamus. A similar decrease of ir-dyn content in the anterior lobe (AL) was ascertained only after lesion of the medial basal hypothalamus. On the contrary, an increase occurred in AL ir-dyn levels following ovariectomy, an effect reversed by estrogens.*

Riassunto (Indagini sui peptidi oppioidi strutturalmente correlati alla dinorfina. I. Impatto sulla trasmissione nocicettiva. II. Regolazione del contenuto ipofisario). – *Sono stati indagati gli effetti sulla trasmissione nocicettiva e sulla funzione motoria di una serie di peptidi derivanti dalla prodinorfina: dinorfina 1-17 (Dyn A), dinorfina 1-32 (Dyn 1-32), dinorfina B (Dyn B), somministrati per via intratecale (i.t.) nel ratto. Parallele manipolazioni farmacologiche e chirurgiche hanno consentito di ottenere informazioni sui meccanismi di regolazione del sistema dinorfinergico ipofisario e sulle sue possibili funzioni biologiche. I peptidi in esame influenzano, a livello spinale, sia la trasmissione nocicettiva sia la funzione motoria. Ambedue gli effetti provocati dai peptidi dinorfino-simili sono mediati da un recettore di tipo k, dal momento che sono antagonizzati dal pretrattamento con un k-antagonista (MR 1452) e non si manifestano, inoltre, in animali resi tolleranti ad un agonista relativamente selettivo per il recettore k quale l'etilchetociclazocina. Tuttavia, poiché il k-antagonista MR 1452, somministrato* dopo *il peptide Dyn A, è in grado di rimuovere solamente gli effetti a carico della soglia nocicettiva, è possibile che differenti meccanismi, probabilmente non oppioidi, contribuiscano alla prolungata depressione della funzione motoria. Le indagini riguardanti il sistema dinorfinergico ipofisario hanno evidenziato come l'immunoreattività dinorfino-simile (ir-dyn) della neuroipofisi venga ridotta dalla lesione a radiofrequenza dei nuclei ipotalamici sopraottico e paraventricolare nonché dell'ipotalamo anteriore e mediobasale. Per quanto concerne l'adenoipofisi (AL), si osserva diminuzione della ir-dyn solamente dopo la lesione dell'ipotalamo mediobasale. Al contrario, l'ovariectomia si traduce in un aumento dell'ir-dyn nella sola AL: il fenomeno è prevenuto dalla somministrazione di estrogeni.*

Introduction

During the past few years a series of new opioid peptides have been detected in the central nervous system of laboratory animals.

In actual fact, after the isolation and identification of dynorphin A (Dyn A) [1, 2] other sequences endowed with opioid activity, structurally-related to Dyn A, have been found to derive from the same precursor, prodynorphin [3]. So far, at least five dynorphin-related peptides have been recognized: α- and β-neo-endorphin, dynorphin 1-8, dynorphin B and leumorphin (or dynorphin B-29) [4]. In addition, two larger dynorphins have been isolated, dynorphin (1-32) comprising Dyn A and dynorphin B (Dyn B) and dynorphin (1-24) which contains the structure of Dyn A and that of Leu-enkephalin [5].

By means of radioimmunological and immunohistochemical methods, a widespread distribution of dynorphin-related peptides has been described throughout the central nervous system of rats [6-9] and humans [10]. At this regard, one of us has previously reported the presence of immunoreactive-dynorphin (ir-dyn) in rat peripheral tissues and plasma [11]. High ir-dyn levels have been found to occur in pituitary and accumulating evidence suggests the existence of two separate pools in anterior and neurointermediate lobes, characterized by different molecular forms [12, 13]; however little is known about their regulation and possible functions.

In vitro studies have shown that prodynorphin-derived peptides behave like k-opioid receptor agonists, Dyn A being the most potent [14].

