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TECHNICAL NOTE

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Demonstration of morphine in ganglion cells of the hippocampus from victims of heroin overdose by means of anti-morphine antiserum

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Abstract To investigate the topography of morphine distribution in the human brain, a method has been developed to detect morphine immunohistochemically. In this study hippocampus tissue from victims of heroin overdose (blood morphine concentrations 220 ng/g–1500 ng/g; 6-MAM positive urine sample), known for its high concentration of μ-opiate receptors was used. The immunohistochemical staining was performed with an anti-morphine antiserum originally developed for radio-immunoassays. In comparison with control specimens from cases of sudden death without morphine exposition or a history of heroin abuse, the brains from victims of heroin overdose showed selectively stained ganglion cells, axons and dendrites, suggesting a massive concentration of morphine in the neuronal structures.

Key words Ganglion cells · Hippocampus · Immunohistochemistry · Mean optical density (MOD) · Morphine

Introduction

To gain a deeper understanding of the distribution of morphine in brain tissue, it seemed useful to carry out an immunohistochemical study of the topography of the morphine distribution in the brain, especially since the time dependency of the concentration gradients between different parts of the brain observed (Vycudilik 1988), remains to date unexplained. A major prerequisite for the study of the regional morphine distribution is the localisation using a staining method. The immunohistochemical process described in this article has been modified from an immunotoxicological method.

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Materials and methods

The brain samples used in this study were taken from corpses when the cause of death of the victim was clearly a heroin overdose. The selection criteria were a lethal blood concentration of morphine and a positive result for 6-monoacetylmorphine (6-MAM) in the urine sample. The detection of 6-MAM, the first hydrolysis product of heroin, in the urine proves heroin consumption (Cone et al. 1991) and was accomplished by thin-layer chromatography, whereby the limit of detection was around 50 ng/g.

Because no anti-morphine antiserum which has been tested for the immunohistochemical staining of paraffin-embedded specimens was available, an antiserum for radio-immuno-assays was used in this study (Sigma Immuno Chemicals, Deisenhofen). Due to the chemically similar structures of morphine and codeine, a strong cross-reaction of the antiserum with codeine is unavoidable and exists for all anti-morphine antisera. Because the antiserum used in the immunohistochemical detection of morphine is said to cross-react (50%–100%) with codeine, a relevant codeine consumption was excluded by accepting only cases where the codeine concentration in the blood was less than 10% that of morphine. Cross-reactions of the antiserum with chlordiazepoxide, nitrazepam, THC, amphetamines, paracetamol and cocaine have also been described (Sigma Immuno Chemicals, Deisenhofen), but with less than 0.01% these are so weak that no further exclusion criteria were necessary.

The brains of four cases could be classified into a case group which fulfil the abovementioned criteria (Table 1, cases 1–4). A history of heroin abuse was known to the police in all cases. The brains of four certain non-drug consumers negative for these criteria formed the control group (Table 1, cases 5–8) where death was due to a traffic accident (case 5), a pulmonary artery embolism (case 6), pneumonia (case 7), or a myocardial infarction (case 8).

The brains were generally autopsied within 2 days post mortem. In accordance with the known opiate receptor distribution (Atweh and Kuhar 1977 a–c; Bero et al. 1988; Bidlack et al. 1988; Hassan et al. 1989; Roy et al. 1988; Schmidt et al. 1994), tissue was taken from one side of the hippocampus, which is rich in μ-opiate receptors (Kuhar et al. 1973; Mansour and Watson 1993) but not fixed

Table 1 Criteria of the examined brains for the case group and the control group

	No.	Sex	Age (years)	Morphine (Blood) ng/g	6-MAM (Urine)	Codeine (Blood) ng/g	Screening criteria
Case group	1	m	22	220	+	10	Injection mark
	2	m	32	590	+	40	Injection needle found next to corpse
	3	m	22	480	+	20	Injection mark
	4	m	24	1500	+	60	Heroin consumption witnessed
Control group	5	m	27	n. d.	n. d.	n. d.	Traffic accident
	6	m	52	n. d.	n. d.	n. d.	Pulmonary embolism
	7	m	33	n. d.	n. d.	n. d.	Pneumonia
	8	m	57	n. d.	n. d.	n. d.	Myocardial infarction

