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Opioid Receptors in the Rat Spinal Cord after Longlasting Deafferentation

I. HÁJEK, J. BUŘITOVÁ[1], N. KŘÍŽ[1]

Laboratory of Cellular Neurophysiology, Institute of Experimental Medicine, Czechoslovak Academy of Sciences, [1]Department of Physiology, 3rd Medical Faculty, Charles University, Prague

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Summary

In vitro binding of specific opioid ligands to their respective sites in membrane fractions and the contribution of individual receptor classes (mu, delta, kappa) was studied in rats after longlasting (up to 22 months) section of spinal dorsal roots at the cervical (C_{5-8}) or thoracic (Th_{1-4}) level. This procedure leads to autotomy or scratching of the skin on the operated side. The total number of receptors in the cervical and thoracic spinal cord was more than doubled in both operated and contralalteral part of the cord in comparison with intact controls of the same age. In the cervical region, this increase mainly represented a rise in the number of free receptors, whilst in the thoracic region both free and saturated receptors were increased. On the deafferented side, receptor selectivity, especially in the delta and kappa types was decreased.

Key words
Opioid receptors – Spinal cord – Deafferentation

Introduction

The spinal cord, being the first stage in the processing of pain signalization from the periphery, has been implicated in the generation of pain and other dysaesthesias following root and nerve damage. It was observed that in animals after spinal root section the abnormal firing of interneurones in the dorsal horns takes place (Loeser and Ward 1967, Lombard *et al.* 1979, Albe-Fessard and Lombard 1983, Lombard and Besson 1989). A similar situation also occurs in man (for review see Ovelmen-Levitt 1988).

It has generally been accepted that opioid peptides and their receptors play an important modulatory role in neural processing of pain (for review see Kosterlitz and McKnight 1981, Yaksh 1981, Besson and Chaouch 1987, Herz and Millan 1990). After deafferentation, a marked reduction in the number of opiate receptors in the superficial dorsal horn occurs in mammals and amphibians (LaMotte *et al.* 1976, Nincovic *et al.* 1981, Hájek *et al.* 1985). The time course of the decrease in binding of specific opioid ligands (for mu and delta receptors) correlates with the degeneration of damaged presynaptic elements and also with transynaptic degeneration of other constituents.

Dorsal root section may, however, lead to the longlasting changes in the opioid tone. We therefore tried to investigate the fate of opioid receptors after dorsal root section and to clarify, if the change in their number might be connected with the abnormal behaviour of animals that during the whole 22-month' period following the operation continuously attacked the deafferented body areas.

Material and Methods

Female rats of the Wistar strain were deafferented at the age of 2 months under pentobarbital anaesthesia (50 mg/kg of Pentobarbital Spofa, i.p.). Unilateral deafferentation was performed by cutting 4 roots at the cervical level (C_{5-8}, four rats), or at the thoracic level (Th_{1-4}, six rats). After the laminectomy, a minimum opening of the dura was made above the point of root entry into the spinal cord and the dorsal root was cut proximal to the dorsal ganglion. The animals recovered completely from anaesthesia in about 4-6 hours, the operation wound healed *per primum* and no infection was observed. The animals showing any early signs of spinal cord damage during the operation were immediately discarded. A few sham-operated rats (laminectomy without rhizotomy) have never shown any behavioural

Table 1
Percentual distribution of total binding into the individual classes of opioid binding sites of rat spinal cord 22 months after deafferentation.

	Cervical region			Thoracic region		
	mu	delta	kappa	mu	delta	kappa
Intact	41.1	44.4	14.5	39.3	39.5	21.2
Deafferented						
– ipsilateral	38.5	47.2	14.3	50.9	34.1	15.0
– contralateral	43.0	46.0	11.0	38.8	34.9	26.3

