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Controlled superfusion of the rat spinal cord for studying non-synaptic transmission: an autoradiographic analysis

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Abstract

Recently, evidence has been raised that long-term changes in the central nervous system are mediated by extrasynaptic spread of neuropeptides ('volume transmission'). To study the effects of volume transmission in the spinal cord we developed the technique of controlled superfusion of the rat cord dorsum. This paper presents quantitative data about the spread, local spinal tissue concentration and redistribution of (2-[^{125}I]iodohistidyl)neurokinin A, applied for 15, 30 or 60 min to the spinal cord dorsum in concentrations of 0.05 or 50 μM (10 μl). Analysis of autoradiograms of sagittal and transverse spinal cord sections was done by computer-assisted densitometry. Under all experimental conditions, the spread of radiolabel into the superfused spinal cord segments reached Rexed's laminae V and VI; maximal spread (1.6 ± 0.3 mm) was measured after superfusion for 30 min. The amount of radiolabel decreased in ventral direction as a function of distance. Highest tissue concentrations of neurokinin A (NKA) were obtained within the superficial spinal cord up to a depth of 0.5 mm and ranged from 700 to 2000 pmol/g following superfusions for 15 or 30 min with 50 μM NKA. Thus, these tissue concentrations were 25–70 times lower than the concentration of NKA in the superfusate. Since pool content was not exchanged, the radioactivity within the spinal cord was lower after superfusion periods of 60 min than after 15 or 30 min. Detection of radiolabel in blood and urine suggests that capillary clearance is relevant and limits the accumulation of the peptide within the spinal cord tissue and the spread into deeper laminae. The controlled superfusion of the rat cord dorsum is a useful method to mimick the spinal release of endogenous neuropeptides such as NKA during intense noxious stimulation, and it can be employed for versatile investigations of the effects of neuroactive molecules on the processing of sensory information in the intact spinal network.

Keywords: Controlled superfusion; Spinal cord; Neurokinin A; Volume transmission; Non-synaptic transmission; Neuropeptide distribution; Autoradiography; Densitometry

1. Introduction

Neurons in the central nervous system (CNS) communicate in different ways. One way is coupled to membrane specializations called gap junctions, which create a cytoplasmatic connection and thereby an electric and metabolic link between adjacent cells. This mode of signal transmission is very rapid (Dudek et al., 1983).

The second pathway also depends upon specialized membrane sites and is mediated by classical neurotransmitters which cause rapid opening and closing of ion channels for fast synaptic signal transfer. Neurotransmitters, stored in small synaptic vesicles, are quickly inactivated after their release, which allows transmission of high-frequency signals.

Another pathway operates more slowly via neuropeptides and may involve non-synaptic release and/or spread of the chemical signal (Duggan et al., 1988; Thureson-Klein and Klein, 1990). The extrasynaptic way of communication within the nervous system has been called 'volume transmission' because the mediators, primarily neuropeptides, but also extrasynaptically acting classical neurotransmitters or ions, diffuse in the volume of the extracellular space (Agnati et al., 1986; Matteoli et al., 1991; Nicholson and Rice,

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1991). Volume transmission affects a population of neurons rather than a single postsynaptic neuron. An extrasynaptic release of neuropeptides and a long half-life in the range of several minutes allows diffusion in the extracellular fluid where their persistence is probably not terminated by re-uptake or by fast degradation, due to their qualified resistance to enzymatic cleavage (McKelvy and Blumberg, 1986). Furthermore, an extrasynaptic spread of synaptically released neuromediators may result if inactivation mechanisms are saturated, e.g., after excessive release. Despite the diffuse presence and low concentration of the neuropeptides at distant sites, a high spatial order of action results by binding to the appropriate, high-affinity receptors.

In the spinal cord, the mismatch of the sites of release and binding (Helke et al., 1990; Herkenham, 1991), the existence of non-synaptic contacts (Ridet et al., 1992, 1993; Brown et al., 1993; Liu et al., 1994) and the presence of neuropeptides at elevated levels in the cerebrospinal fluid of the spinal cord after endogenous release (Go and Yaksh, 1987) provides evidence that volume transmission exists. Primary afferents or central fibers which are immunoreactive for the neuropeptides substance P (SP), neurokinin A (NKA), or somatostatin (SOM) terminate predominately in the superficial laminae of the dorsal horn, few in laminae V, and around the central canal (for review see Duggan and Weihe, 1991). Noxious stimulation or inflammation of the skin or other tissues which strongly excites peptidergic primary afferents result in the simultaneous release and spread of a number of neuropeptides, e.g., SOM (Morton et al., 1988), SP (Duggan et al., 1987; Go and Yaksh, 1987), NKA (Duggan et al., 1990; Hope et al., 1990a), CGRP and others (Morton and Hutchison, 1989) in the spinal dorsal horn and cerebrospinal fluid. The co-release of SP and calcitonin gene-related peptide (CGRP) (Hökfelt et al., 1984) may reduce cleavage of SP (Le Greves et al., 1985; Mao et al., 1992). Following release NKA is detectable for at least 30 min in the cerebrospinal fluid (Hope et al., 1990b).

