

Distribution of cocaine- and amphetamine-regulated transcript in the hippocampal formation of the guinea pig and domestic pig

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This study provides a detailed description concerning the distribution of cocaine- and amphetamine-regulated transcript (CART) subunits — CART_{61–102} and rhCART_{28–116} — in the hippocampal formation (HF) of the guinea pig and domestic pig, focussing on the dentate gyrus (DG) and hippocampus proper (HP). Although in both studied species CART-immunoreactive (CART-IR) neuronal somata and processes were present generally in the same layers, some species-specific differences were still found. In the granular layer (GL) of both species, the oval-shaped neurons and some thick varicose fibres were encountered. In the guinea pig there was an immunoreactive “band of dots”, probably representing cross-sectioned terminals within the DG molecular layer (MOL), whereas in the domestic pig, some varicose fibres were detected, thus suggesting a different orientation of, at least, some nerve terminals. Furthermore, some CART-positive cells and fibres were observed in the hilus (HL) of the guinea pig, whereas in the analogical part of the domestic pig only nerve terminals were labelled.

In both species, in the pyramidal layer (PL) of the hippocampus proper, CART-IR triangular somata were observed in the CA3 sector, as well as some positive processes in MOL; however, a few immunoreactive perikarya were found only in the CA1 sector of the guinea pig. As regards the localization patterns of two isoforms of CART in the guinea pig, both peptide fragments were present simultaneously in each of the labelled neurons or fibres, whereas in the domestic pig three types of fibres may be distinguished within the area of the DG. In the hilus and MOL of the dentate gyrus, there were fibres expressing both isoforms of CART in their whole length (fibres of the first type). Fibres of the second type (in GL) coexpressed both peptides only on their short segments, and the last ones (in MOL) expressed solely rhCART_{28–116}.

These results indicate that the distribution of the two CART isoforms are specifically related, thus the relationship between the two CART isoforms may imply different metabolic profiles of CART-expressing neurons. (Folia Morphol 2009; 68, 1: 23–31)

Key words: CART isoforms, immunocytochemistry, dentate gyrus, hippocampus proper

INTRODUCTION

Cocaine- and amphetamine-regulated transcript (CART) peptides have been studied for over 20 years and were first described in the ovine hypothalamus by Spiess [6, 13]. Nowadays CART has been found in many brain structures and it is thought to have various physiological functions. For example, these neuropeptides were labelled in the nucleus accumbens [5, 7, 8, 22], a structure that plays an important role in the reward and reinforcement of psychostimulants [8], and CART may have an effect on reward and drug abuse [5]. In the addiction process, other brain structures are involved such as the amygdala and cortex, in which CART was also detected [8]. CART peptides are also widely expressed in the hypothalamus [11, 12–14, 18, 32] where they are thought to be involved in the feeding process [2, 12–14], stress response [11, 13, 14], and endocrine control [12]. In feeding, CART plays an anorexic role and it is a cotransmitter for nitric oxide (NO) [12]. Moreover, CART neurons in the hypothalamus possess receptors for leptin hormone, also involved in body-weight regulation in mammals [6]. The role of CART in stress response was proven when peptides were discovered in all three levels in the hypothalamo–pituitary–adrenal axis [11], in which CART is probably tissue-specific transformed. On the other hand, some investigations showed that fragments of CART_{55–102} in the hippocampal formation can promote the survival of neurons; this process is not well understood, but probably the peptide regulates mRNA expression of the brain-derived neurotrophic factor [33]. Other studies suggest that the neuroprotective mechanism of CART may also be linked to the preservation of mitochondrial function [20]. Those various above-described biological roles of CART and its expression in different nuclei of the brain as well as in the peripheral nervous system are probably connected with CART action at the level of mitochondria, which are central to both energy supply and expenditure in cells.

Hitherto, the CART-IR structures in the guinea pig and domestic pig hippocampal formation have remained unknown because no studies have been carried out except one report presented in poster form [10]. CART-immunoreactive structures have only been described in the dentate gyrus and hippocampus proper of man, rat [27], and vole [7]. Thus, the aim of this study was to investigate the distribution and morphology of the CART peptides in two different mammals: the guinea pig and the domestic pig (prenatal and perinatal developers, respectively). The most investigated species (rat) is, in this regard,

different from those investigated by us, because its brain develops postnatally.