abnormalities such as scratching, nibbling or depilation of limb areas, which indicates the absence of abnormal sensation after laminectomy only. The animals were housed in individual cages with free access to food and water. Since the conditions of deafferentation include a certain amount of suffering, the number of operated rats used was kept to a minimum (according to the Committee for Research and Ethical Issues of the IAPS, 1980). All animals were sacrificed at the age of 24 months. There was a statistically significant decrease in body weight of the experimental groups with cervical and thoracic deafferentation in comparison with the controls ($P < 0.05$ and $P < 0.01$, respectively, using the one way analysis of variance ANOVA). The average body weight (± S.E.M.) of the groups was 388.0 ± 14.6 g in the control group, 314.7 ± 13.3 g in that deafferented at the cervical level and 288.8 ± 20.7 g in the group deafferented at the thoracic level. These results indicate that rats of both experimental groups were profoundly affected by the spinal root section even after a very long time after the operation and that all groups were homogenous. For our biochemical studies, 12 intact controls of the same age (i.e. 24 months) were used (8 for the thoracic and 4 for the cervical region of spinal cord). The deafferented segments of the spinal cord were removed and divided into the deafferented and contralateral part using a razor blade and homogenized. Crude membrane fractions were isolated as described previously (Hájek *et al.* 1985).

Binding studies were performed in triplicate in 0.5 ml of 50 mM TRIS-HCl buffer at pH 7.2. One hundred microliters of the membrane suspension were used, containing 50 – 100 μg of protein. Binding was performed at 0 – 4 °C for 2 hours and the unbound ligands were separated by filtration through Whatman GF/B filtres using suction and washed three times with 5 ml aliquots of a cold Tris buffer. Bound radioactivity was estimated in Bray scintillation fluid using a Beckman scintillation counter with an efficiency of about 30 %.

The non-selective ^{3}H-labelled ligand 15,16 ^{3}H diprenorphine (45 Ci/mmol, New England Nuclear, Dreieich, F.R.G.) was used at a concentration of 0.5 nM. Non-selective "cold" levorphanol (1,3 hydroxy-N-methylmorphinan-tartrate, Sigma) was used for the determination of nonspecific binding at a concentration of 10 μM. DAGO (D-Ala^2-MePhe^{4}Glyol^5 enkephalin, Bachem Feinchemikalien AG, Switzerland) and DADLE (D-Ala^2-D-Leu^5 enkephalin, Sigma) served as specific ligands for mu and delta sites, respectively, at concentrations of 1 μM. In several cases, U-50, 488H ([trans-(dl)-3,4-dichloro-N-methyl-N-(2-[1-pyrrolidinyl] cyclohexyl)-benzene acetamide] methyl sulphonate hydrate (Upjohn, Kalamazoo, USA) for kappa sites was used at the same concentration. Specific binding was calculated per mg of protein based for the estimation employing the method of Lowry *et al.* (1951). Using the method of subsequent subtraction from the total specific binding (the method also called "receptor masking" – Williams and Wood 1986), obtained on the basis of diprenorphine binding, values were obtained for all three receptor classes (e.g. "cold" DAGO saturates mu receptors, therefore the binding in its presence represents unsaturated delta + kappa receptors, in the presence of DADLE mu and kappa receptors bind the labelled ligand and in the presence

of U-50,488H mu and delta sites are labelled). Binding was estimated for "free" sites, i.e. those immediately available for binding and for "total" binding, i.e. those revealed after preincubation of membrane fraction (45 min at 37 °C, and recentrifugation, see Hájek *et al.* 1985). The statistical significance of differences between groups was calculated using Student's t-test.

Results

The total number of receptors (binding sites) in the cervical and thoracic regions of deafferented spinal cord increased both on the operated and contralateral sides (Fig. 1) by more than twice in comparison with the intact rats of the same age (i.e. 24 months). In the cervical region, this increase mainly represented a rise in the number of free receptors. In the cervical region, the number of free receptors increased to 309 % in deafferented ipsilateral and to 214 % in contralateral side (number of receptors blocked by endogenous ligands was roughly the same, 102 and 114 %). In the thoracic region the number of both free and saturated receptors was increased, free receptors to 315 and bound to 328 % on the ipsilateral side and to 184 and to 585 %, on contralateral side, respectively.