The functional role of extrasynaptic signaling in the spinal cord is presently unknown. Volume transmission cannot transmit high-frequency information but may mediate long-term effects, e.g., by activation of second-messenger systems and alterations in gene expression. Probably, long-lasting alterations in the strength of pain pathways which follow inflammation or peripheral trauma (Kangrga et al., 1990), and the recovering of reflexes after spinal shock (Illis and Bach y Rita, 1993) can be induced by extrasynaptic neuropeptides.

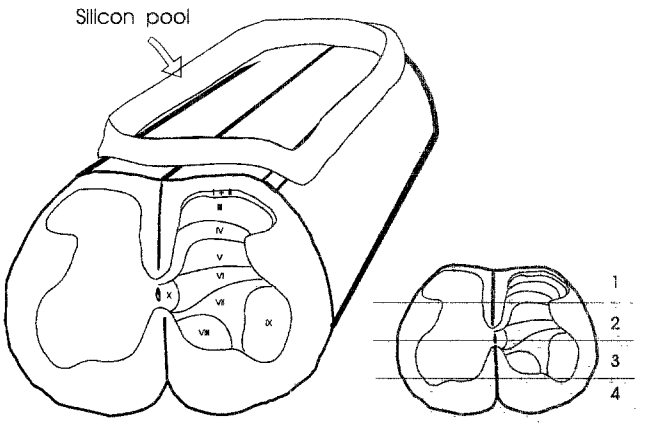

Fig. 1. Schematic illustration of the spinal cord dorsum superfusion technique. A pool is formed on the spinal cord surface with a specially synthetized silicon rubber and filled with the peptide solutions. The silicon rubber can be used to form a pool of any shape and size. In the present study the pool was about 6 mm long and 3 mm wide, and the pool content was about 10 μl. Roman numerals mark the Rexed's laminae I–X. At the right, the arbitrary cuts of an approximately 0.5-mm-thick section in 4 horizontal strips are shown which were prepared for liquid scintillation counting.

To examine the role of extrasynaptic neuropeptides in selected rat spinal segments in vivo, we have employed the technique of controlled superfusion of the cord dorsum (Sandkühler et al., 1991). We have choosen this model because the site of major endogenous release after noxious stimulation, which is the superficial dorsal horn, is very close to the site of the superfused cord surface. Here, we examined the localization and concentration of a radiolabeled neuropeptide, (2-[^{125}I]iodohistidyl)neurokinin A ([^{125}I]NKA), within the spinal cord after controlled superfusion of the cord dorsum by autoradiography. The redistribution of the radiolabel in other organs of the body was quantified by liquid scintillation counting.

2. Materials and Methods

All experiments were performed on male Sprague-Dawley rats (weighing 240–340 g; $n = 21$), initially anesthetized by inhalation of a mixture of halothane/nitrous oxide/oxygen (1:70:29). A femoral artery and