Thus, information about the expression of CART peptides is interesting. To consider that CART showed different post-translational changes and two isoforms of CART e.g. CART_{61–102} and rhCART_{28–116} have been checked in the hippocampal structures. The anatomical and histological nomenclature of hippocampal formation was adopted from the work of Amaral and Witter [1].

MATERIAL AND METHODS

Tissue preparation

Six sexually immature (approximately 8 weeks old) female domestic pigs obtained from the commercial fattening farm in Niedźwiedź, Poland, and 6 young (aged 10 days postnatally, P10) male guinea pigs (strain: Dunkin-Hartley) obtained from the Research Institute of the Polish Mother's Health Centre in Łódź, Poland were used in the present study. In both cases, the animals were taken from two unexposed litters. All experiments were carried out in accordance with Local Ethical Committee rules.

The domestic pig

Thirty minutes before the main anaesthetic, sodium pentobarbital (Vetbutal, Biowet, Poland; 25 mg/kg b.w.) was given intravenously, and the animals were pre-treated with propionyl promazine (Combelen, Bayer, Germany; 0.4 mg/kg b.w. i.m.). Then, after the cessation of heart beating, they were transcardially perfused with one litre of pre-perfusion solution containing 0.9% sodium chloride (Chemia, Gliwice, Poland), 2.5% polyvinylpyrrolidone (Sigma, Deisenhofen, Germany), 0.5 procaine hydrochloride (Polfa, Warsaw, Poland), and 20,000 i.u. of heparin (Heparinum; Polfa, Warsaw, Poland; added *ex tempore*), followed by four litres of 4% ice-cold buffered paraformaldehyde (pH 7.4). No animals used in this study were injected with colchicine. Following perfusion, small tissue blocks consisting of the whole hippocampus (along septo-temporal axis) were post-fixed by immersion in the same fixative for 4 hours, washed twice in 0.1 M phosphate buffer (pH 7.4), and then stored in 30% sucrose until sectioning.

The guinea pig

All animals were anaesthetized by lethal dose of sodium pentobarbital (Morbital, Biowet, Poland; 2 mL/kg b.w). Then the animals were transcardially perfused with 4% paraformaldehyde (50–70 mL) for

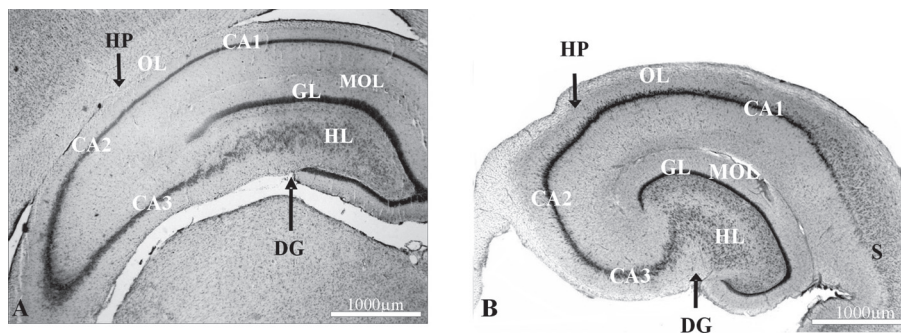


Figure 1. Hippocampal formation of the guinea pig (A) and domestic pig (B). Nissl staining; MOL — molecular layer; GL — granular layer; HL — hilus; OL — oriens layer; CA1–CA3 — sectors of hippocampus proper; S — subiculum; DG — dentate gyrus; HP — hippocampus proper.

30 min. Following the perfusion, the whole brains were postfixed for 0.5 hours in the same fixative, washed twice in 0.1 M phosphate buffer (pH 7.4) and kept in 30% sucrose until sunk.

Immunofluorescence experiments

Cross sections (10 μm thick) of the whole guinea pig brains and tissue blocks of the domestic pig brain (along the rostrocaudal axis) were cut on a cryostat (HM 525, Microm, Germany). They were processed for routine double-labelling immunofluorescence overnight at room temperature by using a rabbit polyclonal antibody against CART peptide_{61–102} (1:10.000 and 1:20.000; H-003-61, Phoenix, UK), and mouse monoclonal rhCART_{28–116} (1:5000 and 1:4000; MAB163, R&D Systems, USA). The next day, sections were incubated for 1 hour with a mixture of Cy3-conjugated donkey anti-rabbit IgG (1:9000; code 712-165-153, Jackson ImmunoLabs, USA) and FITC-conjugated goat anti-mouse IgG (1:600; code 715-095-150, Jackson ImmunoLabs, USA). To prove the specificity of immunoreaction, the control sections underwent replacement, omission, and cross-reaction. All control immunostainings resulted in a lack of immunoreactivity.