The intracerebroventricular administration of Dyn A and structurally-related peptides has been shown to induce hypothermia [15] reduction of gastric secretion [16] and catalepsy but, in spite of its very potent opioid activity *in vitro*, only very weak analgesia [17]; on the other hand, following intrathecal administration, Dyn A is able to induce elevation of nociceptive threshold and, further, motor impairment [18-20].

In recent papers we have reported that these effects are still present in morphine-tolerant rats [21] and, moreover, are better prevented by k-agonists than by naloxone [22].

In the present study we have extended our investigations with respect to: 1) the effects on nociceptive transmission of Dyn A and structurally-related peptides administered at spinal level and 2) the mechanism(s) that regulate the pituitary content of immunoreactive-dynorphin, by means of pharmacological and/or surgical manipulations.

Materials and methods

Experiments on antinociception

Animals. - Male Sprague-Dawley rats (300-350 g) were used. They were chronically implanted with intrathecal (i.t.) catheters in the subarachnoid space, using a modification of the method of Yaksh and Rudy [23].

Briefly, under sodium pentobarbital (40 mg/kg i.p.) anesthesia, a polyethylene tubing (PE 10, 0.61 mm o.d.) was inserted through an incision of the atlanto-occipital membrane and entered 8.5 cm down to the rostral edge of the lumbar enlargement. A loose overhand knot fixed with dental cement placed 8.5 cm from the tip was used to prevent further entering of the catheter into the subarachnoid space; after insertion, 10 µl of vehicle were injected to clear the catheter, then the wound was closed. Behavioral testing was delayed until 7-10 days after surgery; only rats showing no evidence of spinal damage were used. At the end of experiments, the rats were randomly injected with 10 µl of dye (Blue Evans) and sacrificed to verify the correct position of the catheter.

Assessment of antinociceptive activity. - The animals' responsiveness to noxius stimuli was determined using the tail flick [24] and the vocalization [25] tests. In the tail flick test the strength of the radiant heat was adjusted so as to obtain a tail flick latency of 2-4 s in control animals and values were recorded by an automated device; a cut-off time of 10 s was used. For determination of vocalization threshold (measured in mA), two stainless steel 30 gauge electrodes were inserted in the middle section of the tail and electrical stimulation consisted in the application of trains of 1 s duration, containing 125 shocks of 1.6 ms width delivered from a high-frequency square wave constant current generator. The maximal intensity of the current delivered was 2 mA. Individual baseline tail flick latency and vocalization threshold were determined in three pre-tests 20 min before drug administration.

Surgical lesions

Animals. - Male Sprague-Dawley rats (200-250 g) were used; they were anesthetized and positioned in a Kopf small animal stereotaxic apparatus. Bilateral radiofrequency lesions were performed by nickel-chrome electrodes insulated with epoxylite except for the tip, according to the Cushman stereotaxic coordinates: medialbasal hypothalamus: anterior (−) 0.2, lateral (±) 0.8, depth (−) 9.5; supraoptic nucleus: anterior (+) 1.2, lateral (±) 2.2, depth (−) 9.2; paraventricular nucleus: anterior (+) 0.6, lateral (±) 3.0, depth (−) 8.5, < 19°; anterior hypothalamus: anterior (+) 1.0, lateral (±) 0.7, depth (−) 9.1.

Sham lesions were performed in an identical manner with unactivated electrodes. Histological examinations, carried out in some animals, ascertained the correct position and size of lesions.

For a separate set of experiments two groups (8 animals each) of female rats (200-220 g, Sprague-Dawley strain) were ovariectomized (OVX) by lumbar approach, under ether anesthesia; a third group was sham operated to serve as controls. Two weeks after surgery one OVX group received β-estradiol-3-benzoate (EB) implants for 7 days. The EB was administered via Silastic tube implants [26]; briefly,

under ether anesthesia, 5 mm-long Silastic tubing (no. 602-235, Dow Corning, Midland, MI) containing EB was inserted through an incision in the skin of the back into a s.c. pocket. The other group of OVX rats was implanted with empty tubes. After a week all animals were killed by decapitation, their pituitary glands removed and processed as reported (see below).