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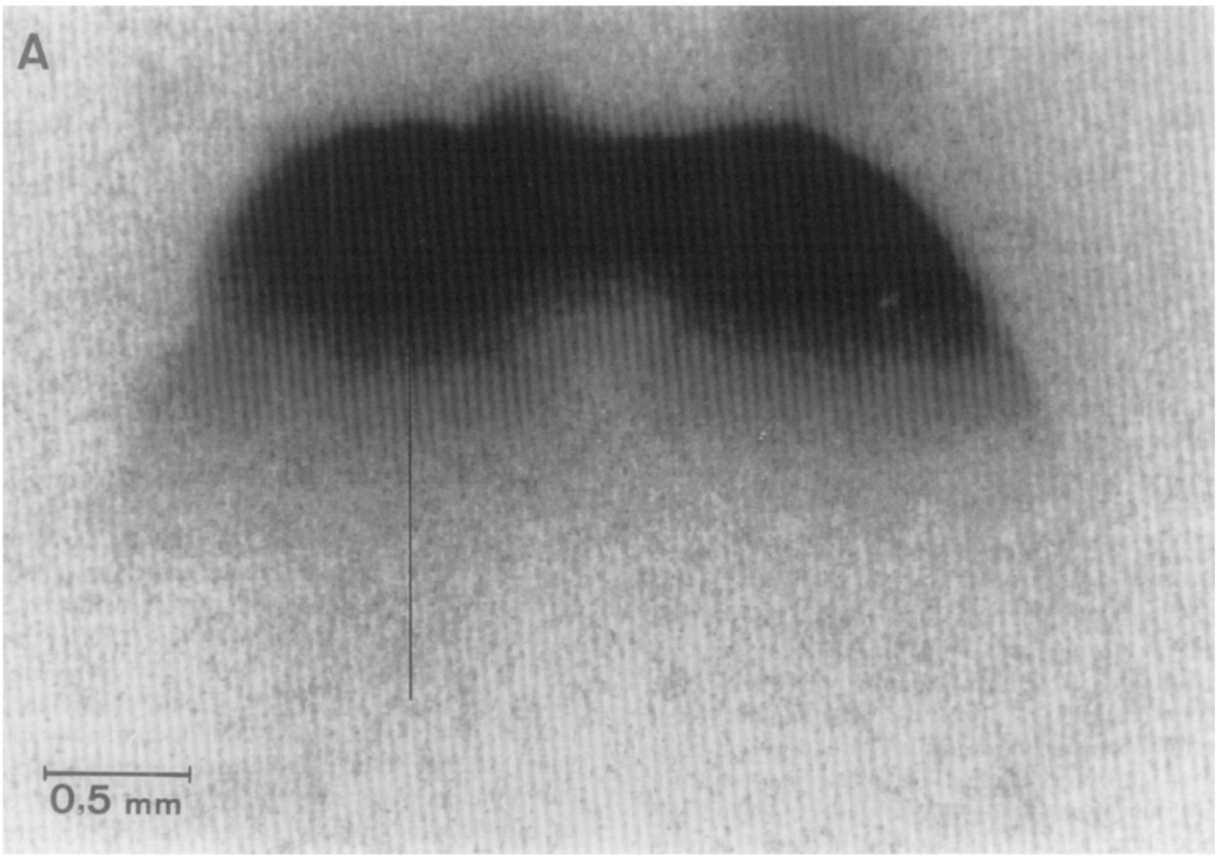
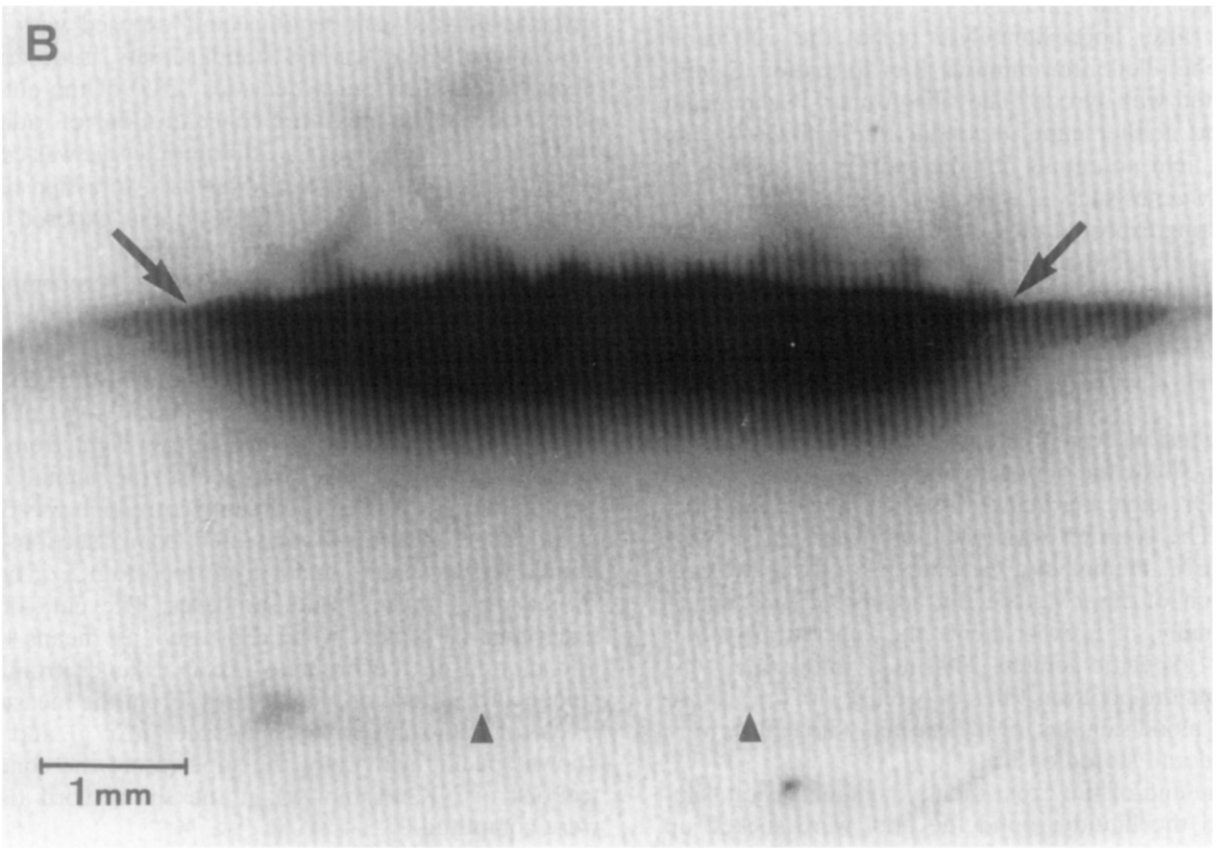
Fig. 2. Representative autoradiograms from transversal (part A) and longitudinal (part B) 20-μm sections through the superfused spinal cord were digitized and show the spread of radiolabel after 30 min of superfusion with [^{125}I]NKA at 50 μM. The black arrows in B indicate the ventral surface of the spinal cord. The black arrow heads in B mark the inner anterior and posterior borders of the silicon pool. The quantitative measurements of absorbance were done at 0.25-mm intervals along the vertical axis plotted in A. The lumbar spinal cord diameter in dorso-ventral direction was on an average 3.1 mm.


A
0,5 mm


B
1 mm

vein were cannulated for monitoring the blood pressure, the sampling of blood and infusion of saline and sodium pentobarbital (5–10 mg/kg/h). Arterial blood was sampled several times during the course of the experiments to evaluate the blood gases and acid-base status of the animals. The body temperature was kept at 37 ± 0.5°C throughout the experiment. All animals prepared for spinal superfusion breathed spontaneously. A laminectomy was made to expose the dorsal surface of the lumbar spinal cord from segments L2 to L6, and the vertebral column was fixed in a horizontal position with two holders. The exposed tissue was covered by agar-agar, except the spinal cord. The dura mater of the lumbar cord was opened and retracted, and the spinal cord surface covered with tyrode.