Cresyl violet staining. One guinea pig's brain and one domestic pig's block of brain tissue were processed for standard Nissl method staining to analyse the anatomical and histological structure in detail, prior to immunostaining. The images were taken using an Olympus SZ61 stereoscope equipped with an ARTCAM-300MI camera connected to a PC.

RESULTS

Morphology of the hippocampal formations in studied species in Nissl sections (Fig. 1)

In the investigated species, both the hippocampus proper (HP) and dentate gyrus (DG) has been shown

to have a three-layered structure. In the DG of both animals, the principal cell layer (granular layer — GL) consisted of oval-shaped neurons. Above the GL was the molecular layer (MOL), devoid of neurons, and below the GL was the hilus (HL) with numerous dispersed nerve cells of various shapes. In the hippocampus proper the main pyramidal layer (PL) was located between the molecular and oriens layers (OL). The PL was built of triangular cells. In the hippocampus proper of the two investigated mammalian species there were three separated sectors (CA1, CA2, CA3) numbered from the subiculum to the DG.

Distribution of CART immunoreactivity

The dentate gyrus. In both species, each layer of the dentate gyrus contained CART-immunoreactive structures. In the molecular layer of the guinea pig only one band of immunoreactive dots was observed (Figs. 2, 3), whereas in the domestic pig, in the corresponding layer, some single varicose fibres were disclosed (Figs. 4, 5B). The granular layer in both species was very similar and contained some relatively large oval-shaped perikarya (Figs. 6A, 7) in which CART-immunoreactive material was found on the cell periphery. Moreover, thick CART-positive fibres passing perpendicularly through the GL were also detected (Figs. 3, 8).

In the HL, two phenotypes of fibres were observed. The first type consisted of thick, varicose fibres similar to those observed in the domestic pig MOL of DG and GL, but their direction was not regular and they were distributed in the whole hilar region. The fibres of the second type were smooth and arranged in a pathway running from the HL to the hippocampus proper. They passed throughout the CA3 sector of the HP and ended within the end bulb (EB) on the border of the CA3 and CA2 sectors in the guinea pig (Fig. 9). In the domestic pig, such

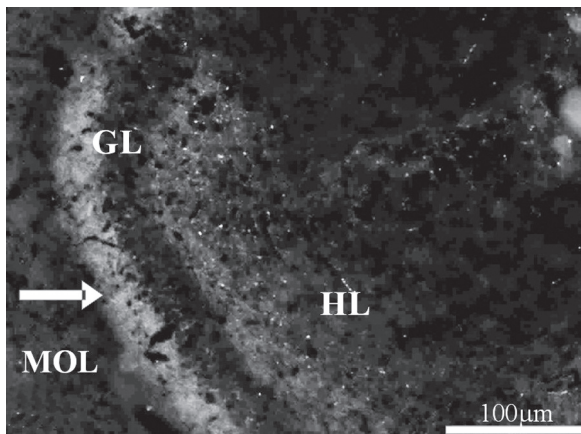


Figure 2. Cocaine- and amphetamine-regulated transcript-immunoreactive (CART-IR) "dots" (arrow) in molecular layer (MOL) of the guinea pig dentate gyrus; GL — granular layer; HL — hilus.

a distinct ending of this pathway was not observed. It was in the form of a wide band of immunoreactive points (Fig. 10). They probably represent the mossy fibres which passed from the DG to the HP and formed the stratum lucidum (LUC). Only in the HL of the guinea pig were a small number of la-

belled perikarya observed, and they were densely surrounded by fibres (Fig. 6B).

The hippocampus proper. In the hippocampus proper, CART-immunoreactivity was weaker than in the dentate gyrus. In the guinea pig, a few triangular neurons localized mainly in the CA3 and CA1 sectors were observed in the pyramidal layer (Fig. 6C). CART-immunoreactivity occupied the periphery of the neuronal somata. In the domestic pig, positive perikarya were found only in the CA3 sector. In this species, CART-immunoreactivity was localized through the cell body and in the initial part of dendrites (Fig. 11). In both species, a small number of varicose fibres were observed. They coursed perpendicularly through almost all layers (Figs. 12–14), apart from the oriens layer of the domestic pig HP, which was devoid of the immunostained processes.