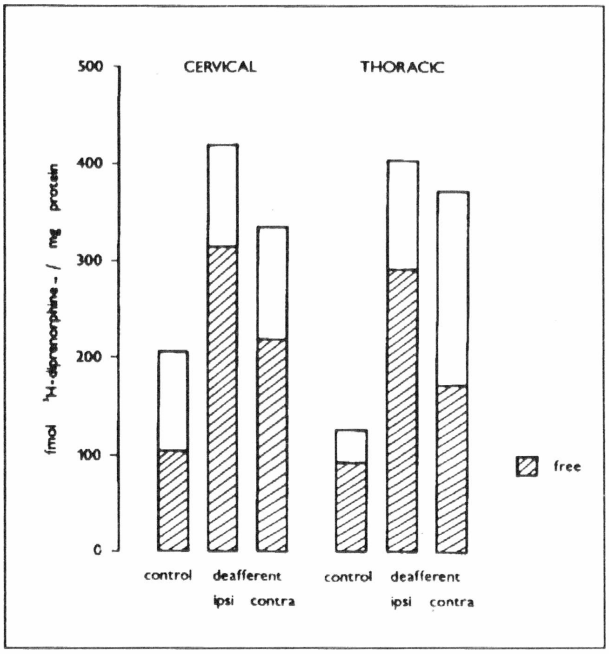

Fig. 1
Total specific ^{3}H-diprenorphine binding to membrane fraction prepared from cervical and thoracic spinal cord segments (in fmol/mg protein). Binding to free receptors is expressed as the hatched portion of the columns. (S.E.M. are not given, but never exceeded 5 %). Binding is given for control unoperated rats and for deafferented (deafferent), both on ipsi- and contralateral sides.

The ipsilateral part was found, accompanied by decreased representation of the other two classes. In contrast to that, the contralateral side showed a higher proportion of kappa sites and lower delta sites.

Table 2
Percentual distribution of binding to free sites into the individual classes of opioid binding sites of rat spinal cord 22 months after deafferentation.

	Cervical region			Thoracic region		
	mu	delta	kappa	mu	delta	kappa
Intact	37.5	54.2	8.3	42.8	28.6	28.3
Deafferented						
– ipsilateral	39.5	51.5	9.0	60.6	18.2	21.2
– contralateral	39.5	42.5	18.0	37.2	32.0	30.8

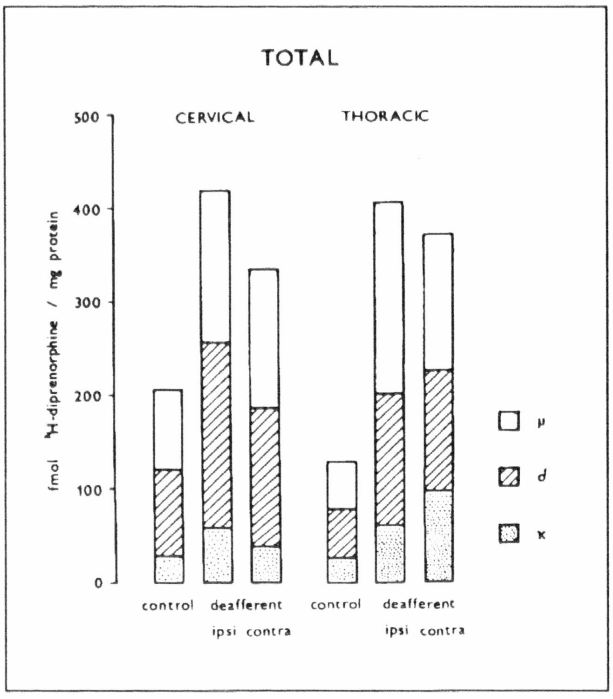

Fig. 2

Total specific binding of ^{3}H-diprenorphine shown as the proportion of mu, delta and kappa classes of opioid receptors. Marking of columns as in Fig. 1.

All three classes of opioid binding sites were affected in a similar way (Figs. 2 and 3). An absolute increase can be seen in the number of all classes of opioid receptors. The percentual distribution of individual classes (total receptors) is given in Table 1. Changes that occur after the deafferentation in the cervical region with respect to mu and delta sites are relatively small and the only marked changes appeared in a higher proportion of kappa sites in contralateral parts of the cord. In the thoracic region, however, the marked increase in the representation of mu sites in the ipsilateral part was found, accompanied by decreased representation of other two classes. In contrast to that, the contralateral side showed a higher proportion of kappa sites and lower delta sites.

The number of free (i.e. non-occupied) binding sites was also increased in all cases (Fig. 3). The percentual distribution of free sites into the individual classes is given in Table 2. It can be seen that in the cervical region the most pronounced changes occurred in the proportion of kappa sites. In the thoracic region the highest increase in the proportion of mu receptors occurred in the deafferented (ipsilateral) cords, accompanied by a decrease in the representation of other two classes.