2.1. Silicon pool for superfusion

For controlled superfusion a ring (3 × 6 mm) of a specially prepared silicon rubber was formed on the cord dorsum (Sandkühler et al., 1991). The rubber material contained magnesium silicate and silicon oil (S49; BIODUR, Heidelberg, Germany) to which two hardeners (S3, KSA40; BIODUR) were added, initiating fusing of the silicon molecules by end-to-end polymerisation and cross-linking. The mass was pasty for about 20 min after adding the hardeners. Within this time the rubber was applied to the dried cord dorsum using a 1-ml syringe connected to a stretched polyethylene tubing. A pool was formed by superposing 2 or 3 rings which rapidly merged. The rubber became solid and dry after approximately 2 h, but the well could already be filled a few minutes after formation. Sealing was tested with tyrode. The silicon mass is chemically inert and neither impulse conduction in fibers of passage or field potentials nor the expression of immediate early genes such as c-*fos* or *jun* B were affected by long-lasting exposure of the cord dorsum to this mass. A schematic of the spinal cord with the superfusion pool on the dorsal surface is shown in Fig. 1.

2.2. Controlled superfusion with (2-[^{125}I]iodohistidyl) neurokinin A

Superfusions were made for 15, 30 and 60 min (each $n = 5$) by filling the pool with about 1 μCi of [^{125}I]NKA (NEN, Dreieich, Germany) dissolved in 10 μl of 0.9% saline. The solution was mixed with unlabeled NKA (BACHEM, Heidelberg, Germany) to obtain the concentration of 50 μM (specific activity: 2 nCi/pmol). Additionally, in 5 experiments the concentration was 0.05 μM (specific activity: 2000 nCi/pmol). In all experiments the pool was filled only once with [^{125}I]NKA. Arterial blood samples for measuring radioactivity were collected and stored on ice.

At the end of the experiments the superfusion solution and the liquid around the pool were sucked up and analyzed for radioactivity by liquid scintillation counting (Packard Tri-Carb 4000 series; scintillation cocktail: Opti-Fluor, Canberra Packard). The animals were killed with an i.v. overdose of sodium pentobarbital and the spinal cord was quickly removed, dipped for 1 s in saline to remove excessive superficial radioactivity and frozen immediately in 2-methylbutane chilled to −45°C. The piece of spinal cord was covered with chilled embedding medium (Lipshaw M1) and stored at −80°C in plastic bags until it was cut serially in a cryostat into 20 μm transverse or sagittal sections. The sections were immediately thaw-mounted on glass cover-slips and dried on a hot plate at 60°C. The dried sections were exposed for 3 weeks to X-ray film (Kodak MR-1) together with precalibrated ^{14}C-labeled methylmetacrylate standard sets (Amersham, Braunschweig, Germany).

For precalibration of the ^{14}C-labeled plastic standards, which was done for quantitative autoradiography of ^{125}I in subsequent experiments, approximately 2 g of rat brain were homogenized in a mortar after ^{125}I had been added (purchased as NaI; NEN, Dreieich, Germany). An aliquot of the homogenate was weighed and assayed for radioactivity in a liquid scintillation counter using external standardization for quench correction. The remainder was centrifuged for 10 min to remove air bubbles and rapidly frozen. This procedure was repeated 6 times with increasing amounts of ^{125}I to obtain increasing concentrations of radioactivity in the homogenate. The frozen homogenates were cut in a cryostat into 20 μm sections and exposed with ^{14}C-labeled methylmetacrylate standard sets as described above. From the optical densities (OD) of the autoradiograms and the measured concentrations of radioactivity in the homogenates a calibration curve was established. This calibration curve was used to assign tissue concentrations of ^{125}I in nCi/g to the exposed ^{14}C-labeled methylmetacrylate standard sets.

Computer-assisted densitometry (Microcomputer Imaging Device, MCID, Imaging Research, Ontario, Canada) was used to measure the optical density in 2 autoradiograms per experiment. The measurements of local tissue concentrations were made in steps of 0.25 mm by scanning about 0.5 mm left or right from the midline in ventral direction across the spinal cord autoradiograms (Fig. 2A). Shading correction was done in all images. The results obtained from 2 sections per animal were averaged and transformed to nCi/g tissue by means of the exposed standards. The data of all experimental groups were expressed as means ± SE and plotted on the ordinate versus distance from the spinal cord surface on the abscissa. From the measured levels of radioactivity in the tissue (nCi/g) and the known specific activity of the superfusate the concentrations of NKA (pmol/g) in the spinal cord tissue were computed.

In 4 experiments a thick section (≈ 0.5 mm) was cut from the center of the superfused segment, then transversally divided into 4 pieces (Fig. 1), which were weighed and placed in vials. From 3 animals, tissue samples of the heart, liver, lung, femoral skeletal muscle, cerebral cortex, cervical spinal cord and kidney were taken, weighed, solubilized in 0.5 M NaOH by heating, and assayed for radioactivity with liquid scintillation counting technique. Additionally, aliquots of final urine, plasma and blood were assayed for radioactivity. The radioactivity was expressed in decays per minute (dpm) per gram sample. Quench correction was done by external standardization. Samples of the urine and boiled acetic acid extracts from the spinal cord of 3 animals were analyzed by thin-layer chromatography (ethyl acetoacetate/pyridine/acetic acid/H_2O (6:5:1:3); silica gel glass plates 60 Å, Merck, Darmstadt, Germany).