Co-localization pattern of two truncated forms of the CART peptides: CART_{61–102} and rhCART_{28–116}

The dentate gyrus. In the guinea pig, both fragments of CART were co-expressed in every detected cell body and fibre, whereas in the domestic pig,

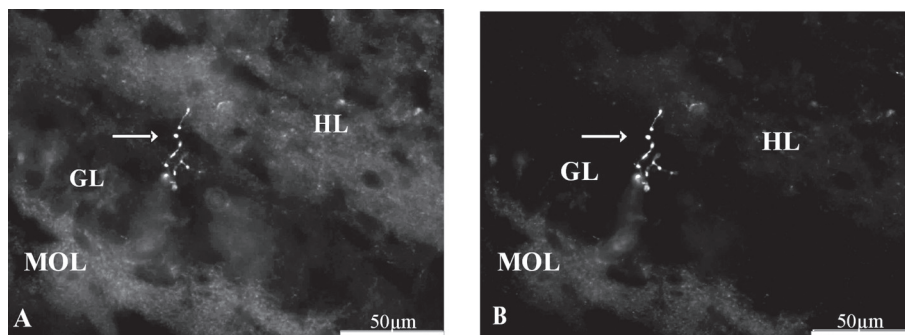


Figure 3. Cocaine- and amphetamine-regulated transcript-immunoreactive (CART-IR) nerve terminal in the granular layer (GL) of the guinea pig (arrow), immunopositive for both CART_{61–102} (A) and rhCART (B); MOL — molecular layer; HL — hilus.

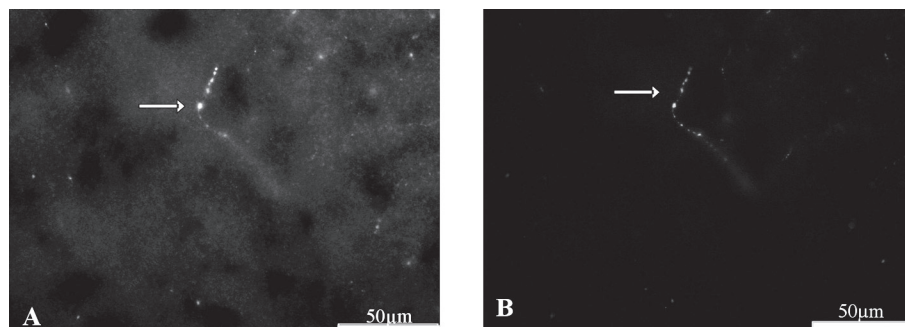


Figure 4. Cocaine- and amphetamine-regulated transcript-immunoreactive (CART-IR) nerve terminal in the domestic pig molecular layer (MOL; arrow), immunopositive for both CART_{61–102} (A) and rhCART (B).

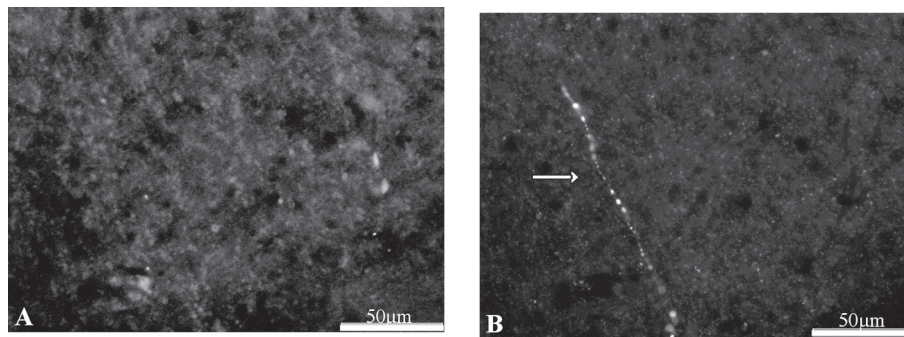


Figure 5. Cocaine- and amphetamine-regulated transcript (CART_{61-102})-immunonegative (A), but rhCART-immunopositive (B; arrow) nerve fibre in the domestic pig molecular layer.