Determination of immunoreactive dynorphin. - Rats were killed by decapitation and pituirary glands were divided into anterior (AL) and neurointermediate (NIL) lobes within two minutes. Immunoreactive-dynorphin in pituitary extracts was determined as described previously [11] by means of the high-titer sensitive antiserum "Lucia" (kindly supplied by Dr. A. Goldstein) with full cross reactivity to dynorphin (1-17), dynorphin (1-24), dynorphin (1-32) and negligible cross- reactivity to dynorphin (1-8).

Drugs

The following drugs were used: Dyn A, Dyn B, dynorphin (1-32) (Dyn 1-32), des-tyr-dynorphin A (des-tyr-Dyn A) (Bachem Feinchemikalien, Bubendorf, Switzerland); ethylketocyclazocine methanesulfonate (EKC) (gift of Sterling Winthrop), and (−)-N-(3-furylmethyl)-α-normetazocine methanesulfonate (MR 1452) (gift of Boehringer Ingelheim). These drugs, dissolved in an osmotically balanced solution adopted as vehicle [22], were intrathecally (i.t.) injected in a volume of 15 µl; the catheter was cleared by subsequent injection of 8 µl of vehicle.

β-estradiol-3-benzoate (Sigma Chemical Co., St. Louis, MO) was administered by Silastic tubing (see Animals).

Results

Experiments on antinociception - Fig. 1 shows the effects of the i.t. administration of dynorphin structurally-related peptides, as assessed by the tail flick (top panel) and vocalization (bottom panel) tests, together with the results obtained with 25 nmol/rat i.t. Dyn A as a comparison.

As previously reported [27] this dose of Dyn A induced a short- lasting elevation of vocalization threshold associated with hindlimb paralysis and tail flaccidity which caused a maximal elevation of tail flick latencies lasting the entire period of observation.

Dyn 1-32 elicited, at the dose of 25 nmol, effects of the same intensity but longer duration than Dyn A, whereas Dyn B appeared to be less potent than Dyn A both in the elevation of nociceptive threshold and in the production of motor impairment. As regards these parameters, significant effects were observed only with a dose of 100 nmol Dyn B.

Only a very high dose of des-tyr-Dyn A (100 nmol/rat) was able to reproduce the effects of Dyn A (Fig. 1).

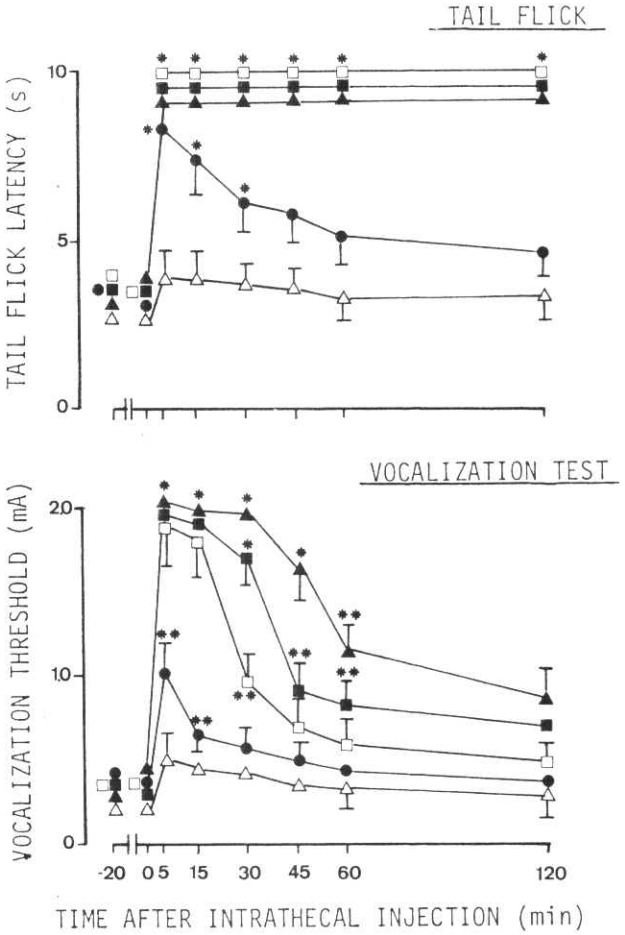

Fig. 1. - Effects of intrathecal dynorphin-related peptides on tail flick (top) and vocalization threshold (bottom).