2.3. Statistics

Significance of differences between means was analyzed with the Wilcoxon 2-sample test; $P < 0.05$ was considered significant.

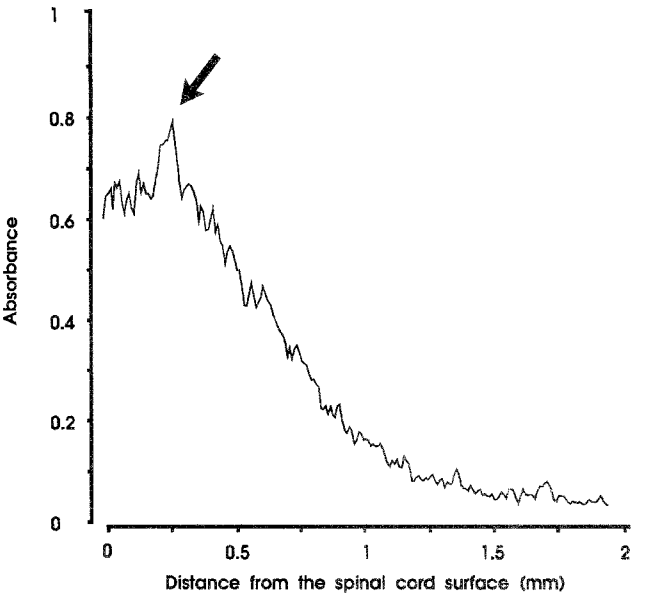


Fig. 3. The dorsoventral distribution of absorbance in 1 sagittal section was measured along a line as shown in Fig. 2A and was plotted on the ordinate versus the distance from the spinal cord surface on the abscissa. The superfusion with NKA at 50 μM lasted 30 min. Note the peak in absorbance at the border line between white and gray matter at the depth of approximately 0.25 mm (black arrow).

3. Results

Results were obtained from 21 rats. Mean arterial blood pressure was stable (90–105 mm Hg) and physiological values of blood gases (pO_2 (mm Hg): 72.0–86.5 [79.4] (minimum-maximum [arithmetic mean]); pCO_2 (mm Hg): 49.2–63.1 [45.7]), pH: 7.25–7.36 [7.30], base excess (mM): −2.1–2.8 [+1.2], heart rate (min^{-1}): [345] and hematocrit (%): 40.5–43.5 [42.6] were verified throughout the course of the experiments.

3.1. Rostro-caudal and dorso-ventral distribution of [^{125}I]NKA after controlled superfusion of the rat spinal cord dorsum

The characteristic pattern of distribution of the radiolabel within the superfused spinal cord and adjacent areas that was seen under all experimental conditions in autoradiograms of longitudinal and transversal sections is illustrated in Fig. 2A,B. The radiolabel was concentrated within the dorsal half of the spinal cord (Fig. 2A), rostral and caudal to the exposed spinal cord the radioactivity was substantially absent (Fig. 2B). Within the dorsal half, the area of the superficial laminae of the gray matter was more densely marked than the overlaying white matter or the deeper laminae. The measurement of the absorbance along a vertical line in the autoradiogram of a single sagittal section is shown in Fig. 3. The white matter corresponded to a depth up to 0.25 mm with a uniformly high absorbance. The superficial dorsal horn was marked by a notable increase in the optical density. Beyond this peak the density decreased continuously as a function of distance up to the depth of approximately 1.5 mm reaching background values at the level of laminae V/VI.

3.2. Spread of neuropeptide as a function of superfusion duration

To quantitatively study the dynamics and the extent of the spread of [^{125}I]NKA applied to the cord dorsum the cord tissue was analyzed by densitometry (Fig. 4; $n = 5$ in each group) following superfusions for 15, 30 or 60 min.

Independent of the duration of superfusion, radiolabel peaked at a distance of approximately 0.25 mm corresponding to the superficial layers of the dorsal horn, then the radiolabel decreased continuously reaching background values at a distance of 1.0–1.5 mm from the dorsal spinal cord surface. The absolute levels of radioactivity in the tissue did, however, depend upon the duration of superfusion. In the superficial spinal cord up to a depth of 0.5 mm the highest level of radiolabel was reached within 15 min of superfusion and was slightly lower after 30 min of superfusion. After 60 min the level of radioactivity in the superficial spinal cord was significantly reduced to about one-fourth of the level reached within 15 min. In the deeper dorsal horn the highest concentration was reached after 30 min of superfusion. The radiolabel at