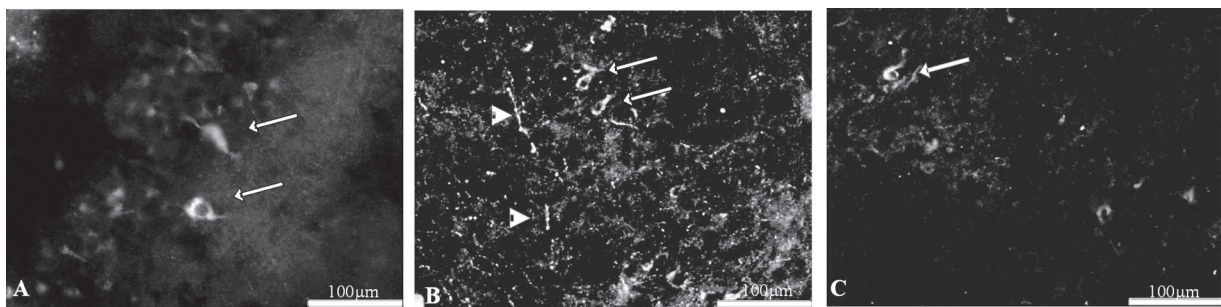


Figure 6. Cocaine- and amphetamine-regulated transcript-immunoreactive perikarya (arrows) and fibres (arrowheads) in the granular layer of the guinea pig (A), hilus (B) and CA3 sector (C).

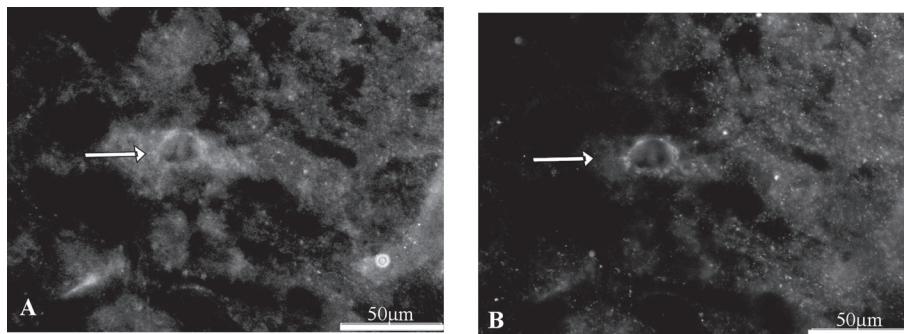


Figure 7. Cocaine- and amphetamine-regulated transcript-immunoreactive (CART-IR) perikarya in the domestic pig granular layer (arrow), immunoreactive for both CART_{61-102} (A) and rhCART (B).

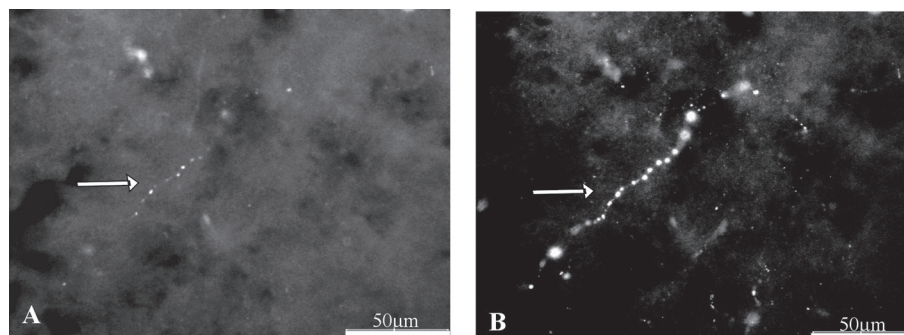


Figure 8. Cocaine- and amphetamine-regulated transcript-immunoreactive (CART-IR) nerve fibre in the domestic pig granular layer (arrow) immunoreactive for both CART_{61-102} (A) and rhCART (B).

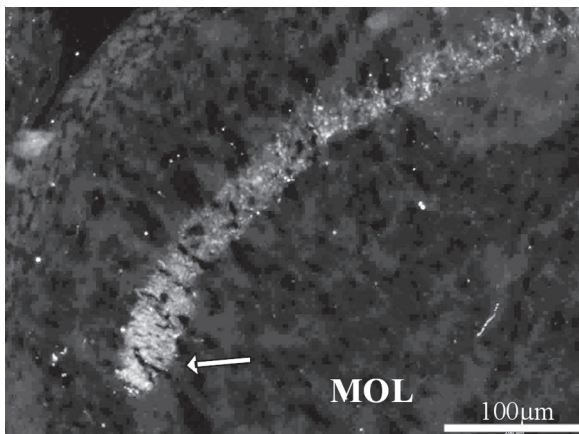


Figure 9. The end of mossy fibres in the guinea pig (the end bulb — arrow); MOL — molecular layer.