□ Dyn A 25 nmol
▲ Dyn 1-32 25 nmol
● Dyn B 100 nmol
■ Des-tyr-Dyn A 100 nmol
△ Des-tyr-Dyn A 25 nmol
* $p < 0.01$, ** $p < 0.05$ vs pre-drug values

The purported k-antagonist MR 1452 (30 nmol/-rat) i.t. administered before Dyn A (25 nmol/rat), fully prevented the effects of the peptide in both tests adopted (Fig. 2); however, when administered 5 min *after* Dyn A, only the elevation of the vocalization threshold was antagonized while tail flick latencies remained unmodified (Fig. 2). The administration of Dyn A (25 nmol/rat) in rats chronically i.t. treated with the putative k-ligand EKC (100 µg/rat i.t. twice daily for 7 days) was no longer able either to increase the nociceptive threshold or to induce motor impairment (Fig. 3).

Effects of surgical and pharmacological manipulations on the pituitary content of ir-dyn - As shown in Table 1, radiofrequency lesions of the supraoptic and paraventricular nuclei or in the anterior hypothalamic area reduced the ir-dyn content of the NIL only. On the contrary, rats bearing lesions of the medial basal hypothalamus displayed a depletion of immunoreactivity in both the pituitary lobes.

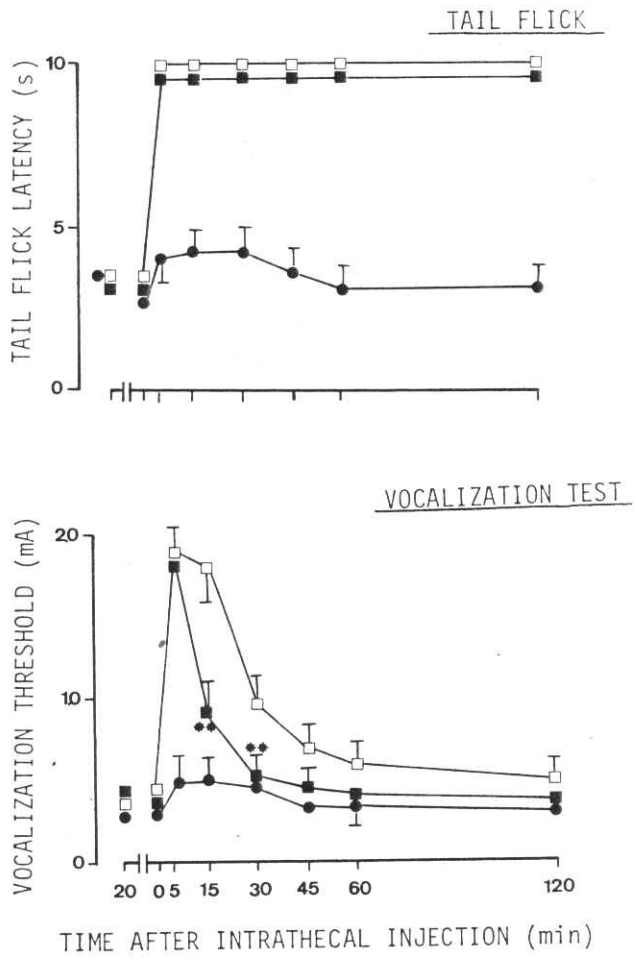

Fig. 2. - Effects of intrathecal Dyn A on tail flick (top) and vocalization threshold (bottom) in rats treated 5 min before or 5 min after the peptide with intrathecal MR 1452.