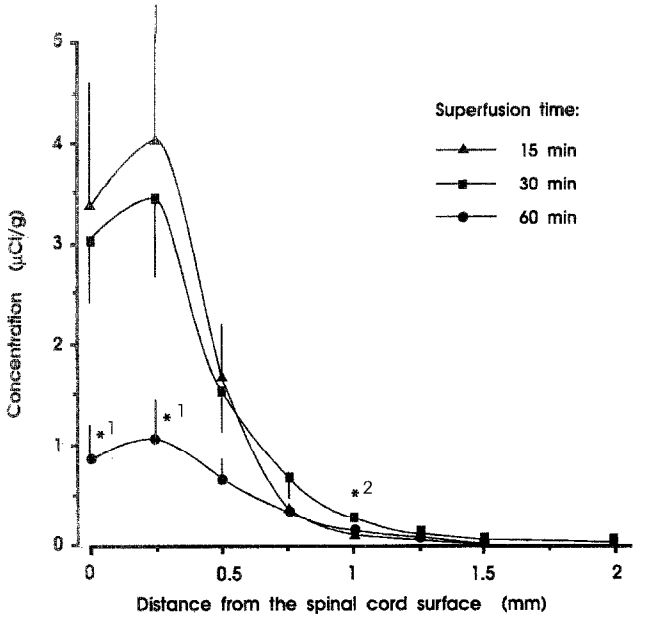


Fig. 4. The concentration of radiolabel (μCi/g) was measured at 0.25-mm intervals along the axis as shown in Fig. 2A after 15-, 30- and 60-min periods of superfusion with 50 μM [^{125}I]NKA. Mean values ($\pm$SEM, $n = 5$ in each group) are plotted versus the distance (mm) from the spinal cord surface. [1]At the depths of 0 and 0.25 mm, the tissue concentration measured after 60 min is significantly lower than in the other groups. [2]At a depth of 1 mm, the tissue concentration measured after 30 min is significantly higher than the concentration reached after 15 min.

a depth of 1 mm was significantly higher after superfusions of 30 min than following 15 min of superfusion (Fig. 4).

As an arbitrary measure for the maximal depth reached by radiolabel, we measured the distance from the superfused cord surface to the level where the radioactivity was reduced to 50 nCi/g, which is 0.05% of the radioactivity in the superfusate. The measured distances were 1.3 ± 0.3 mm (mean $\pm$ SD) after 15 min, 1.6 ± 0.3 mm after 30 min and 1.4 ± 0.2 mm after 60 min of superfusion.

The tissue concentrations of NKA (pmol/g), listed in Table 1, were calculated from the levels of radioactivity measured by densitometry and the known specific activity of the superfusate. Following superfusions with 50 μM NKA (i.e., 50 nmol/ml) for 15 or 30 min, the concentration gradient of NKA ranged from 700 to 2000 pmol/g in the superficial spinal cord (0–0.5 mm), which is 25–70 times lower than the concentration of NKA in the superfusate. Thus, in the superficial spinal cord, the tissue concentration of [^{125}I]NKA was 1.5–4.1% of the concentration in the superfusate. Since the superfusate was not exchanged, the concentration of NKA within the superficial spinal cord following superfusions of 1 h was reduced to 0.6–1.0% of the original concentration in the superfusate.

In the deeper spinal cord (0.75–1.25 mm) the maximal concentrations of NKA (50–300 pmol/g) were markedly lower than in the superficial spinal cord. After 30 min of superfusion the NKA concentration in the deep dorsal horn was approximately 2-fold higher than after 15 or 60 min of superfusion (see Table 1). At a depth beyond 1.5 mm, the NKA concentration in all groups was below 30 pmol/g, which is more than 3 orders of magnitude lower than the concentration in the superfusate.

We have also measured the concentrations of NKA by means of liquid scintillation counting in thick transversal sections cut in 4 horizontal strips (see Fig. 1). After 15 min of superfusion the concentration of NKA from dorsal to ventral was 0.8, 0.04, 0.02 and 0.01 nmol/g. After 30 min of superfusion the concentration gradient was 0.71, 0.12, 0.02 and 0.01 nmol/g, and

Table 1
Mean tissue concentrations of [^{125}i]NKA after controlled superfusion of the rat lumbar spinal cord dorsum

Depth (mm)	NKA superfusate concentration (μM)							
	50			0.05	50			0.05
	Superfusion time (min)							
	15	30	60	30	15	30	60	30
	NKA tissue concentration (pmol/g)				Tissue conc. in % of superf. conc.			
0	1683 ± 606	1512 ± 336	419 ± 172	0.68 ± 0.15	3.4	3.0	0.8	1.4
0.25	2037 ± 712	1640 ± 410	524 ± 196	0.72 ± 0.19	4.1	3.3	1.0	1.4
0.5	827 ± 282	756 ± 217	325 ± 107	0.34 ± 0.07	1.7	1.5	0.6	0.7
0.75	174 ± 47	328 ± 100	174 ± 58	0.14 ± 0.03	0.3	0.7	0.3	0.3
1.0	52 ± 16	120 ± 32	75 ± 20	0.06 ± 0.01	0.1	0.2	0.1	0.1
1.25	31 ± 7	54 ± 12	35 ± 6	0.04	0.06	0.11	0.07	0.08
1.5	22 ± 4	29 ± 6	22 ± 3	0.03	0.04	0.06	0.04	0.06
2	< 20	< 20	< 20	–	–	–	–	–

The concentrations of NKA in the superfused spinal cord tissue as measured by densitometry in the autoradiograms depend upon the time of superfusion and the concentration of NKA in the superfusate (left part). The tissue/superfusate ratio is given in % (right part of the table). All values are means ($\pm$SEM) of 5 experiments per group. The depths from the cord dorsum is given in millimeters in the left row. The specific activity of NKA in the superfusates was 2 nCi/pmol (50 μM) and 2000 nCi/pmol (0.05 μM).