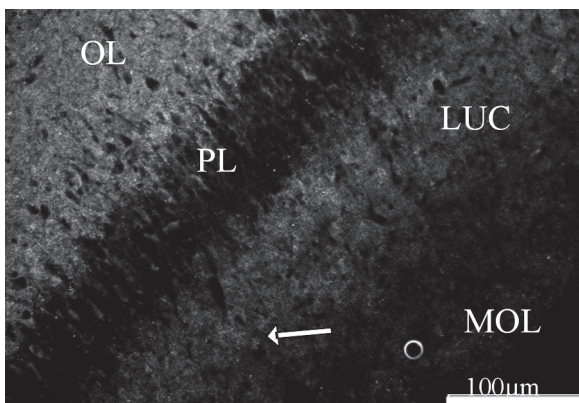


Figure 10. The end of mossy fibres in the domestic pig (arrow); OL — oriens layer; PL — pyramidal layer; LUC — stratum lucidum; MOL — molecular layer.

certain differences were observed. In the domestic pig granular layer, some oval-shaped neurons were found and all of them expressed both isoforms of the peptide (Fig. 7). Moreover, in the

domestic pig, three kinds of varicose fibres were differentiated: the first, most numerous, kind immunostained for both studied peptides in the whole length of the process. They were found in the hilus (Fig. 15) and molecular layer of the dentate gyrus (Fig. 4). The second kind expressed immunoreactivity for both CART peptides, but colocalization was observed only in the short course of the processes. These fibres were observed in the granular layer (Fig. 8). The last type was identified in the molecular layer (Fig. 5). They were not numerous and expressed only rhCART isoform.

The hippocampus proper. The present study showed expression of both isoforms of CART in all the above-described hippocampal structures of the domestic pig and guinea pig. Generally, the immunoreactive material occupied entire cell bodies as well as the whole length of the processes, independently of the layer (Figs. 11–14).

DISCUSSION

Hippocampus formation in the guinea pig and domestic pig is composed of the subiculum, hippocampus proper, and dentate gyrus, although only the dentate gyrus and hippocampus proper were investigated. Its general anatomical organization and three-layered structure appeared to be similar in primates: human [2], rhesus [25], and macaque [23], as well as non-primates mammals such as the pig [4], cat [31], hedgehog [15, 20], shrew [9], rat [17], and mouse [29].

The study showed that distribution of CART_{61–102}- and rhCART_{28–116}-immunoreactivity in the guinea pig and domestic pig was similar in this respect, i.e. that both peptides were shown in the same layers of hippocampal formation. However, some species differences were observed. In the molecular layer of the guinea pig dentate gyrus a band of immunostained

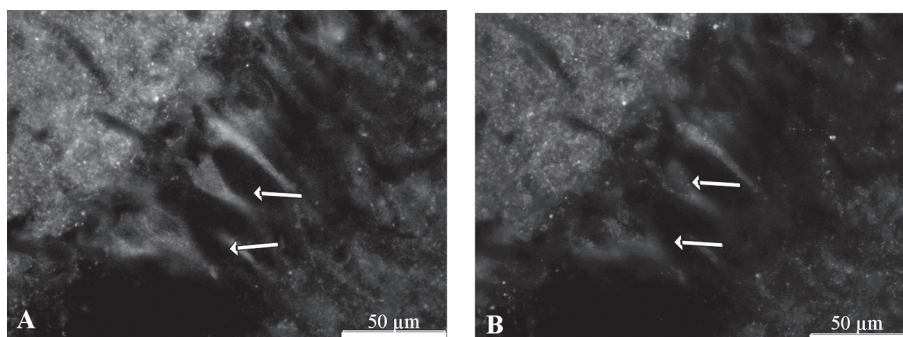


Figure 11. Cocaine- and amphetamine-regulated transcript-immunoreactive (CART-IR) neurons in the domestic pig pyramidal layer (arrows), immunoreactive for both CART_{61–102} (A) and rhCART (B).