The total binding capacity, calculated from the total specific binding (per mg of protein), multiplied by the total protein yield in individual preparations is shown in Table 3. This result indicates the overall number of binding sites in the whole section of the spinal cord and how it was changed after deafferentation. It was found that the highest increase in the diprenorfine binding sites (per 4 segments of the spinal cord) occurred in the number of free sites in the deafferented cervical spinal cord (by 95 % in comparison with intact controls). The total number of sites increased both in the cervical and thoracic regions on the ipsilateral side (by 34 and 58 %, respectively). This increase was higher than that observed on the contralateral side (by 23 and 28 %, respectively).

A decrease in receptor selectivity was observed in some cases on the deafferented side (in about one third of cases), especially in the delta and kappa types. This can be concluded from the finding that the sum of delta + kappa was higher than would be expected from the total binding capacity minus the mu binding. Newly synthesized receptors were apparently less discriminative towards specific ligands.

Table 3

Number of opioid binding sites (in fmol/spinal cord segment) as calculated from total specific ^{3}H-diprenorphine binding (see Fig. 1) multiplied by total protein yield. Binding to "free" and total receptors.

Cervical region:

Intact rat		Deafferented rat			
		Ipsilateral side		Contralateral side	
Total	Free	Total	Free	Total	Free
201.9	104.4	271.3	203.5	248.7	146.0

Thoracic region:

Intact rat		Deafferented rat			
		Ipsilateral side		Contralateral side	
Total	Free	Total	Free	Total	Free
198.4	143.8	314.2	225.7	254.2	117.6

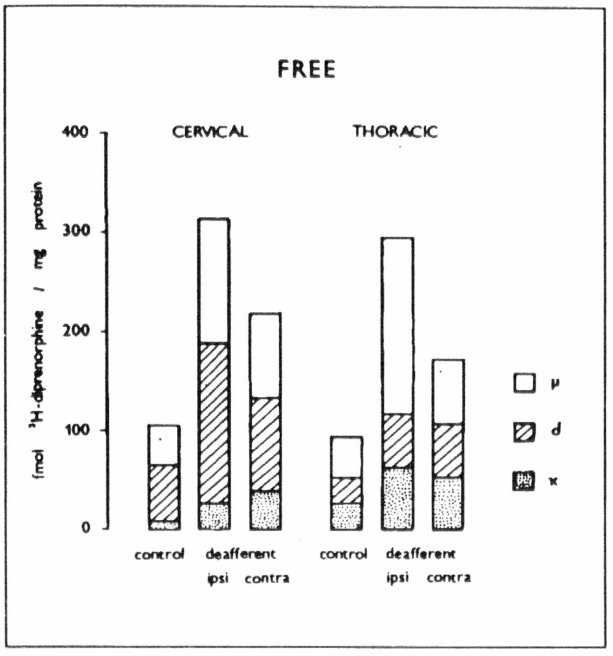

Fig. 3

Binding of ^{3}H-diprenorphine to free receptors (see Method section for definition) shown as the proportion of mu, delta and kappa classes of opioid receptors. Marking of columns as in Fig. 1.

Discussion

The changes in binding of labelled opioid ligands, representing the number and availability of specific receptors after the elimination of peripheral input by deafferentation, seem to have several phases. During the first phase which takes place several hours or the first few days (the differences are due to interspecific variations) after the operation, the section of dorsal roots (or spinalization) results in increased binding to free receptors (Hájek *et al.* 1985). This is apparently due to decreased signalization (from the periphery or CNS). During this stage no degeneration of terminals can be observed. In the second phase, the degeneration of transected primary afferent fibres leads to a decrease in the total number of binding sites (LaMotte *et al.* 1976 – monkey, Zajac *et al.* 1989, Besson *et al.* 1990, Svoboda and Hájek, unpublished results – rat, Hájek *et al.* 1985 – frog). During the third phase, binding sites (located on surviving interneurones and probably also on glial cells, Hájek and Syková 1985) which do not receive adequate signalization due to interrupted inputs, are subjected to the phenomenon of up-regulation. We can assume that in our experiments we have seen the results of such a process in the increased number of both total and free binding sites of all three classes.