□ Dyn A 25 nmol
● MR 1452 30 nmol + Dyn 25 nmol
■ Dyn A 25 nmol + MR 1452 30 nmol
** $p < 0.05$ vs Dyn A-treated rats

Two weeks after ovariectomy a significant increase of ir-dyn was observed only in the AL; immunoreactivity did not change in the NIL and HYP. In animals ovariectomized two weeks before, the administration of EB through Silastic tube implants for 7 days reversed the ir-Dyn increase (Table 2).

Discussion

The results of the present investigations demonstrate, in agreement with studies of other laboratories [18,19] that dynorphin related peptides (Dyn A, Dyn 1-32, Dyn B) cause, at spinal level, alterations in nociception and motor function, clearly distinguishable in time by means of two different analgesimetric procedures.

The duration of effects of Dyn 1-32 is longer than that of Dyn A whereas Dyn B exhibits an activity far below that of the other two peptides: signs of motor dysfunction, in particular, appear only with high doses of Dyn B. The comparison of the activity of Dyn A by means of the two different tests, allows to confirm that the prolonged depression of the tail flick reflex should be ascribed to a motor dysfunction and does not completely reflect the animal's response to painful stimuli.

Both alterations induced by the dynorphin-related peptides are mediated by an opioid receptor of the k-type, since they are antagonized by a pre-treatment with a k antagonist. Moreover, a cross-tolerance to these effects is shown with the relatively pure k agonist ethylketocyclazocine.

However, different mechanism(s), possibly non opioid in nature, may contribute to the prolonged depression of motor function, since the k-receptor blocker MR 1452 fully *prevented* Dyn A's effect but, when administered *after* the peptide, it was capable to reverse the elevation of the vocalization threshold only, without removing motor impairment.

This agrees with our previous observations [27] that a few minutes after intrathecal injection, monoiodinated Dyn A is largely brokendown: the

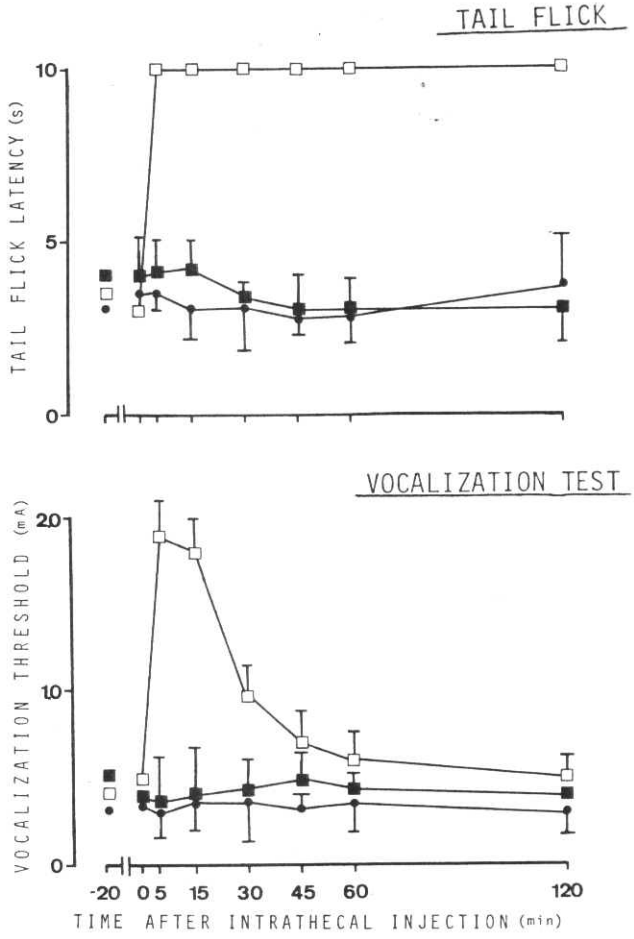

Fig. 3. - Effect of intrathecal Dyn A on tail flick (top) and vocalization threshold (bottom) in EKC-tolerant rats.