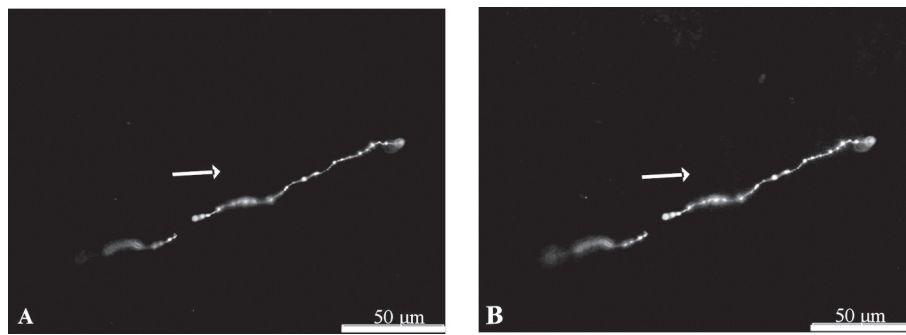


Figure 12. Cocaine- and amphetamine-regulated transcript-immunoreactive (CART-IR) nerve fibre in the oriens layer of the guinea pig (arrow), immunoreactive for both CART₆₁₋₁₀₂ (A) and rhCART (B).

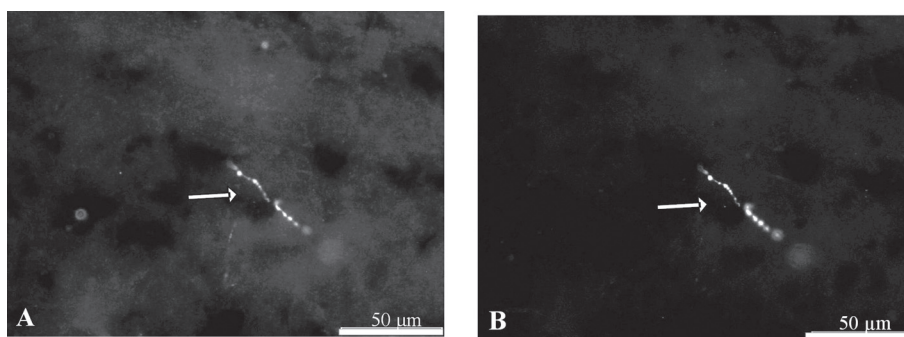


Figure 13. Cocaine- and amphetamine-regulated transcript-immunoreactive (CART-IR) nerve fibre in the pyramidal layer of the guinea pig (arrow), immunoreactive for both CART₆₁₋₁₀₂ (A) and rhCART (B).

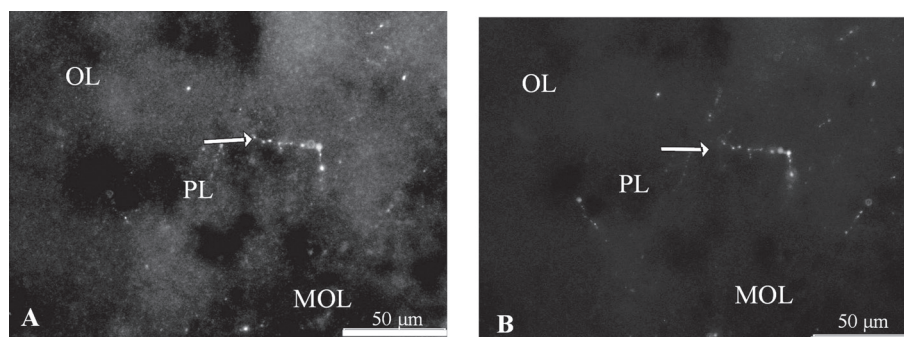


Figure 14. Cocaine- and amphetamine-regulated transcript-immunoreactive (CART-IR) nerve terminal in the domestic pig pyramidal layer (PL; arrow), immunoreactive for both CART₆₁₋₁₀₂ (A) and rhCART (B); OL — oriens layer; MOL — molecular layer.

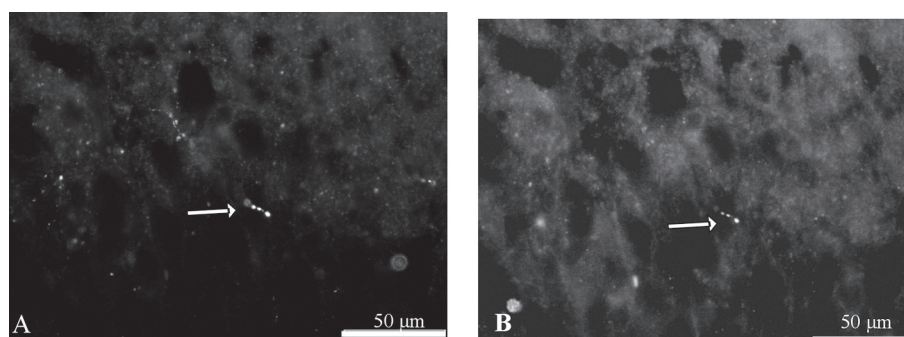


Figure 15. Cocaine- and amphetamine-regulated transcript-immunoreactive (CART-IR) nerve terminal in the domestic pig hilus (arrow), immunoreactive for both CART₆₁₋₁₀₂ (A) and rhCART (B).