+: positive detection
n. d.: not detectable, concentration below limit of detection

in formalin and subjected to a quantitative morphine and codeine analysis by means of GC/MS. These measurements were performed in a Hewlett Packard gas chromatograph (HP 5890 GC Series II) and a Hewlett Packard mass spectrometer (MSD HP 5971 Series), whereby the limit of detection was around 2 ng/g and the limit of quantitation around 5 ng/g.

After 14 days fixation in 4% buffered formaldehyde, tissue sections from the contralateral hippocampus region were prepared for both routine examination (HE staining) and for immunohistochemical examinations. For immunohistochemistry the endogenous peroxidase activity was blocked with methanol-H_2O_2 (30% H_2O_2) and to reduce non-specific binding to Fc receptors, the specimens were treated with buffered pig serum (dilution 1:10 in TBS buffer). Finally, after rinsing with 0.05 M TBS buffer (pH 7.6), the tissue sections were ready for the immunohistochemical examination. TBS buffer was prepared by dissolving 8.0 g of NaCl, 0.2 g of KCl, and 3 g of Tris base in 80 ml of distilled water. After adjusting the pH to 7.6 with 1M HCl the volume was made up to 1 l with distilled water.

The detection of morphine was carried out using a polyclonal rabbit anti-morphine antiserum (Sigma Immuno Chemicals, Deisenhofen) as primary antiserum (dilution 1:1000 in TBS-buffer) and biotinylated anti-rabbit-F(ab)2-fragment (DAKO, Hamburg, dilution 1:200 in TBS-buffer) served as the secondary antiserum. The detection of specific antiserum binding was performed with the avidin-biotin-peroxidase-complex method (DAKO, Hamburg) using 3,3′-diaminobenzidine as chromogen (Hsu et al. 1981). The specimens were counterstained with hematoxylin for 10 s.

A first control of the positive immunoreaction was effected by blocking the antiserum with 0.1% aqueous morphine solution before applying them to the specimen and further controls were provided by successively omitting each reagent from the reaction.

The quantitative assessment of the extent of the immunoreaction was effected by TV densitometry and computer-assisted determination of the mean optical density (MOD) (Fritz et al. 1996).

For the TV densitometry method, each neuron was displayed on a monitor by means of a camera and circumscribed with a mouse cursor. The computer (Sun Work Station) then calculated the gray-value pixel by pixel in the blue-channel of the red/green/blue (RGB) camera signal for the circumscribed cell area. The integration of these measurements over the surface of the cell produced the integrated optical density (IOD). The MOD is obtained by dividing the IOD by the circumscribed area (in pixels). The MOD is measured at 470 nm to reach the optimal ratio between the absorbencies of diaminobenzidine and hematoxylin (1.01:0.37) (Fritz et al. 1992). After encoding the cases and without knowing to which group they belonged, the MOD of 60 randomised neurons from the bond of neurons in the area of the gyrus dentatus was determined for each specimen. The cell density of the examined regions was in both the case group and the control group 94.06 ± 6.73 cells/mm^2. A uniformity of the cell measurement was provided by only considering those cells where the nucleoli had been transected. The mean of these 60 determinations produced the mean optical density.

Results

The results are summarized in Table 2. In the non-formalin fixed hippocampus sections of the case group, the morphine concentrations were between 134 ng/g and 298 ng/g and the codeine concentrations between 19 ng/g and 54 ng/g, whereas neither morphine nor codeine were detected in the control cases.