The tissue concentration of various endogenous opioids, their release, synthesis or the concentration of respective m-RNAs in deafferentation has not been studied so far. However, from the amount and proportion of receptors, saturated by endogenous ligands, that can be calculated from the comparison of binding to total and free receptors, it would be possible to estimate the probable composition of the endogenous ligand. In the control cervical spinal cord, we found the following theoretical "composition": mu ligands 46.3 %, delta 34.2 % and kappa 19.5 %. On the ipsilateral side of the deafferented cord, the following result was obtained for individual ligands 35.0, 32.5 and 32.5 %, and on the contralateral side 55.1, 44.9 and 0(!), respectively. In the thoracic spinal cord, the theoretical composition of the endogenous ligand in control rats was 26.7 % of the mu type and 73.3 % of the delta type (with no kappa present). In deafferented rats, mu on the ipsilateral side represented 21.7 % and delta 78.3 %, with no kappa and on the contralateral side the proportion of individual ligands was 39.8 %, 38.5 and 21.7 % (mu, delta and kappa, respectively). From these results we can summarize that, in the cervical region, the most pronounced changes in the composition of endogenous ligand(s) occurred in the ipsilateral deafferented spinal cord in proportion of mu (decrease) and kappa fraction (increase), whereas both mu and delta ligands increased and no kappa ligand was produced on the contralateral side. In the thoracic region the control endogenous ligand was composed of mu and delta ligands (ratio 1:3, and no kappa ligand was produced). In the deafferented cord on the ipsilateral side no dramatic change occurred (ratio approx. 1:4), however on contralateral side all types of ligands including kappa could be found (ratio approx. 2:2:1).

The observed differences in the reaction of spinal cord of rats on section of dorsal roots at different levels might be responsible for the heterogenous behaviour of operated rats. More severe reactions to deafferentation in our experiments were always observed in rats with cervical root section. Changes in the relative representation of individual receptor classes could be responsible for changes in the sensitivity of deafferented animals to the antinociceptive effect of morphine (see Besson *et al.* 1990).

It has been shown by Loeser and Ward (1967) that longlasting deafferentation in the cat produced abnormal firing in dorsal horns persisting for as long as 180 days. These authors also described the appearance of neurones that became "hyperactive" to stimulation from adjacent spinal cord segments. A similar increase in the excitability of extensor, but not of flexor motoneurones, was observed in rats by Hník *et al.* (1981). Bursting activity was not recorded only in the deafferented spinal cord but also at the thalamic level (Lombard *et al.* 1979b). This continuous neuronal activity causes an increase in the concentration of extracellular potassium in specific thalamic nuclei (Kříž *et al.* 1991). These findings may be related to the

decreased opioid tone that has manifested itself in our experiments by increased number of both total and free sites of all classes, based on the mechanism of up-regulation.

The finding that even the contralateral sides are affected after longlasting deafferentation similarly as the operated sides was rather surprising. It is possible, however, to assume that there is a relatively large number of crossed afferent fibres. This has been indicated by Matsushita and Tanami (1983) in caudal and sacral segments in cats. Vierck *et al.* (1986) also confirmed that the conduction of pain can occur through contralateral axons of ipsilateral cells (the contralateral spinothalamic and contralateral spinoreticular tracts), through ipsilateral axons of ipsilateral cells (the ipsilateral spinothalamic tract) or by contralateral or ipsilateral conduction from cells in the contralateral dorsal horn. The increased opioid binding present even on the side contralateral to the lesion, observed in our experiments, may thus be attributed to the longlasting alteration of cellular elements, which were deprived of normal signalization from the periphery for a very long time.

It is still far from clear, if deafferentation causes dysaesthesia or evokes pain sensation (see Coderre and Melzack 1986). From the present results, and also from our previous findings (Hájek *et al.* 1985), it seems that the opioid tone plays an important role in pain sensitivity by regulating the proportion of free and total opioid receptors. As longlasting deafferentation causes a dysbalance of this relation, it is more likely that rhizotomy alters pain sensitivity.

In addition, there are certain indications that even the spectrum of endogenous ligands, produced under the effect of deafferentation, differs both between the ipsilateral and contralateral sides, but also between various regions of the spinal cord.

It might be only speculated that similar phenomena observed in our experiments on rats also take place in human pathology and are responsible for the pain syndromes due to deafferentation or peripheral nerve injury, such as phantom pain.

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Reprint Requests

I. Hájek, PhD. Laboratory of Cellular Neurophysiology, Institute for Experimental Medicine CAS, CS-180 85 Prague 8, Bulovka, Pav. 11.