"dots" was revealed, whereas in the analogical layer of the domestic pig only single varicose fibres were found. This may indicate a different spatial organization of nerve fibres running through the studied layer, where the "dots" most probably reflected the profiles of cross-sectioned nerve terminals. In the granular layer of both species, no significant differences in the distribution of CART peptides were observed in cells or varicose fibres, which crossed that layer. Only in the hilus of the guinea pig were small CART-IR perikarya found. In the HL of both studied mammalian species, two kinds of fibres were observed: smooth fibres and varicose fibres. The smooth fibres formed a track from the hilus to the hippocampus proper and ran lengthwise along the CA3 sector of the HP. It is supposed to be an efferent pathway [27, 30], which ends on the border between sectors CA3 and CA2, forming an end bulb in the guinea pig [28], or just as an indefinite fibre ending in the domestic pig. Probably they were the mossy fibres which lay in the stratum lucidum.

In both species, a few CART-IR perikarya were identified in the pyramidal layer of sector CA3. Additionally, in the guinea pig, some positive somata in region CA1 were disclosed.

Generally, distribution of CART-immunoreactivity in the rat and in humans [27] resembled those of the guinea pig and domestic pig, although they differed in some details. In the rat, MOL of dentate gyrus CART₄₁₋₈₉ immunoreactivity was weaker than in humans, whereas in the present studies both CART isoforms were observed and they were weaker in the HP than in the DG. In the border of the MOL and GL in rat dentate gyrus, single CART-positive cells were detected [27], but they were not found in the guinea pig or domestic pig. The pattern of immunoreactivity in the human DG was similar to that observed in the guinea pig (band of immunostained dots in MOL and CART-IR perikarya in the hilus), which was different from the rat and domestic pig. In our study, oval CART-IR perikarya in the guinea pig hilus were detected, but whether they were mossy cells, like multipolar ones in the guinea pig [26] and in humans [27] and form a separate group of hilar cells [27, 28], remains unknown.

CART-IR neurons were detected in various layers of HP in different mammals. They were presented in the CA1 sector in humans [27], CA3 sector in the domestic pig, and in both CA1 and CA3 sectors in the guinea pig (present studies), whereas in the rat, IR neurons were detected only in the oriens layer [27].

The observed discrepancies may thus reflect either interspecies differences or neurochemical variance of

discrete neuronal circuitries, in which many other biological active substances are presented. The hippocampal formation has been reported to express neuropeptide Y [21], substance P [29], nitric oxide synthase [21], glutamic acid decarboxylase [18], acetylcholinesterase [3, 16], and calcium binding proteins [18, 19, 24, 34]. The substances were localized in the interneurons, the projecting neurons, and in fibres and were also labelled in various immunoreactive puncta [3, 18, 19, 21, 24, 29, 34]. Some investigations showed the co-localization of CART with other peptides in such nuclei as accumbens [5, 7, 8, 22] and paraventricular [11–14, 18, 22, 32]. However, there was not any information about their coexistence in the hippocampal structures, but their distribution is similar suggesting a possible co-localization of those substances with CART [18, 19, 24, 34]. Earlier reports did not provide information concerning the co-localization pattern of two forms of CART peptide, the CART₆₁₋₁₀₂ and rhCART₂₈₋₁₁₆. Our results showed slight differences of the co-incidence pattern between these peptides in the area of hippocampal formation. Most of the CART-IR structures showed full or partial co-localization (positive neurons and fibres), but single fibres in the molecular layer of the dentate gyrus expressed only one peptide (longer isoform). These results indicate that the relationship between the two CART isoforms may imply different metabolic profiles of CART expressing neurons.

Thus, the present study provides detailed immunohistochemical and morphological data dealing with the presence, distribution, and co-incidence pattern of two forms of the CART peptide within the domestic pig and guinea pig hippocampal formations. However, these data should be considered as preliminary, being the first step in the characterisation of the chemical coding of neuronal somata and nerve terminals located in the domestic pig and guinea pig hippocampal formations. Future studies will deal with the co-localization pattern of CART-IR structures with other neuropeptides.

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