Figure 1 shows in addition to negative, numerous positive immunoreactions of neurons from a case group hippocampus (case 2, lethal morphine concentration in the blood of 590 ng/g and a morphine concentration in the hippocampus of 255 ng/g) after staining with anti-morphine antiserum. Figure 2 shows a corresponding specimen from the control group (case 6, death by pulmonary embolism). In contrast with the controls, the immunohistochemical stains of the sections from the morphine victims showed a clear positive immunoreaction within the boundaries of the neuron cytoplasm, axons and dendrites. No diffuse staining of the tissue or of other structures was seen. A one-tailed student-t-test showed that the average MOD of the stained case group neurons was significantly greater than that of the control group by a ratio of 1.04:0.46 ($p = 0.001$), showing that the nerve cells of the hippocampus areas from victims of heroin overdose clearly stained more intensively than those of the control group.

Table 2 Morphine and codeine concentrations in the hippocampus versus mean optical density (MOD) of the hippocampus neurons of the case group and control group

	No.	Morphine (Hippocampus) ng/g	Codeine (Hippocampus) ng/g	MOD (Hippocampus)
Case group	1	134	19	0.92 ± 0.39
	2	255	54	1.09 ± 0.30
	3	199	30	1.20 ± 0.58
	4	298	48	0.95 ± 0.42
Control group	5	n. d.	n. d.	0.49 ± 0.15
	6	n. d.	n. d.	0.48 ± 0.11
	7	n. d.	n. d.	0.36 ± 0.11
	8	n. d.	n. d.	0.51 ± 0.19

n. d.: not detectable, concentration below limit of detection

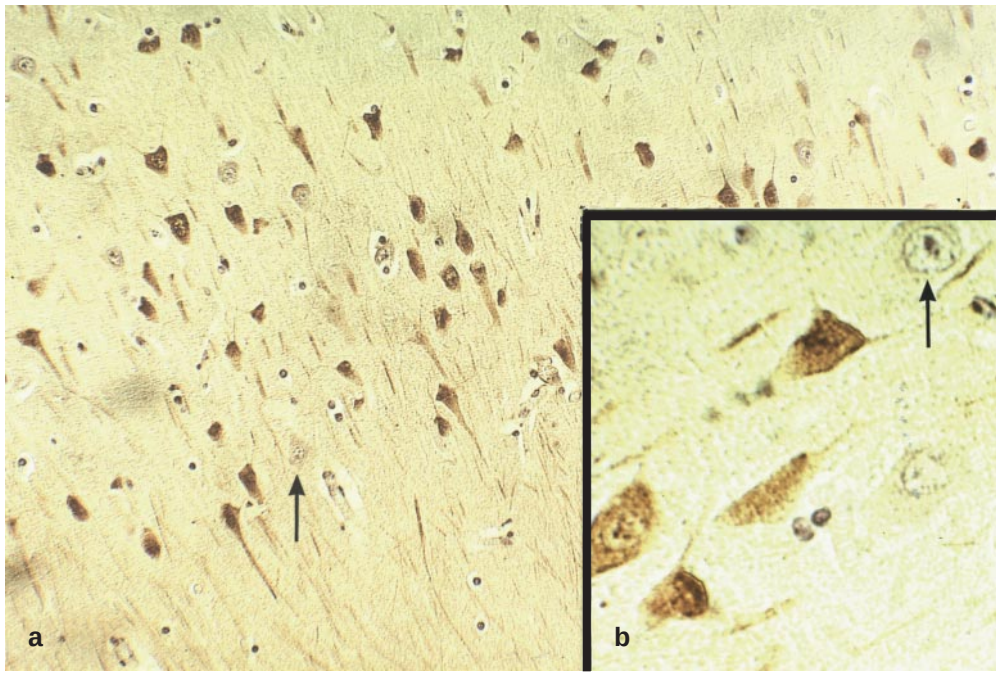

Fig. 1 a–b Selective staining of nerve cell bodies and partly of dendrites and axons of the hippocampus after immunohistochemical treatment with an anti-morphine antiserum (heroin victim; morphine concentration in the blood: 590 ng/g, morphine concentration in the hippocampus: 255 ng/g) (↑: immunohistochemical negative ganglion cell), magnification **a** 85 ×, **b** 170 ×