Table 2
Distribution of radioactivity in various organs, body fluids and the superfusate after controlled superfusion of the rat spinal cord dorsum

	dpm/g	dpm/animal	%
Superfusate	–	1,790,000	100
Rest in pool	–	413,500	23.1
Superfused spinal cord	–	388,800	21.7
Outside pool	–	8300	0.5
Urine	31,000	55,900	3.1
Blood	1200	90,100	5.0
Spinal cord	1100	1800	0.1
Brain	3300	7100	0.4
Heart	4800	5100	0.3
Lung	3600	5000	0.3
Kidneys	15,000	31,200	1.7
Liver	6000	71,500	4.0
Skeletal muscle	2900	320,900	17.9
Σ Total body	–	596,900	33.3
Undefined	–	390,800	21.9

In 3 rats (mean body weight: 280 g) radioactivity was measured in different organs, body fluids and the superfusate after 30 min of superfusion. The mean radioactivity in the superfusates (50 μM NKA) in these experiments was 1,790,000 dpm which is equivalent to 0.8 μCi. From tissue and fluid samples, the counts for the whole organs (3rd row) were calculated from the tissue concentrations (2nd row) basing upon average organ weights and fluid volumes.

after 60 min it was 0.54, 0.08, 0.03 and 0.01 nmol/g. These results were in accordance to the analogous data obtained by densitometry (Table 1), when comparing the mean concentrations of NKA at the depth from 0 to 0.75 mm with strip 1, and from 0.75 to 2.0 mm with strip 2.

3.3. Effect of different NKA concentrations in the superfusate

We have measured tissue concentrations following 30 min of superfusion with [^{125}I]NKA at 2 different concentrations (0.05 μM and 50 μM) in 5 animals per group. The results are listed in Table 1 and indicate, that a smaller fraction diffused into the spinal cord when the lower NKA concentration was employed.

3.4. Redistribution from the site of superfusion

In 3 animals the distribution of the radioactivity in various organs was estimated after 30 min of superfusion with [^{125}I]NKA at 50 μM (Table 2). Twenty-two percent of the initial radioactivity in the pool were detected within the superfused spinal cord area. Twenty-three percent were still recovered from the pool. At least one-third, but less than 55% of the total radioactivity applied, was cleared from the spinal cord and redistributed in various organs. Twenty-two percent of the applied radiolabel could not be localized with our procedure, a part of this which may have been distributed in the body (we did not investigate bone, skin and gut). An additional major amount was probably still attached to the silicon pool and the spinal cord surface, but was not recovered. Thus, the uptake rate of radioactivity from the superfusate was between 50 and 80% within 30 min.

Absorption into the blood is probably a major way to clear the radiolabeled compound from the spinal cord, since a high portion of radioactivity was found in the blood. A major transport of radiolabel by liquor can be excluded as adjacent spinal cord segments were unlabeled. The concentration of radioactivity in the heart, the lungs and especially in the brain (3300 dpm/g) was low and several times smaller than the concentration detected in the kidneys. Three percent of the counts were found in the final urine. Using thin-layer chromatography, at least 3 radiolabeled compounds were discovered in the urine. By the same method, only 1 labeled compound was found in the boiled acetic acid extract from the superfused spinal cord segments. These findings indicate that elimination and degradation followed the uptake in the blood circulation.

4. Discussion

The study provides quantitative data about the distribution of [^{125}I]NKA following the controlled superfusion of the rat spinal cord dorsum. The available evidence suggests that the source of radioactivity in the superfused spinal cord is the radiolabeled NKA. First, NKA is highly resistant to enzymatic degradation (Theodorsson-Norheim et al., 1987), and second, thin-layer chromatograms of the spinal cord extract demonstrated a single peak after 30 min of superfusion. Finally, no radioactivity was found in the thyroid gland, suggesting that ^{125}I was not cleaved from NKA. We can, however, not exclude that fragments of NKA are present in the spinal cord after superfusion periods longer than 30 min.

Diffusion of molecules can be described by Fick's law. Since NKA like other neuropeptides cannot pass through cells, Fick's law has to be modified by the tortuosity of the extracellular clefts (Cserr, 1971) and the volume fraction (Kessler et al., 1976d), both limiting the rate of diffusion. The fact that highest tissue concentrations of NKA were reached within 15–30 min suggests that indeed no major diffusion barriers exist. Substance-specific sink properties (active uptake into cells and degradation) which reduce the migration within the spinal cord tissue is unlikely because neuronal uptake and recycling of neuropeptides has not be shown, and the degradation of NKA is slow. The uptake of NKA and/or its fragments into the blood stream is a major factor limiting the spread of NKA

into deep layers of the spinal cord during long (60 min) superfusions. The pharmacological effects of NKA, e.g., enhancement of vascular permeability, increase of heart rate and a biphasic change of mean arterial blood pressure (Hassessian et al., 1987), may further increase clearance by the blood stream. During the controlled superfusions with NKA, however, we observed no change in the blood pressure and only a small change in the heart rate with an initial short decrease followed by a long-lasting small increase (results not shown).