● vehicle
□ Dyn A 25 nmol
■ Dyn A 25 nmol in EKC-tolerant rats

Table 1. – *Ir-dyn in the AL and NIL of rats with lesions of the supraoptic and paraventricular nuclei (SON + PVN), anterior hypothalamus (AH) and of the medial basal hypothalamus (MBH). Values represent the mean ± S.E.M.*

Experimental group	ir-dyn (pmol/gland)	
	AL	NIL
Sham-lesioned	1234 ± 74.9	1471 ± 60.1
SON + PVN-lesioned	1212 ± 50.7	973 ± 70.5*
Sham-lesioned	1108 ± 44.7	1123 ± 72.8
AH-lesioned	1357 ± 160.3	829 ± 90.8*
Sham-lesioned	1138 ± 131.5	1545 ± 100.9
MBH-lesioned	652 ± 80.5*	760 ± 30.8*

* $p < 0.05$ vs sham-lesioned rats (Student's t test).

rapid degradation could explain, at the same time, the short antinociceptive activity whereas the longer lasting effect on motor function could represent the result of perseverative activity on the complex spinal neural circuits, no longer dependent on the integrity of the Dyn A molecule.

This hypothesis is further supported by our data showing the weaker and shorter antinociceptive action of the peptide des-tyr-Dyn A.

That the effects of intrathecal Dyn A derive from an action in the spinal cord is confirmed by data showing that after intrathecal administration of monoiodinated Dyn A, no radioactivity was detected in brain areas in the time period corresponding to maximal antinociceptive effect of the peptide [27]. However further research is necessary to clarify whether the action of dynorphin related peptides on nociception and motor function is simply pharmacological or reproduces a physiological effect of endogenous peptides, of different molecular forms, present at spinal level.

As regards experiments on the regulation of dynorphin content in pituitary, we observed a reduction of the neurohypophyseal pool of ir-dyn in rats lesioned in the supraoptic, paraventricular and anterior hypothalamic area. Thus it could be assumed

Table 2. – *Effect of ovariectomy and estrogen replacement on the pituitary content of ir-dyn. Values represent the mean ± S.E.M.*

Treatment	ir-dyn (pmol/gland)	
	AL	NIL
SHAM	746 ± 76.2	811 ± 52.8
OVX	1727 ± 171.1*	863 ± 24.0
OVX + EB	1049 ± 48.1	1025 ± 49.5

* $p < 0.05$ vs sham-operated rats (Student's t test).

that neurons originating from these nuclei, or fibers in passage through, may contribute to ir-dyn in the NIL. These findings are in line with immunohistochemical studies showing the presence of dynorphin-positive perikarya and fibers within these nuclei [9]. A depletion of ir-dyn occurs in the NIL of rats lesioned in the medial basal hypothalamus. This might be in consequence to the destruction of the hypothalamo-neurohypophyseal tract. Moreover, since lesions of this area lead, at the same time, to a significant loss of immunoreactivity in the AL, it is suggested that the absence of one or more factors, still unknown, here produced and/or stored may cause the observed decrement of ir-dyn.

Adenohypophyseal ir-dyn seems to be selectively modulated by the ovary. This influence seems to be mediated by ovarian steroids since EB treatment reversed the post castration rise of immunoreactivity.

In conclusion, the results here reported indicate that dynorphin structurally-related peptides may play a role in the modulation of sensory input at spinal level. In addition, investigations on dynorphinergic system show the existence of two distinct pools for dynorphin peptides in anterior and posterior lobes which are differently regulated by hypothalamic nuclei. Moreover ovarian estrogens seems to participate in this regulation of adenohypophyseal dynorphin content.

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