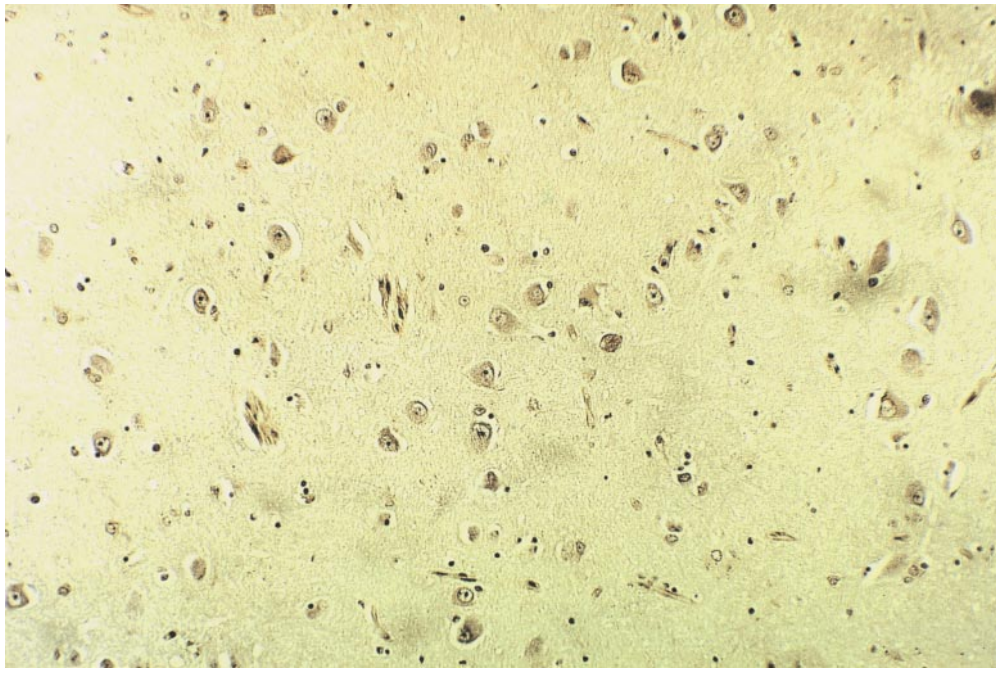

Fig. 2 Control staining after immunohistochemical treatment with an anti-morphine antiserum (sudden death from pulmonary embolism, no morphine detectable in blood or brain tissue), magnification 85 ×

Discussion

The presented paper shows the development of a method, which selectively demonstrates morphine by immunohistochemistry in ganglion cells. The findings are in agreement with those of Liu et al. (1996), in which however a description of the methods is not available.

The results show that neuron bodies, axons and dendrites of hippocampus regions from victims of heroin overdose with lethal morphine concentrations in the blood and 6-MAM positive urine, can be immunohistochemically marked with an anti-morphine antiserum selectively. The hippocampus regions from corpses without detectable morphine concentrations in the blood and brain tissue showed either no reaction or a subliminal reaction. The specificity of the antiserum excludes cross-reactions but a reaction with the similar endogenous morphine is possible, which would explain the subliminal reaction of the morphine negative hippocampus areas. The cross-reactivity also implies a reaction with codeine, but this does not seem to be relevant as the codeine concentrations were much lower than the morphine concentrations. It is thus reasonable to assume that the positive immunoreac-

tion is with morphine and not with codeine even for non-receptor-bound morphine. The method described cannot differentiate whether the antiserum detect free morphine or receptor-bound morphine. However the staining of the nerve cell bodies, axons and dendrites was topographically disjunct, thus suggesting receptor binding of the morphine. If this is the case, then an interference by codeine would be highly unlikely as the affinity of codeine to the μ-opiate receptors of the hippocampus is approximately 200 times less than that of morphine (Chen et al. 1991). Therefore this method could be considered as immunohistochemical evidence of morphine poisoning.