With a given ratio of cellular to extracellular space of 4:1 (Kessler et al., 1976a), the actual NKA concentration in the extracellular space is 5 times higher than the concentration measured in the total tissue volume. The characteristic high level of radiolabel in the gray matter of the superficial dorsal horn is not due to a different self-absorption of radiation from ^{125}I in white versus gray matter (Herkenham and Sokoloff, 1984), but corresponds to high tachykinin receptor density; thus, receptor binding may enhance local tissue concentration of the ligand. Furthermore, the cell density is higher in the gray matter than in the white matter which results in a 4–5% larger extracellular space in the gray matter (Kessler et al., 1976a), a correspondingly larger distribution compartment and, thereby, larger local tissue concentration of NKA. Together, these factors reduce the access of NKA to the deepest laminae of the dorsal horn.

A similar gradient of the radiolabel from the surface to the depth was also seen under steady state conditions during superfusion of the circumference of the spinal cord of rhesus monkeys with [^{3}H]homovanillic acid, [^{14}C]5-hydroxy-3-indoleacetic acid or [^{3}H]3-methoxy-4-hydroxyphenylethyleneglycol for 2–6 h (Kessler et al., 1976b–d). The decline in tissue levels was caused by capillary uptake, and is a function of distance from the spinal cord surface. In the present study, a steady state of tissue infiltration was most likely not reached. Bulk flow, which would facilitate the extracellular transport and which would contribute to the clearance of a substance from the superfused tissue, has never been demonstrated, and it is reasonable to gauge the distribution of various intrathecally applied substances independently of bulk flow.

Iontophoresis and pressure microinjections of picoliter quantities are often used to study drug effects on single neurons close to the site of injection. Microinjections of nanoliter quantities are frequently used to study drug effects on larger populations of neurons in deep brain or spinal cord structures. Injections of 50–100 nl [^{3}H]bicuculline (Segura et al., 1992) or [^{3}H]thyrotropin-releasing hormone (Vonhof et al., 1990) resulted in a spherical distribution of the injected tracers, 90% of which were located within a radius of 0.3–0.7 mm around the site of injection. The punctuate or spherical distribution of substances applied by iontophoresis or microinjection into the spinal cord may mimick the spread from a site of high terminal density, if the terminals are selectively activated. The present results illustrate that the controlled superfusion may, however, mimick the effect of spinal release after extensive afferent stimulation as described with antibody microprobes (Duggan et al., 1990). Even though the precise distribution and tissue concentration of released neuropeptides is not known, the studies by Duggan et al. (1987, 1990) suggest that strong noxious stimuli may produce the release throughout the superficial dorsal horn and a widespread distribution of the peptides may result, similar to the distribution achieved by the superfusion of the cord dorsum. This technique provides the possibility to study the effects of single, extrasynaptic chemicals on the neuronal population of selected cord segments, e.g., in electrophysiological or immunohistochemical studies in anesthetized or in decerebrated, unanesthetized, spinalized animals.

In awake animals, the chronic catheterization of the spinal subarachnoid space (Yaksh and Rudy, 1976; Hylden and Wilcox, 1980) can be used to topically apply substances to the spinal cord. The actual site of application through the catheter is, of course, more variable, and the spread of substances is more extend including several segments and probably also the ventral spinal cord. Experiments in which 10 μl of dye was injected intrathecally indicate that 10–15 min after injection the substances spread within the subarachnoid space of rats 2.5–3.5 cm from the tip of the catheter (Yaksh and Rudy, 1976; Bryant et al., 1983; Tseng and Fujimoto, 1984). Using [^{3}H]naloxone (Yaksh and Rudy, 1976; Tang and Schoenfeld, 1978; Tseng and Fujimoto, 1985), [^{14}C]5-serotonin (Schmauss et al., 1983), [^{125}I]SP (Wolf and Mohrland, 1984) in cats, mice and rats, rapid distribution to further rostral segments was found. At brain sites, the level of radiolabel measured 15 or 30 min after the injections depended upon lipophilicity. Substances with high lipid solubility reached supraspinal sites by uptake into circulation and redistribution. Substances with lower lipid solubility resulting in slower absorption into the blood may gain access to brain sites by diffusion in the arachnoidal space after intrathecal injection as reported for SP 30 min after injection. In contrast, following the controlled superfusion of the cord dorsum with [^{125}I]NKA, a very low level of radioactivity was found both in the adjacent spinal cord segments and in the brain. Thus, the radiolabeled area was largely restricted to the exposed spinal cord segments.

Of course, the controlled superfusion of the cord dorsum may be used to apply any molecule (neuropeptides, receptor agonists, receptor antagonists, ligands, oligonucleotides, etc.) to selected spinal cord segments (Sandkühler et al., 1988, 1990, 1994; Gillar-

don et al., 1994). When using substances other than NKA in the superfusate, it must, however, be considered that the lipophilicity, the size and form of the molecule, the stability in the tissue, binding properties and clearance by effective uptake mechanisms may influence the distribution, clearance and the neurobiological effects and may lead to deviations from the present results. It should taken into account that substances with a considerable higher lipophilicity than NKA can cross cell membranes more readily and may enter systemic circulation more rapidly, resulting in a faster redistribution and clearance from the cord.

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