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References

Atweh SF, Kuhar MJ (1977a) Autoradiographic localization of opiate receptors in rat brain. I. Spinal cord and lower medulla. Brain Res 124:53–67

Atweh SF, Kuhar MJ (1977b) Autoradiographic localization of opiate receptors in rat brain. II. The brain stem. Brain Res 129:1–12

Atweh SF, Kuhar MJ (1977c) Autoradiographic localization of opiate receptors in rat brain. III. The telencephalon. Brain Res 134:393–405

Bero LA, Roy S, Lee NM (1988) Identification of endogenous opioid receptor components in rat brain using a monoclonal antiserum. Mol Pharmacol 34:614–620

Bidlack JM, O'Malley WE, Schulz R (1988) Comparison of [125I]-endorphin binding to rat brain and NG 108-15 cells using a monoclonal antiserum directed against the opioid receptor. Mol Pharmacol 33:170–177

Chen ZR, Irvine RJ, Somogyi AA, Bochner F (1991) Mu receptor binding of some commonly used opioids and their metabolites. Life Sci 48:2165–2171

Cone EJ, Welch P, Mitchell JM, Paul BP (1991) Forensic drug testing for opiates. I. Detection of 6-acetylmorphine in urine as an indicator of recent heroin exposure; drug and assay considerations and detection times. J Anal Toxicol 15:1–7

Fritz P, Klein C, Mischlinski A, Hage C, Dittel KK, Laschner W (1992) Morphometric analysis of the angioarchitecture of the synovial membrane in rheumatoid arthritis and osteoarthritis. Zentralbl Pathol 138:128–135

Fritz P, Behrle E, Zanger UM, Mürdter T, Schwarzmann P, Kroemer HK (1996) Immunohistological epoxide hydrolase in primary and secondary liver neoplasm: a quantitative approach. Xenobiotica 26(1):107–116

Hassan AHS, Almeida OFX, Gramsch Ch, Herz A (1989) Immunocytochemical demonstration of opioid receptors in selected rat brain areas and neuroblastoma × glioma hybrid (NG 108-15) cells using a monoclonal anti-idiotypic antiserum. Neuroscience 32(1):269–278

Hsu SM, Raine L, Fanger H (1981) The use of antiavidin antiserum and avidin-biotin-complex in immunoperoxidase technics. Am J Clin Pathol 75:816–821

Kuhar JM, Pert CB, Snyder SH (1973) Regional distribution of opiate receptor binding in monkey and human brain. Nature 245:447–450

Liu M, Wu J, Wang W (1996) Immunohistochemical study of morphine in human tissues from opiate associated death. Hua Hsi I Ko Hsueh Hsue Pao 27:151–154

Mansour A, Watson SJ (1993) Anatomical distribution of opioid receptors in mammalians: an overview. In: Herz A (ed) Handbook of experimental pharmacology, vol 104/I. Springer, Berlin Heidelberg New York, pp 79–106

Roy BF, Bowen WD, Frazier JS, Rose JW, McFarland HF, McFarlin DE, Murphy DL, Morishia JM (1988) Human anti-idiotypic antiserum against opiate receptors. Ann Neurol 24 (1):57–63

Schmidt P, Schröder H, Maderspach K, Staak M (1994) Immunohistochemical localization of kappa opioid receptors in the human frontal cortex. Brain Res 654(2):223–233

Vycudilik W (1988) Vergleichende Morphinbestimmung an Gehirnteilen mittels GC/MS. Eine Möglichkeit zur Eingrenzung der Überlebenszeit. Z Rechtsmed 99:263–272